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RESEARCH ARTICLE

# Habitat explained microgeographic variation in Little Penguin agonistic calls

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## ABSTRACT

Vocalizations in birds play a significant role in species and mate recognition as well as sexual selection. Geographic variation in vocalization is well studied in male songbirds but largely unexplored in seabirds and in females. We investigated variation in male and female agonistic and advertising calls between 4 populations of Little Penguins (*Eudyptula minor*) in South Australia. We also determined whether call similarity was better explained by the geographic distances between the colonies, by microhabitat variation, or by variation in the physical characteristics of the individuals. Further, we used playback experiments testing male and female responses to determine the biological importance of geographic call variation. Both agonistic and advertising calls differed between individuals and sexes, with males producing calls at higher frequencies than females. Our results also reveal significant variation in agonistic calls across the colonies, best explained by variation in microhabitat. However, resident birds did not discriminate between calls originating from different colonies. The behavioral patterns are discussed in relation to gene flow and population differentiation.

**Keywords:** acoustic divergence, female choice, non-vocal-learning species, vocal discrimination

## L'habitat a expliqué une variation micro-géographique dans les cris agonistiques d'*Eudyptula minor*

## RÉSUMÉ

Les vocalisations chez les oiseaux jouent un rôle important dans la reconnaissance des espèces et des partenaires, ainsi que dans la sélection sexuelle. La variation géographique de la vocalisation est bien étudiée chez les oiseaux chanteurs mâles, mais elle demeure relativement inexplorée chez les oiseaux marins ou les femelles. Nous avons examiné la variation dans les cris agonistiques et d'avertissement des mâles et des femelles de quatre populations d'*Eudyptula minor* dans le sud de l'Australie. Nous avons aussi déterminé si la similarité des cris était mieux expliquée par les distances géographiques entre les colonies, la variation du micro-habitat ou par la variation dans les caractéristiques physiques des individus. Par ailleurs, nous avons utilisé une repasse expérimentale des réponses des mâles et des femelles afin de déterminer l'importance biologique de la variation géographique des cris. Les cris agonistiques et d'avertissement ont différencié entre les individus et les sexes, les mâles produisant des cris à plus haute fréquence que les femelles. Nos résultats ont aussi révélé une importante variation dans les cris agonistiques entre les colonies, ce qui était davantage expliqué par la variation dans le micro-habitat. Cependant, les oiseaux résidents n'ont pas fait de discrimination entre les cris provenant des différentes colonies. Les patrons comportementaux sont discutés en relation au flux génétique et à la différenciation des populations.

**Mots-clés:** espèce à apprentissage non vocal, divergence acoustique, choix de la femelle, discrimination vocale

## INTRODUCTION

Divergence in mating signals between populations is an important evolutionary process that can facilitate the development of behavioral barriers to gene flow (Uy et al. 2009, Brumm et al. 2010). In birds, vocalizations play a significant role in species and mate recognition as well as in sexual selection (Darwin 1871, Andersson 1994, Catchpole and Slater 2008) and there is evidence that female preference for particular songs can increase genetic differentiation between populations (Baker 1983, Fleischer

and Rothstein 1988, Ellers and Slabbekoorn 2003). However, most studies exploring the importance of acoustic divergence in birds have focused on songbirds, in which individuals learn their songs; very few studies have investigated these questions in non-vocal-learning species (exceptions include Bretagnolle and Lequette 1990, Bolton 2007, Miyazaki and Nakagawa 2015).

In songbirds, song divergence is influenced by both genetic (Nowicki et al. 1992, Podos 2001, Huber and Podos 2006) and cultural inheritance (Grant and Grant 1996, Greig et al. 2012). By contrast, vocalizations in non-vocal-

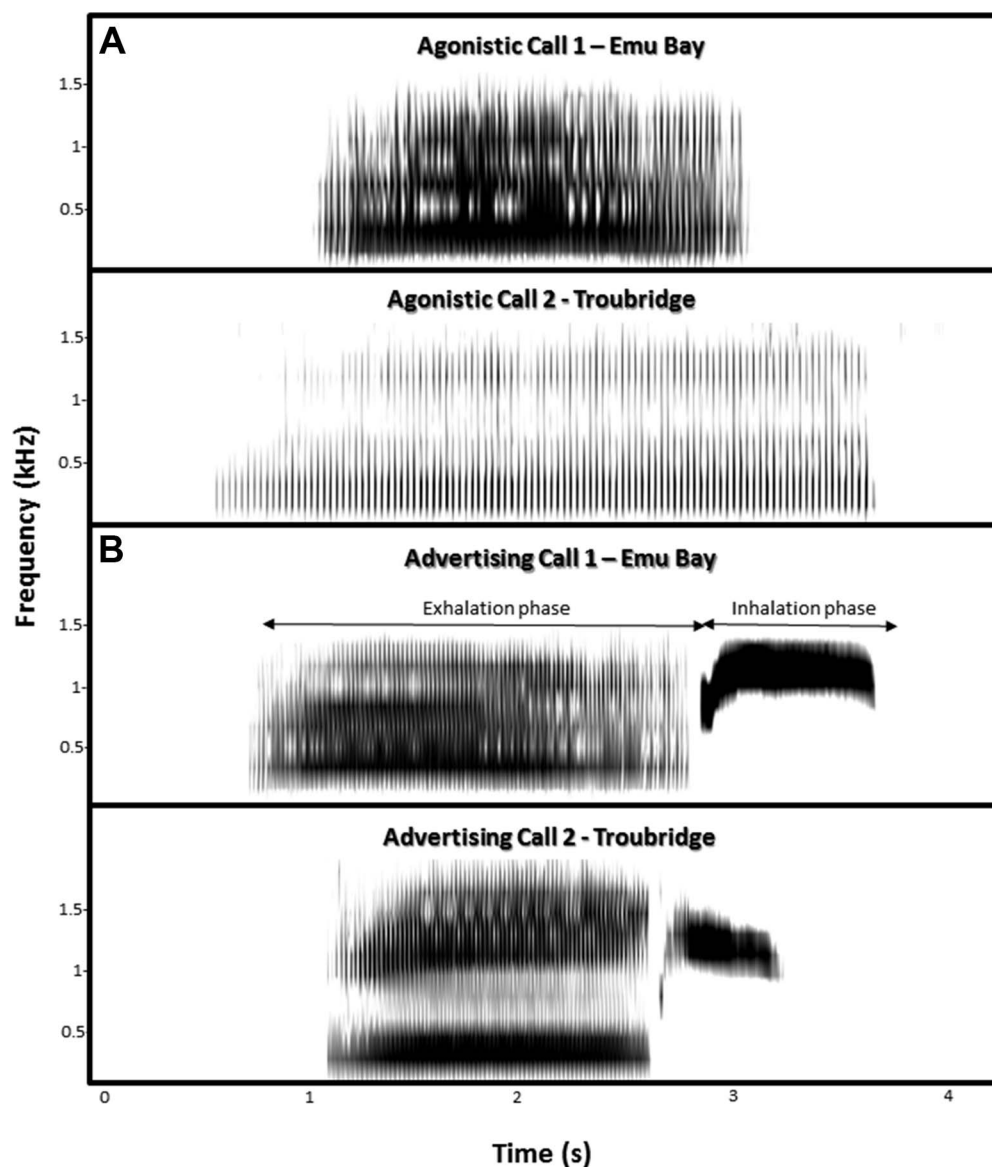
learning species are essentially inherited (Kroodsmas 2005, Catchpole and Slater 2008), and divergence in the vocalizations of such species is largely the result of evolutionary and ecological factors. Geographic variation in vocalizations is therefore expected to reflect genetic differences between populations (Smith and Friesen 2007). Variation will either increase continuously with increasing distance, as a consequence of high connectivity among neighboring populations and limited connectivity between distant populations (Isler et al. 2005, Budka et al. 2014), or be diffuse (with significant differences occurring between the populations regardless of the geographic distances) as a consequence of local genetic differences (Bretagnolle and Genevois 1997, Odom and Mennill 2012). Geographic variation in vocalizations can also arise as a byproduct of variation in habitat (Slabbekoorn and Smith 2002, Brumm and Naguib 2009) or in individual physical characteristics (Barbraud et al. 2000, Miyazaki and Waas 2003b, Favaro et al. 2015). However, geographic variation in vocalizations of non-vocal-learning species is more often studied at a macrogeographic scale (e.g., Peake and McGregor 1999, Odom and Mennill 2012, Budka et al. 2014, Favaro et al. 2016), and very little is known about which factors influence microgeographic variation in these species (Catchpole and Slater 2008).

Once divergence in vocalizations has arisen, the probability that these differences will be biologically relevant for the individuals depends on the discriminatory capacities and behavioral responses of the intended receivers. This is because variation in the acoustic structure of vocalizations is irrelevant if conspecifics do not perceive and distinguish such variation or do not respond differently to the variation (e.g., Schibler and Manser 2007). There is ample evidence that birds can perceive variation in vocalizations and discriminate among conspecifics solely on the basis of vocalizations (Podos 2007, 2010, Derryberry 2011, Bradley et al. 2013, Kleindorfer et al. 2013). But most of our knowledge on bird responses to geographic variation in vocalizations is restricted to males (e.g., Bronson et al. 2003, Ellers and Slabbekoorn 2003, Greig et al. 2015), with very few studies investigating male and female responses together (but see Searcy et al. 1997, Nelson and Soha 2004a). In songbirds, males generally exhibit a stronger response to local calls or songs than to vocalizations produced by individuals from distant populations (Searcy et al. 1997, Nelson and Soha 2004b, Podos 2007), whereas females do (Baker 1983, Danner et al. 2011) or do not (Nelson and Soha 2004a) show preference for local dialects. Therefore, understanding female response to geographic variation in vocalizations is just as important as understanding male response.

We investigated sex and microgeographic call variation between 4 populations of Little Penguins (*Eudyptula minor*) in South Australia. Little Penguins are highly vocal,

nocturnal seabirds that do not learn their vocalizations and that nest in burrows close to the shore (Klomp et al. 1991, Dann 1994, Hoskins et al. 2008). Like most seabirds, they exhibit high levels of philopatry, most individuals returning to their natal colony to breed (Dann 1992, Pledger and Bullen 1998, Bull 2000, Johannessen et al. 2002). They are strongly territorial (Waas 1988b, 1990, 1991a, 1991b) and vocalize mainly at night; vocalizations and individual recognition are important in mate choice and pair bonding (Miyazaki and Waas 2002, 2003b). Little Penguins' repertoire includes 6 identified calls: quacks, advertising or display calls (also referred to as "bray calls"), agonistic calls (also referred to as "growl calls"), aggressive barks, hisses, and cheeps (Jouventin 1982, Waas 1988b). We focused on agonistic and advertising calls (Figure 1) because they are the most common vocalizations used by Little Penguins (Miyazaki and Waas 2003b). Agonistic calls are low-intensity calls, used both before and after pairs engage in mutual displays (Waas 1988a) and as threats during defensive displays and high-risk aggressions (Waas 1990, 1991a). Advertising calls are high-intensity calls principally used by males to defend their territory against intruders and to court females (Waas 1988a, 1990, 1991a). Males produce highly individualized calls during the pairing stage to attract their mates (Miyazaki and Waas 2003a) and defend their territory (Waas 1990, Mouterde et al. 2012). Females prefer larger males because they produce eggs and chicks earlier in the breeding season, and larger males produce advertising calls with lower frequencies (Miyazaki and Waas 2003a, 2003b, Waas 2006). Very little is known about the information encoded in female calls. Studies in other bird species have shown that low-frequency calls can also be particularly effective in aggressive contexts (Bradbury and Vehrencamp 1998, Fitch et al. 2002, Cardoso 2012) because they likely indicate an opponent with superior fighting abilities (Maynard Smith and Price 1973, Waas 2006). Hence, it would be advantageous for Little Penguins to signal information regarding their body size in their vocalizations for fighting contests and to attract mates.

A recent study by Miyazaki and Nakagawa (2015) showed geographic differences in male advertising calls between the 2 Little Penguin subspecies living in New Zealand and demonstrated that females discriminated between advertising calls on the basis of their geographic origin. South Australia supports several Little Penguin colonies that have been declining considerably over the past decades (Wiebkin 2011, Colombelli-Négrel 2017). Previous studies showed that individuals living on Troubridge Island showed subtle genetic differentiation compared to the other colonies sampled in the area (Burridge et al. 2015, Graff 2015). Substantial morphological variation in body mass and bill measurements was also found between the same colonies, which was

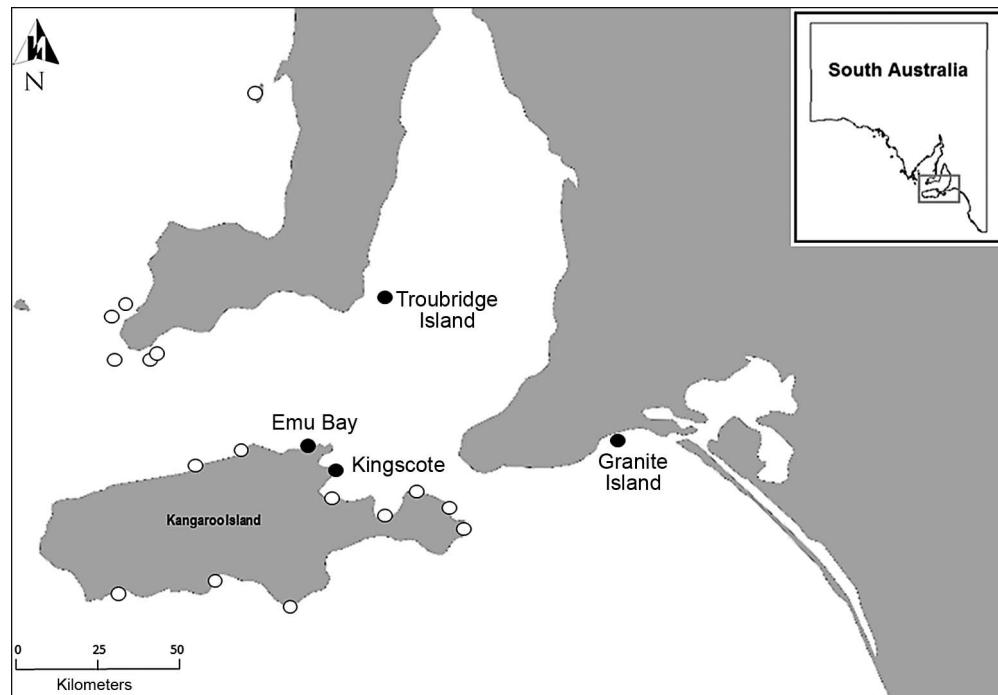


**FIGURE 1.** Examples of agonistic and advertising calls of male Little Penguins: (A) 2 agonistic calls and (B) 2 advertising calls from 4 different males from Emu Bay and Troubridge Island, South Australia (2013–2015).

influenced by both environmental parameters and geographic interpopulation distances (Colombelli-Négrel 2016a). Therefore, it is important to examine whether behavioral reproductive isolating mechanisms, such as variation in vocalizations, exist between the 2 identified genetic populations.

We first investigated variation in calls in relation to individuals, sexes, and colonies to quantify differences in acoustic characteristics between 4 South Australian populations, including Troubridge Island. We then investigated 3 potential causes of acoustic divergence: geographic isolation between the colonies (distances by sea in kilometers), variation in individual physical characteristics

(head length and body mass), and variation in microhabitat (percentage of trees and shrubs cover within a 10 m radius). Finally, we tested male and female discriminatory capacities and behavioral responses in playback experiments with calls from different colonies. The present study differs from that of Miyazaki and Nakagawa (2015) in that we (1) investigated call variation and response to the variation in both males and females, (2) focused on microgeographic variation (rather than macrogeographic variation), (3) explored potential factors influencing acoustic variation, and (4) investigated the existence of reproductive isolating mechanisms between populations of the same subspecies.



**FIGURE 2.** Distribution of the 4 sampled breeding colonies of Little Penguins (black circles) and the other Little Penguin colonies still present (white circles) in this area of South Australia (2013–2015).

## METHODS

### Study Sites

We conducted the study between August and December in 3 yr (2013–2015) at 4 colonies in South Australia: (1) Troubridge Island ( $35^{\circ}4'S$ ,  $137^{\circ}49'33''E$ ), a sandy island located  $\sim 8$  km east–southeast of Edithburgh (Yorke Peninsula) and mostly dominated by nitre bush (*Nitraria schoberi*) and African boxthorn (*Lycium ferocissimum*); (2) Kingscote ( $35^{\circ}37'S$ ,  $137^{\circ}13'E$ ), located on the north coast of Kangaroo Island, where Little Penguins were monitored in the artificial burrows installed along Hospital Beach, on the northern side of the jetty; (3) Emu Bay ( $35^{\circ}35'S$ ,  $137^{\circ}30'E$ ), also located on the north coast of Kangaroo Island,  $\sim 18$  km by land west of Kingscote, where the colony is set along the sandy and rocky beach north of the jetty of Emu Bay; and (4) Granite Island ( $35^{\circ}37'S$ ,  $138^{\circ}36'E$ ), a small island of Victor Harbour with a rocky, granite coastline, connected to the mainland by a bridge causeway and open to pedestrians during the day. During our study, all the colonies included a mix of individuals that were not breeding as well as individuals on eggs or with young. Study sites are shown in Figure 2.

### Burrow Selection

During daytime, we searched for burrows that contained only a single individual and noted the sex and (when applicable) the identity of the adult present. We selected

burrows with only one individual to ensure that recorded calls or playback responses could be correctly assigned to the targeted individual and that playback response was not biased by the presence of another individual in the burrow. Little Penguins in our study sites return to their colony only at night and take turns between remaining in their burrow and foraging at sea, sometimes for several days at a time (e.g., Saraux et al. 2011a, 2011b). To ensure that no mate had returned from foraging since we checked the burrows during the day, the number of individuals within a burrow was reconfirmed directly prior to recording or playback. Any burrows where the mate had returned were not used for playback or recording. For the playback experiments (see below), we also noted the breeding stage of the individual (not breeding, incubating, or with young chicks). We then recorded the location of each burrow using a Garmin GPS 64s so that we could revisit the selected burrows at night, either for audio recordings or for playback experiments. We estimated the sex of individuals on the basis of bill shape when both adults were present during our regular monitoring (e.g., on Kangaroo Island, females have pointy bills while male bills are rounder) and by measuring bill depth as previously described for Little Penguins (Arnould et al. 2004, Overeem et al. 2006, Wiebkin 2012, Colombelli-Négrel 2016a). Each colony was visited every 2 wk for breeding monitoring as part of another study; hence, individuals were observed, measured, sexed, and microchipped



through the monitoring period. The sex of a subset of individuals was also verified with a genetic-based method, which confirmed that sex was correctly assigned in ~83% of the individuals (see Colombelli-Négrel 2016a). To avoid the possibility of replicated sampling, we applied the following rules: (1) Selected individuals were microchipped or received a black mark with a permanent marker on their belly. (2) Burrows were used only once. (3) New burrows were selected far away ( $\geq 100$  m) from previously selected burrows to ensure that different individuals would be tested, because Little Penguins are known to return to the same part of their colony year after year, usually occupying burrows within a few meters of the ones used the previous year (Reilly and Cullen 1981).

### Call Recording and Analysis

We recorded all individuals using a Zoom Handy Recorder H4n (Zoom, Brookvale, Australia) in the evenings, during the first 2 hr after sundown. All recorders had integrated stereo microphones and were placed outside the burrow (approximately 30–50 cm away), facing its entrance and hidden in the vegetation. All calls were recorded as spontaneous calls and not in response to the playback experiments. We recorded all sound files as broadcast wave files (44.1 kHz sampling rate, 16-bit depth) and transferred the recordings to an Apple MacPro to visualize them with Amadeus Pro 1.5 (Hairsersoft, Switzerland) and analyzed them with Raven Pro 1.4 (Bioacoustics Research Program, Cornell Lab of Ornithology, Ithaca, New York, USA). We created spectrograms for each recording using the Hann algorithm (128-bit sample format; discrete Fourier transform, DFT = 256 samples; frequency resolution = 248 Hz; time resolution = 5.80 ms; frame overlap = 50%).

We recorded agonistic and advertising calls from 75 and 102 adults, respectively. Of those, we were uncertain of the sex of 5 individuals for agonistic calls and 26 individuals for advertising calls. Hence, we excluded these recordings from the analysis. We analyzed only calls with a high signal-to-noise ratio ( $>10$  dB) and no overlapping sound to ensure that we would obtain accurate measurements. Little Penguin colonies can be extremely noisy, and recordings were often obstructed by abiotic noise (sea waves and wind) and vocalizations from other seabird species or from conspecifics. As a result, we were able to reliably use 198 agonistic calls from 70 adults and 293 advertising calls from 76 adults for further analysis. Of the 70 adults recorded for the agonistic calls, 44 were also recorded for the advertising calls (13 individuals at Emu Bay, 10 individuals at Kingscote, 3 individuals on Granite Island, and 18 individuals on Troubridge Island).

From each agonistic call recording, we selected 1–5 successive calls individual<sup>-1</sup> (on average,  $3 \pm 0.19$  calls individual<sup>-1</sup>). From each advertising call recording, we selected 1–6 successive calls individual<sup>-1</sup> (on average,  $4 \pm$

$0.19$  calls individual<sup>-1</sup>). All calls were selected using Raven Pro 1.4, based on the fact that there was no overlapping sound. Analyses were conducted on the fundamental frequency following previously used measurements for Little Penguin calls (Jouventin and Aubin 2000, Miyazaki and Waas 2003a, 2003b, Miyazaki and Nakagawa 2015). For each selected agonistic call, we noted (1) total call duration (in seconds), (2) minimum and (3) maximum frequencies (Hz; frequencies at which minimum and maximum powers occurred within the selection), (4) frequency bandwidth or frequency range (Hz; difference between the maximum and minimum frequencies), and (5) the dominant frequency (Hz; frequency at which peak power occurred within the selection). For each selected advertising call, we noted (1) total call duration (in seconds), (2) exhalation phase duration (in seconds), (3) minimum and (4) maximum exhalation frequencies (Hz), (5) exhalation frequency bandwidth (Hz), (6) the dominant exhalation frequency (Hz), (7) inhalation phase duration (in seconds), (8) minimum and (9) maximum inhalation frequencies (Hz), (10) inhalation frequency bandwidth (Hz), and (11) the dominant inhalation frequency (Hz). We created the window selection manually using the cursor in Raven Pro 1.4 at the points where the amplitude of the vocalization reached  $-24$  dB in relation to the maximum amplitude (selection spectrum view).

### Potential Causes of Acoustic Divergence

We investigated 3 potential causes of acoustic divergence: geographic isolation between the colonies, variation in individual physical characteristics (bill size), and variation in microhabitat. To test for the idea that acoustical variation resulted from isolation by distance between the colonies, we estimated the shortest possible distances by sea (range: 35–124 km) between the breeding colonies (Troubridge Island, Emu Bay, Kingscote, and Granite Island) using the measurement tool in Google Earth 7.1 (<http://earth.google.com>). To test for the importance of habitat variation on acoustic divergence, we classified the microhabitat within a 10 m radius of each burrow—based on the percentage of tree and shrub cover (to a maximum of 100%) within the radius—as open (0–33% cover), mixed (34–66%), or closed (67–100%). To test for a relationship between body size and call characteristics, we captured by hand and measured 24 adults (9 males and 15 females) for agonistic calls and 24 adults (12 males and 12 females) for advertising calls. For all captured individuals, we measured (1) head length (with calipers, from the back of the head to the tip of the bill) and (2) body mass (weight recorded to the nearest 10 g). Miyazaki and Waas (2003b) have demonstrated that head length is a good indicator of body size in Little Penguins. The date of capture and the stage of breeding (not breeding, incubating, or with chicks) did not influence body mass (Colombelli-Négrel 2016a).

**TABLE 1.** Sample sizes of male and female Little Penguins for call recordings and playback experiments across the 4 studied colonies in South Australia (2013–2015).

	Males	Females	Total
Agonistic-call recordings ( <i>n</i> = 70)			
Troubridge Island	15	14	29
Emu Bay	8	12	20
Kingscote	5	9	14
Granite Island	4	3	7
Advertising-call recordings ( <i>n</i> = 76)			
Troubridge Island	18	14	32
Emu Bay	12	8	20
Kingscote	6	8	14
Granite Island	6	4	10
Agonistic-call playback experiments ( <i>n</i> = 82)			
Troubridge Island	17	25	42
Emu Bay	6	8	14
Kingscote	7	7	14
Granite Island	6	6	12
Advertising-call playback experiments ( <i>n</i> = 96)			
Troubridge Island	30	20	50
Emu Bay	8	11	19
Kingscote	5	5	10
Granite Island	10	7	17
Control playback experiments ( <i>n</i> = 24)			
Troubridge Island	5	5	10
Emu Bay	3	3	6
Kingscote	2	2	4
Granite Island	2	2	4

### Playback Experiments

We conducted playback experiments between August and October in 2014 and 2015 and tested the responses of 202 adults to male calls (102 individuals from Troubridge Island, 39 from Emu Bay, 28 from Kingscote, and 33 from Granite Island). We tested the individuals (both males and females) with either (1) advertising calls of a male Little Penguin (*n* = 96), (2) agonistic calls of a male Little Penguin (*n* = 82), or (3) contact calls of a Cory's Shearwater (*Calonectris borealis*)—a seabird that does not occur in Australia—as a control (*n* = 24).

We prepared a total of 43 different stimulus tracks (17 advertising-call tracks, 19 agonistic-call tracks, and 7 control tracks). Each track comprised 5 min of pre-playback silence (pre-trial) followed by 5 min of playback (trial) and 5 min of silence (post-trial). The 5 min of each trial consisted of 10 evenly spaced calls (every 30 s). The 10 calls were created using 3 different calls from the same individual presented in different order. For the control stimuli, we used calls from Cory's Shearwaters sourced from Xeno-Canto (<http://www.xeno-canto.org/species/Calonectris-borealis>). We used 7 sets of 3 calls (total 21 calls) from each of 7 individual Cory's Shearwaters. For the Little Penguin stimuli, we used calls from previously

recorded individuals (recorded at 24 kHz sampling rate, 16-bit depth as described above) that were distinct and without overlapping sound. We used advertising calls from 22 different males (4 from Granite Island and 6 for each of the other colonies) and agonistic calls from 15 different males (3 from Granite Island and 4 for each of the other colonies). We used calls from all 4 colonies to create our playback tracks and tested all colonies with calls originating from all colonies. For example, individuals on Troubridge Island were tested with calls from Troubridge Island, Granite Island, Emu Bay, and Kingscote. For all stimuli, we filtered out sounds <1.5 kHz and normalized playbacks at −15 db in Amadeus Pro. All files were saved as uncompressed 16-bit broadcast wave files (.wav). We then transferred these stimuli onto an Apple iPod connected to a Moshi Bassburger speaker (Moshi, San Francisco, California, USA; sensitivity >80 db; frequency response 280 Hz to 16 kHz).

We carried out all playback experiments in the evenings during the first 2 hr after sundown. All tested burrows had only one individual at the beginning of the experiment. Each adult was presented with only one selected stimulus track and therefore was tested only once. We selected the stimulus tracks randomly but ensured that we used agonistic or advertising calls of a male living at least several territories away from the tested individual to avoid any effect of familiarity. We audio recorded all experiments using a Zoom Handy Recorder H4n placed outside the burrows as described above. We played all playback tracks at the same volume ( $\sim 83 \pm 1.0$  db at 1 m), which was equivalent to a naturally calling Little Penguin ( $\sim 85$  db at 1 m; Miyazaki and Waas 2002, 2005, Mouterde et al. 2012). For each experiment, we noted the following: (1) the latency (in seconds) to respond during the trial and (2) the number of advertising and agonistic calls produced during the entire monitoring period (pre-trial, trial, and post-trial; total = 15 min).

### Sample-size Limitation

Because of local population declines on Kangaroo and Granite islands, sample size in some of the colonies was limited by the number of Little Penguins present at the time; hence, sample size was uneven between the colonies. During the study, the numbers of adult Little Penguins estimated to be present on each island were as follows: 300–1,000+ on Troubridge Island (Bool and Wiebkin 2013, Colombelli-Négrel 2016b); 42–70 at Emu Bay (Colombelli-Négrel 2016b); 18–32 at Kingscote (Hospital Beach; Colombelli-Négrel 2016b); and 22–38 on Granite Island (Colombelli-Négrel 2015, 2016b). Sample size was further limited because some Little Penguins did not vocalize during our study, as found in other studies (e.g., Waas 1991a). Sample sizes are presented in Table 1.

## Statistical Analysis

We carried out all statistical analyses using SPSS/PASW 22.0 for Windows (IBM, Armonk, New York, USA). Results are presented as means  $\pm$  SE. We analyzed agonistic and advertising calls separately. Multiple comparisons were corrected with Bonferroni adjustments, and  $\eta^2$  (defined as the proportion of variance attributed to an effect) was used as a measure of effect size (Thompson 2006, Steyn and Ellis 2009). We used multivariate analysis of variance (MANOVA) on all calls to assess the influence of “individuality” vs. “sex” and obtain  $F$ -ratios. Large  $F$ -ratios represent greater between- than within-group variability (Campbell 1989). We then averaged calls per individuals and combined sexes to analyze differences between the colonies. We performed a MANOVA with colony and sex as fixed factors and used Bonferroni post hoc tests to identify which of the pairwise comparisons were significantly different. We then carried out a discriminant function analysis (DFA) on the averaged calls with a leave-one-out cross-validation method to quantify the extent to which individuals could be classified to their colony of origin on the basis of their calls. We calculated Euclidean distances for all pairwise combinations of colonies using the acoustical data. We then used Mantel’s test to examine independence between the acoustic and geographic distance matrices using XLSTAT 2015.4.01 (Addinsoft, New York, New York, USA). We used a MANOVA to assess the relationship between microhabitat and call characteristics. We reduced call parameters using principal component analysis (PCA) and used linear regressions (weighted by sex) to test the relationship between body-size measurements (head length and body mass) and PCA factors.

We used MANOVAs to test for differences in pre-trial vocalizations in relation to playback type (advertising calls, agonistic calls, or control) and found no significant difference in the pre-trial vocalizations (all  $P > 0.67$ ). Therefore, pre-trial vocalizations were excluded from further analysis (data available from the author on request). We also used MANOVA to test for differences in playback response (latency and numbers of advertising and agonistic calls produced during the trial and the post-trial periods) in relation to breeding stage (not breeding, incubating, or with young chicks). Because we found no significant difference in playback response in relation to the breeding stage of the individuals (all  $P > 0.05$ ), all data were combined for the remainder of the analysis. We used independent  $t$ -tests to compare playback response (latency and numbers of advertising and agonistic calls for trial and post-trial periods) to Little Penguin call stimuli vs. control. Because Little Penguins responded more strongly to Little Penguin calls (see below), we excluded responses to control stimuli from the analyses and focused on responses to conspecific calls. Responses to advertising and agonistic

calls were then analyzed separately. We used generalized linear mixed models (GLMM) to analyze playback response, with sex and colony of the tested individual and colony source of the playback calls as fixed effects and individual and playback track as random effects.

## RESULTS

### Acoustic Variation in Calls

**Agonistic calls.** We analyzed a total of 198 agonistic calls ( $3 \pm 0.19$  calls individual<sup>-1</sup>) from 70 adults (32 males and 38 females) across the 4 colonies. Agonistic calls differed significantly between individuals and sexes: Males produced agonistic calls with lower minimum frequencies, higher maximum frequencies, and broader bandwidths than females (Table 2). Comparison of  $F$ -ratios showed that the effect of “individual” was always stronger than the effect of “sex,” except for the maximum frequency (Table 2). There was no effect of the interaction term sex\*colony on any of the agonistic-call characteristics (MANOVA, all  $P > 0.08$ ). Agonistic calls differed between the colonies only in minimum frequency, with individuals on Granite Island and at Emu Bay having the lowest minimum frequencies compared to the other colonies. Mean values ( $\pm$  SE) of the call characteristics between colonies as well as the MANOVA and post hoc test results are presented in Table 3. The DFA revealed significant differences in agonistic calls between the 4 colonies (Wilks’s  $\lambda = 0.76$ ,  $P < 0.0001$ ). Cross-validated DFA classified 39% of agonistic calls to the correct colony, which was higher than the percentage of correct classification by chance (i.e.  $1/4 = 25\%$ ; Table 4).

**Advertising calls.** We analyzed a total of 293 advertising calls ( $4 \pm 0.19$  calls individual<sup>-1</sup>) from 76 adults (41 males and 35 females) across the 4 colonies. Advertising calls differed significantly between individuals, and males produced advertising calls with higher dominant exhalation frequencies than females (Table 2). Comparison of  $F$ -ratios showed that the effect of “individual” was always stronger than the effect of “sex,” except for the dominant exhalation frequency (Table 2). There was no effect of the interaction term sex\*colony on any of the advertising-call characteristics (MANOVA, all  $P > 0.15$ ). Advertising calls did not differ between the colonies (all  $P > 0.05$  after Bonferroni corrections).

### Potential Causes of Acoustic Divergence

**Agonistic calls.** We found no significant correlation between differences in agonistic-call characteristics and the geographic distances between the colonies (Mantel test:  $r = 0.41$ ,  $P = 0.37$ ). However, we found that Little Penguins breeding in open habitat produced agonistic calls with lower minimum frequencies than those breeding in mixed or closed habitat ( $F_{2,69} = 12.57$ ,  $P < 0.0001$ ,  $\eta^2 =$



**TABLE 2.** Results from MANOVA analysis testing the influence of “individuality” and “sex” on the agonistic-call and advertising-call characteristics of Little Penguins in the sampled colonies in South Australia (2013–2015). Large *F*-ratios represent greater between- than within-group variability. *P* values in bold were significant after Bonferroni correction.

Call parameter	<i>F</i> -ratio	<i>P</i>	Eta <sup>2</sup>	<i>F</i> -ratio	<i>P</i>	Eta <sup>2</sup>
Agonistic calls						
	Individuality ( <i>F</i> <sub>69, 197</sub> )			Sex ( <i>F</i> <sub>1, 197</sub> )		
Duration (s)	23.49	< <b>0.0001</b>	1.00	3.72	0.05	0.48
Minimum frequency (Hz)	15.31	< <b>0.0001</b>	1.00	14.93	< <b>0.0001</b>	0.97
Maximum frequency (Hz)	14.48	< <b>0.0001</b>	1.00	18.60	< <b>0.0001</b>	0.99
Frequency bandwidth (Hz)	18.72	< <b>0.0001</b>	1.00	7.14	<b>0.01</b>	0.76
Dominant frequency (Hz)	4.53	< <b>0.0001</b>	1.00	0.03	0.87	0.05
Advertising calls						
	Individuality ( <i>F</i> <sub>75, 292</sub> )			Sex ( <i>F</i> <sub>1, 292</sub> )		
Total duration (s)	8.52	< <b>0.0001</b>	1.00	0.02	0.89	0.05
Minimum frequency exhalation (Hz)	42.67	< <b>0.0001</b>	1.00	0.002	0.97	0.05
Maximum frequency exhalation (Hz)	14.92	< <b>0.0001</b>	1.00	1.96	0.16	0.29
Bandwidth exhalation (Hz)	13.53	< <b>0.0001</b>	1.00	2.15	0.14	0.31
Dominant frequency exhalation (Hz)	20.01	< <b>0.0001</b>	1.00	20.13	< <b>0.0001</b>	0.99
Duration exhalation (s)	6.36	< <b>0.0001</b>	1.00	0.62	0.43	0.12
Minimum frequency inhalation (Hz)	11.60	< <b>0.0001</b>	1.00	3.83	0.05	0.50
Maximum frequency inhalation (Hz)	10.50	< <b>0.0001</b>	1.00	3.69	0.06	0.48
Bandwidth inhalation (Hz)	7.46	< <b>0.0001</b>	1.00	1.33	0.25	0.21
Dominant frequency inhalation (Hz)	7.18	< <b>0.0001</b>	1.00	4.85	0.05	0.59
Duration inhalation (s)	10.46	< <b>0.0001</b>	1.00	5.41	0.05	0.64

0.99; all other *P* > 0.05 after Bonferroni corrections). The PCA provided 2 components for agonistic calls with eigenvalues >1, which explained 66% of the variance (PC1: Maximum Frequency accounted for 45% of the variance, and PC2: Minimum Frequency accounted for 21%; Table 5A). Larger and heavier individuals produced agonistic calls at higher maximum frequencies and with larger bandwidths than smaller individuals (PC1: Maximum Frequency and body mass: *t* = 2.28, *P* = 0.04; PC1: Maximum Frequency and head length: *t* = 2.74, *P* = 0.02; PC2: Minimum Frequency: all *P* > 0.97).

**Advertising calls.** We found no significant correlation between differences in advertising-call characteristics and the geographic distances between the colonies (Mantel test: *r* = 0.83, *P* = 0.06). Advertising-call characteristics did not vary between the different habitats (all *P* > 0.05 after

Bonferroni corrections). The PCA provided 3 components for advertising calls with eigenvalues >1, which explained 83% of the variance (PC1: Frequency Exhalation accounted for 49% of the variance, PC2: Duration accounted for 23.5%, and PC3: Minimum Frequency Inhalation accounted for 10.5%; Table 5B). Neither of the body-size measurements (head length and body mass) were correlated with advertising-call characteristics (all *P* > 0.15).

### Playback Experiments

We tested a total of 202 adults in playback experiments (96 with advertising calls, 82 with agonistic calls, and 24 with the control). Overall, individuals responded faster (*t*-test: *t* = −3.21, *df* = 29, *P* = 0.003) and produced more advertising calls (*t* = 2.010, *df* = 50, *P* = 0.04) and agonistic calls (*t* = 2.47, *df* = 53, *P* = 0.02) in response to Little Penguin calls

**TABLE 3.** Mean values (± SE) of all the acoustic variables measured for Little Penguin agonistic calls for each sampled colony in South Australia (2013–2015), MANOVA results, and Bonferroni post hoc comparisons. *P* values in bold were significant after Bonferroni correction.

Colony ( <i>n</i> individuals)	Duration (s)	Minimum frequency (Hz)	Maximum frequency (Hz)	Frequency bandwidth (Hz)	Dominant frequency (Hz)
Troubridge Island (29)	2.2 ± 0.1	121.9 ± 3.4	922.0 ± 29.1	800.2 ± 28.0	368.3 ± 31.7
Emu Bay (20)	2.1 ± 0.2	91.9 ± 6.6	963.7 ± 42.8	871.8 ± 41.1	407.4 ± 38.1
Kingscote (14)	1.9 ± 0.2	115.3 ± 5.6	965.3 ± 51.7	849.9 ± 49.5	404.1 ± 37.9
Granite Island (7)	2.0 ± 0.3	89.1 ± 18.2	1,046.1 ± 133.5	957.0 ± 129.4	326.4 ± 44.4
MANOVA	<i>F</i> = 1.12	<i>F</i> = 6.77	<i>F</i> = 0.70	<i>F</i> = 1.37	<i>F</i> = 0.74
	<i>P</i> = 0.35	<b><i>P</i> = 0.001</b>	<i>P</i> = 0.55	<i>P</i> = 0.26	<i>P</i> = 0.53
	Eta <sup>2</sup> = 0.29	Eta <sup>2</sup> = 0.97	Eta <sup>2</sup> = 0.19	Eta <sup>2</sup> = 0.35	Eta <sup>2</sup> = 0.20
Post hoc tests (only significant differences are shown) <sup>a</sup>		EB–TB GI–TB			

<sup>a</sup>In the post hoc results, the colony that is being compared to the other colonies is on the left and a colony that is significantly different from that colony is on the right (abbreviations: EB = Emu Bay, TB = Troubridge Island, GI = Granite Island).

**TABLE 4.** Assignments (%) from the discriminant function analysis of Little Penguin agonistic calls to the 4 sampled colonies in South Australia (2013–2015).

	Emu Bay	Granite Island	Kingscote	Troubridge Island
Emu Bay	15.0	45.0	25.0	15.0
Granite Island	28.6	14.3	28.6	28.6
Kingscote	14.3	7.1	21.4	57.1
Troubridge Island	3.4	6.9	20.7	69.0

than to the control. Therefore, we focused on responses to conspecific-call playback only.

**Agonistic calls.** We tested 82 individuals (36 males and 46 females) with playback of Little Penguin agonistic calls. We found no effect of sex, colony, colony source of the playback calls, or their interaction terms on the latency response or the number of advertising and agonistic calls produced in the post-trial period (GLMM: all  $P > 0.08$ ). There was a residual effect of individual and playback track on latency ( $P < 0.0001$ ) and the number of agonistic calls produced in the post-trial period ( $P < 0.0001$ ), but not on the number of advertising calls produced in the post-trial period ( $P = 0.70$ ). We found an effect of sex ( $F_{1,59} = 11.87$ ,  $P = 0.001$ ) and sex\*colony ( $F_{1,59} = 3.67$ ,  $P = 0.02$ ) on the number of advertising calls produced in the trial period (all other  $P > 0.10$ ): Males responded to the agonistic-call playback with more advertising calls than females, particularly at Emu Bay and Kingscote. We found an effect

of sex ( $F_{1,59} = 4.71$ ,  $P = 0.03$ ) and colony ( $F_{1,59} = 2.78$ ,  $P = 0.05$ ) on the number of agonistic calls produced in the trial period (all other  $P > 0.15$ ): Males responded to the agonistic-call playback with more agonistic calls than females, and individuals from Emu Bay produced more agonistic calls than individuals from the other colonies. There was a residual effect of individual and playback track on the number of agonistic calls produced in the trial period ( $P < 0.0001$ ), but not on the number of advertising calls ( $P = 0.51$ ).

**Advertising calls.** We tested 96 individuals (43 males and 53 females) with playback of Little Penguin advertising calls. Males produced more agonistic calls than females in the post-trial period in response to playback of advertising calls (GLMM:  $F_{1,73} = 3.97$ ,  $P = 0.05$ ). We found no effect of sex, colony, colony source of the playback calls, or their interaction terms on any of the other playback responses (all  $P > 0.11$ ). There was a residual effect of individual and playback track on latency and on the number of agonistic and advertising calls produced in the trial period (all  $P < 0.0001$ ), but not on the number of agonistic and advertising calls produced in the post-trial period (all  $P > 0.54$ ).

## DISCUSSION

We found that Little Penguin agonistic calls, but not advertising calls, showed microgeographic variation, which

**TABLE 5.** Factor loadings from principal component analysis (PCA) of Little Penguin (A) agonistic-call and (B) advertising-call characteristics. High PCA scores indicate larger parameters (in bold). Eigenvalues and percentages of the variance explained by each factor are given in parentheses.

(A)			
	PC1: Maximum Frequency (2.24, 45%)	PC2: Minimum Frequency (1.06, 21%)	
Agonistic-call parameters			
Minimum frequency	0.292	− <b>0.813</b>	
Maximum frequency	<b>0.958</b>	0.003	
Frequency bandwidth	<b>0.932</b>	0.138	
Dominant frequency	0.309	<b>0.597</b>	
Duration	− <b>0.525</b>	0.149	
(B)			
	PC1: Frequency Exhalation (5.40, 49%)	PC2: Duration (2.58, 23.5%)	PC3: Minimum Frequency Inhalation (1.13, 10.5%)
Advertising-call parameters			
Total duration	−0.084	<b>0.961</b>	0.216
Minimum frequency exhalation	<b>0.715</b>	−0.258	−0.225
Maximum frequency exhalation	<b>0.853</b>	0.265	−0.272
Bandwidth exhalation	<b>0.828</b>	0.300	−0.265
Dominant frequency exhalation	<b>0.757</b>	0.158	−0.233
Duration exhalation	−0.371	<b>0.878</b>	0.002
Minimum frequency inhalation	0.639	−0.385	<b>0.683</b>
Maximum frequency inhalation	<b>0.932</b>	−0.091	0.058
Bandwidth inhalation	<b>0.764</b>	0.125	−0.288
Dominant frequency inhalation	<b>0.798</b>	−0.135	0.507
Duration inhalation	0.511	<b>0.665</b>	0.384

was best explained by differences in microhabitat. Both agonistic-call and advertising-call characteristics differed between individuals and sexes, with males and larger individuals producing calls at higher frequencies than females or smaller individuals. During playback experiments, males were more vocal than females. However, resident birds did not discriminate between calls originating from different colonies, which suggests that advertising and agonistic calls do not serve as an isolating mechanism between the 2 previously identified genetic populations.

Microgeographic variation in bird vocalizations can have important implications for species conservation. In songbirds, song dialects can act as a reproductive isolating mechanism between populations and generates novel phenotypes (e.g., Greig and Webster 2013). Such a mechanism would be of great concern in small or declining species, by furthering dividing populations into smaller units when they might otherwise have been considered large enough for conservation purposes. Although previous genetic analyses suggested that Australian Little Penguins form a single clade (Banks et al. 2002, 2008, Peucker et al. 2009), more recent studies have shown subtle but significant genetic differentiation within the South Australia populations (BurrIDGE et al. 2015, Graff 2015). Given that some of these populations have declined significantly over recent decades (Wiebkin 2011, Colombelli-Négrel 2015, 2016b), there are concerns as to whether additional management measures may be needed to preserve behaviorally, morphologically, or genetically unique phenotypes. In the present study, we found some microgeographic variation in South Australian Little Penguin agonistic calls. However, the DFA analysis assigned only 39% of agonistic calls to the correct colony, which suggests some overlap between the colonies (Table 2). Individuals from Troubridge Island had the highest assignment scores, with 69% of the individuals from Troubridge Island assigned to Troubridge Island. But for all the other colonies, the percentage of individuals that were not correctly assigned to their own colony was higher than the percentage of individuals classified to the correct colony: 45% of the individuals from Emu Bay were classified to Granite Island, 29% of the individuals from Granite Island were classified to Emu Bay, and 57% of the individuals from Kingscote were classified to Troubridge Island (Table 2). Such overlap is expected when there is significant call variation between individuals within colonies (Bretagnolle and Genevois 1997) or when calls are fairly simple in structure, purely because the combination of acoustic parameters between individuals is limited (see Ahonen et al. 2014). In addition, we found that individuals did not discriminate between agonistic calls on the basis of their geographic origin. Such lack of response may be biased by the significant call overlap between the colonies or simply indicate that Little

Penguins are tolerant toward regional immigrants, which is further supported by the presence of gene flow between the colonies (Graff 2015) and would support the idea that there is no reproductive isolating mechanism between the 2 genetic populations previously identified. However, the present study analyzed the combined responses of males and females, and females may have been responding mainly to defend their territory and not to potential mates. Thus, we cannot state whether females would still not preferably select local males as mates (hence creating reproductive isolating mechanisms), an issue that would be worth pursuing in future experiments.

Contrary to the study by Miyazaki and Nakagawa (2015) that showed macrogeographic variation in male advertising calls between 2 Little Penguin subspecies living >1,000 km apart, we did not find any geographic variation in advertising calls. The fact that advertising and agonistic calls show different scales of variation (macro vs. micro) suggests that they may be under different selective pressures. Previous studies have shown that acoustic characteristics and the amount of diversification of vocalizations can depend on their function (Marler 1955; reviewed in Otte 1974). For example, vocalizations that are used in mate attraction have presumably evolved under selection pressures to maximize transmission over long distances (e.g., Brenowitz 1982, Robisson et al. 1993). In Little Penguins (and other penguin species), agonistic calls are used both before and after pairs engage in mutual displays (Waas 1988a), as well as in threats during defensive displays and high-risk aggressions in territory defense (Jouventin 1982, Waas 1990, 1991a, Favaro et al. 2014); whereas advertising calls are used by individuals to defend their territory against intruders and advertise the results of intraspecific agonistic encounters (Jouventin 1982, Waas 1990, Mouderde et al. 2012, Favaro et al. 2014) and are also used by males to court females (Waas 1988a, 1990, 1991a). Therefore, advertising calls seem to function principally to attract mates and advertise quality—and hence they could be under strong intersexual selection for mate choice. Agonistic calls, by contrast, seem to function principally in territory defense and to maintain the pair bond—and thus would be more likely under intrasexual selection.

Studies investigating call variation in birds generally found that male calls were more distinct than those of females (e.g., Zann 1984, Bretagnolle and Genevois 1997) and that males and females encoded distinctiveness within their calls using different characteristics (Dentressangle et al. 2012). Our results were similar to those of other studies on Little Penguins (Jouventin and Aubin 2000, Miyazaki and Waas 2003a) and on other penguin species (e.g., Aubin and Jouventin 2002a, Favaro et al. 2015, 2016) in finding that both males and females produce highly individualized calls. We also found that they used similar cues to encode

distinctiveness within their calls, but that male Little Penguins produced calls at higher frequencies than females. Such sex difference in frequencies seems to be a common characteristic in seabirds (Taoka et al. 1989, Aubin et al. 2007, Cure et al. 2009). Frequencies are important cues for sexual selection and mate recognition (Taoka and Okumura 1990, Miyazaki and Waas 2003a), and it has been suggested that they are mainly due to differences in body size and syrinx structure (Ryan and Brenowitz 1985, Ballintijn and Cate 1997, Barbraud et al. 2000). However, our results also show that the individuality information encoded within the calls generally varied more within individuals (and hence was predominant) compared to the sex information (see Table 2). To our knowledge, only 2 other studies have reported similar results: one in a songbird species, the Superb Fairywren (*Malurus cyaneus*; Colombelli-Négrel and Evans 2017), and one in a squirrel species, the yellow-bellied marmot (*Marmota flaviventris*; Blumstein and Munos 2005). In Superb Fairywrens, calls varied between individuals but not between sexes, and it was suggested that individuality information allowed individuals to adjust their investment in relation to their levels of relatedness with other conspecifics because most individuals within a population were somewhat related (Colombelli-Négrel and Evans 2017). In yellow-bellied marmots, calls varied between individuals and sexes, but individuals paid more attention to the individuality information when responding to playback, supposedly as a mean to assess and remember callers' reliability (Blumstein and Munos 2005). The ability to discriminate individuals plays an important role for social behaviors (Tibbetts and Dale 2007, Wiley 2013), especially in long-lived and colonial birds such as penguins (e.g., Jouventin et al. 1999, Lengagne et al. 1999, Jouventin and Aubin 2002). In long-lived species, individual recognition can help mediate interactions with conspecifics by increasing the recognition of long-term mating partners (Carter and Roberts 1997, Jouventin and Aubin 2002) or reducing aggressive interactions between neighbors (Godard 1991, Waas 2006), thus saving time for other activities. Such recognition seems to develop early in a penguin's life (Nakagawa et al. 2001, Aubin and Jouventin 2002b, Jouventin and Aubin 2002), potentially to allow sufficient time to learn about other individuals within a colony (see Waas 2006). Hence, in Little Penguins, individuality information may be more important to signal to conspecifics than sex information. Conspecifics may also inherently extract sex information from the individuality information, or pay attention to other characteristics of the calls that specifically signal sex information when individuals are unfamiliar. For example, the *F*-ratios for the agonistic calls showed that the impact of "sex" was higher than "individual" on the maximum frequencies, indicating that this call characteristic might be the most useful in

distinguishing between sexes. For Little Penguin advertising calls, Miyazaki and Nakagawa (2015) suggested that some call characteristics may be used to encode information for mate choice, while others may be used to encode other information, such as geographic or sex variation. Combined, our results therefore highlight the need to further explore how seabirds encode different information within their calls.

In seabirds, geographic variation in calls is expected to be shaped by evolutionary and ecological factors, in particular genetic drift (Bretagnolle and Genevois 1997, Budka et al. 2014) or variation in the morphological characteristics of individuals (Barbraud et al. 2000, Miyazaki and Waas 2003b). In Little Penguins, individuals from colonies located farther from each other showed greater morphological (Colombelli-Négrel 2016a) and genetic (Graff 2015) divergence than adjacent colonies. By contrast, geographically more distant individuals did not have more pronounced differences in their calls. This may be due to our small sample size (only 4 colonies) or to the lack of discrimination of foreign calls exhibited by the individuals, which remains to be tested further. Moreover, the relationship between divergence in vocalizations and geographic distances in Little Penguins may not follow a straight and continuous pattern (as expected for clinal divergence) but instead follow a radiation divergence pattern as seen for other island systems (e.g., Grant and Grant 2002, Blackburn et al. 2013, Warren et al. 2016). Troubridge Island is believed to be a recent island (see Wiebkin 2010, Burridge et al. 2015). During 2 visits in the early 1900s, the prominent ornithologist S. A. White did not record any presence of Little Penguins on Troubridge Island, despite taking careful notes on all the birds present there (see Dann 2016). The earliest South Australian Museum records of Little Penguin presence on Troubridge Island are from 1982. This suggests that Troubridge Island was colonized after Granite and Kangaroo islands, and it could also perhaps explain why individuals from Troubridge Island had the highest call-assignment scores (69% compared to <22% for the other colonies).

Furthermore, given that Little Penguin agonistic-call structure is influenced by bill size, body mass (Miyazaki and Waas 2003b, present study), and habitat (present study), the observed effects of distance could be reduced. Indeed, we found that morphological variation between individuals influenced agonistic-call (but not advertising-call) characteristics. Specifically, larger and heavier individuals (regardless of sex) produced calls at higher frequencies (maximum frequencies and bandwidths) than smaller ones, similar to what was found by Miyazaki and Waas (2003b). In many species, the frequency of vocalizations is correlated negatively with body size (Reby and McComb 2003, Hall et al. 2013, Linhart and Fuchs 2015, Favaro et al. 2017). However, such a relationship is not



always consistent and has been found to be weak or nonexistent in others species (Cardoso and Mota 2007, Sanvito et al. 2007, Cardoso et al. 2008, Peters et al. 2009). Little Penguins settle disputes by using an “escalation process” in which individuals increase their aggressive behaviors—generally by increasing the intensity and amplitude of their calls, grading from agonistic to advertising calls—until one retreats (Waas 1991a, 2006). Therefore, Waas (2006) suggested that the higher frequencies of the larger individuals may reflect a stronger motivational state or willingness to escalate disputes, as found in other bird species in which more dominant or aggressive males produced vocalizations at higher frequencies (Nelson 1984, Leonard and Horn 1995, Araya-Ajoy et al. 2009). It should also be noted that agonistic calls’ maximum frequencies and bandwidths did not differ between the colonies, but differed between individuals and sexes. This suggests that the impact of morphological variation between individuals on acoustic variation in Little Penguins may be more pronounced at an individual level than at the colony level.

Environmental factors can also be powerful drivers of acoustic differentiation in birds (Brumm and Naguib 2009). Studies in songbirds have shown that individuals living in habitats with dense vegetation produced calls with lower frequencies than those living in open habitats that were likely to carry signals over long distances (acoustic adaptation hypothesis; reviewed in Boncoraglio and Saino 2007). Very little is known about the impact of habitat on call variation in seabirds. Here, we found that microgeographic variation in agonistic calls was influenced by variation in microhabitat: Little Penguins breeding in open habitats produced calls with lower minimum frequencies than those breeding in closed habitats. This contrast with the results in songbirds may be explained by the fact that agonistic calls are used in close-distance encounters, either during territory defense or within pairs (Waas 1988a, 1990, 1991a), and not over long distances, and hence could be under different selection pressures from the environment. Additionally, variation in minimum frequencies may have been influenced by variation in abiotic noises (such as sea waves and wind) and biotic differences (such as individual density). Indeed, it has been shown that microgeographic variation in calls may arise as a consequence of social interactions among males or territory occupancy (Rek 2013; see also Budka and Osiejuk 2017). Troubridge Island is a small, sandy island covered with dense boxthorn bushes and one of the largest Little Penguin colonies in South Australia. By contrast, the other colonies (particularly Kingscote and Granite Island) are a lot more open and smaller and tend to have individuals widespread along much larger distances. Therefore, differences in calls may be explained by differences in penguin density, which could be exacerbated by differences in ambient noise because individuals need to

compete with both conspecifics and environmental noises. In support of this idea, agonistic calls from Troubridge Island (the loudest colony) also had the highest minimum frequencies. Low frequencies are also particularly effective in aggressive contexts (Bradbury and Vehrencamp 1998, Fitch et al. 2002, Cardoso 2012), and male seabirds in better body condition generally have lower call frequencies (Mager et al. 2007, Marks et al. 2010, Klenova et al. 2011). Therefore, males in better condition may have secured burrows in more open habitat, perhaps to enhance vigilance for predators or competitors.

In conclusion, the results presented here increase our understanding of the mechanisms behind seabird population differentiation and of the factors influencing this process. To our knowledge, variation in vocalizations over a few kilometers has mainly been reported in passerines (e.g., Leader et al. 2000, MacDougall-Shackleton and MacDougall-Shackleton 2001) and in one seabird species (Bretagnolle and Genevois 1997). There is therefore a critical need for additional studies investigating these questions across more seabird species. Our results also highlight the necessity of investigating an array of parameters (such as dispersal capacity, site fidelity, body size, and/or environmental features) to fully understand geographic variation in calls in non-vocal-learning bird species.

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**Data deposits:** Data are deposited in Flinders University share drive.

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