

**Gentoo Penguin Behavioral Ecology:
Vocalizations, Aggression, and Stress within the Colony**

A Dissertation Presented

by

Maureen Anne Lynch

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in Partial Fulfillment of the

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in

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Abstract of the Dissertation

Gentoo Penguin Behavioral Ecology:

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Colonial species face a unique set of costs and benefits, where mate bonding and territory defense are necessary for success in a system without a defined social structure or cooperative actions. Using gentoo penguins as study species, I examined how colony structure affects behavior, examining both interactions within the colony and differences between colonies across a broad geographic range. I addressed these questions using observations of vocalizations and agonistic behaviors, as well as measures of corticosterone stress responses. Results show that even though penguins have no known social group structure, interactions within a colony shape a large amount of individual behavior, and that these intra-colony effects may shape regional variation. Vocal differentiation between individuals is seen at the local level, which drives random cultural drift when comparing at regional scales. Individuals wait for a quiet pause before producing an ecstatic display call while group calling behavior, which I found to be similar in form but acoustically distinct, does not observe this pause. Conspecific encounters are

frequent and usually result in aggressive reactions, which are influenced by how many previous interactions an individual has had. Though there is much concern that tourism activity may have negative consequences for gentoo penguin colonies, some of which are among the most heavily visited in the Antarctic, I found no evidence of an increase in hormonal stress due to tourism activities, but a high degree of individual variation. These results provide a more nuanced picture of gentoo penguin behavior within the colony, with important implications for other colonial seabird species.

Table of Contents

List of Figures.....	vi
List of Tables.....	vii
Acknowledgments.....	viii
1 – Introduction	1
2 – Variation in the ecstatic display call of the gentoo penguin across regional geographic scales	7
2.1 - Abstract.....	7
2.2 - Introduction	8
2.3 - Methods	13
2.4 - Results	19
2.5 - Discussion.....	25
3 – Patterns of vocal behavior in gentoo penguin colonies	28
3.1 - Abstract.....	28
3.2 - Introduction	28
3.3 - Methods	30
3.4 - Results	35
3.5 - Discussion.....	39
4 – Conspecific aggression in gentoo penguin colonies	41
4.1 - Abstract.....	41
4.2 - Introduction	41
4.3 - Methods	44
4.4 - Results	51
4.5 - Discussion.....	59
5 – Tourism and stress hormone measures in gentoo penguins on the Antarctic Peninsula	63
5.1 - Abstract.....	63
5.2 - Introduction	64
5.3 - Methods	67
5.4 - Results	73
5.5 - Discussion.....	77
6 – Conclusion	80
Bibliography.....	83

List of Figures

Figure 2-1: Sampling locations on the Antarctic Peninsula, South Georgia, the Falkland Islands, and Argentina.....	11
Figure 2-2: Spectrograms of the ecstatic display call of the gentoo penguin.....	12
Figure 2-3: Principal Components Analysis of acoustic measurements of ecstatic display calls...21	
Figure 2-4: Boxplots demonstrating variation at each site in 5% frequency center frequency based on latitude and relative distance.....	22
Figure 3-1: Visualization of calls in time, histograms of pause duration prior to any given call, and the calculated L statistic following any given call..	37
Figure 3-2: Principal Components Analysis of acoustic measurements of individual ecstatic display calls compared to group calls..	38
Figure 4-1: Plot of focal subject encounter rate and reaction frequency.....	54
Figure 4-2: Spectrograms of an ecstatic display call and two separate agonistic calls from the same individual..	55
Figure 4-3: Principal Components Analysis of acoustic measurements of ecstatic display calls compared to agonistic calls.....	56
Figure 4-4: Histograms of permuted MANOVA F statistics on a suite of acoustic measurements.....	57
Figure 5-1: Sample locations on the Antarctic Peninsula and the South Shetland Islands	71
Figure 5-2: Scatterplot showing IAATO tourist landings and boxplots showing variation in corticosterone concentrations in guano samples.....	75

List of Tables

Table 2-1: Sampling locations, colony abundance, and number of ecstatic calls used in the analysis.	17
Table 2-2: Descriptions of spectrogram measurements used in analysis..	18
Table 2-3: Variable loadings for each acoustic measurement for the first five principle components.	23
Table 2-4: The five most important variables from Random Forest machine learning for subspecies classification.....	24
Table 3-1: Sampling locations for wild gentoo penguin breeding colonies along the Antarctic Peninsula	33
Table 3-2: Descriptions of spectrogram measurements used in analysis.	34
Table 4-1: Names and locations of gentoo penguin breeding colonies, sampling season, and which analyses were performed.....	48
Table 4-2: Categories of gentoo penguin reactions to conspecific encounters, ordered by aggressiveness.	49
Table 4-3: Descriptions of spectrogram measurements used in analysis..	50
Table 4-4: Model results from generalized estimating equations and ordinal logistic regression.	58
Table 5-1: Sampling locations, colony abundance, with number of samples, mean, and standard error of corticosterone concentration at each colony.	72
Table 5-2: Parameter estimates and credible intervals from a Bayesian model relating log-corticosterone concentrations to tourism, population growth, and population abundance.	76

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1 – Introduction

Approximately 95% of all seabird species are colonial (Schrieber and Burger 2002), and the costs, benefits, and evolution of coloniality have long been discussed in the literature (e.g., Hoogland 1979, Danchin and Wagner 1997, Dubois *et al.* 1998, Varela *et al.* 2007, Ashbrook *et al.* 2014, Evans *et al.* 2016). Coloniality may provide defense from predators, easy access to mates, opportunities for group foraging, and social stimuli has been shown to cue and synchronize breeding behaviors. However, breeding in dense aggregations also increases competition for food, territory, and mates, facilitates disease and parasite transmission, and may actually concentrate the effect of predators, particularly introduced species (Schrieber and Burger 2002).

Yet as we continue to examine these various costs and benefits, we find a large degree of nuance. Reproductive success may increase with breeding density (Pratte *et al.* 2016), but may decrease in high densities (Hill *et al.* 1997, Stokes and Boersma 2000). Predation risk may decrease with the size of the group, but larger congregations also show decreased vigilance (Jungwirth 2015). Some species experience reduced territorial interactions when they nest with another species, while their cohabitant species experiences increased territorial interactions in those same mixed colonies (Pius and Leberg 1997). Much of this nuance derives from species-specific life history and behavior, which are often less well understood because they require detailed monitoring of individuals or interactions over time. By increasing our understanding of a species' natural history, we can better understand the balance of costs and benefits of different breeding strategies and, in doing so, address larger-scale ecological or population-level questions important for conservation.

Colonial seabirds provide an ideal system for studying group living behaviors. For this work, I have focused on the behavioral ecology of gentoo penguin (*Pygoscelis papua*). The gentoo penguin is ostensibly a sub-Antarctic species and has a circumpolar distribution on isolated islands in the Southern Ocean, but also breeds on the Western Antarctic Peninsula and South Shetland Islands. In this region, their breeding range overlaps with the two other *Pygoscelis* species, the Adélie (*P. adeliae*) and chinstrap (*P. antarcticus*) penguins. Gentoo penguins nest in relatively small colonies that range from several hundred to several thousand breeding pairs (Lynch 2013).

The breeding colony is a central aspect of gentoo penguin ecology, as adult penguins live in the colony approximately four to five months of the year (longer in more northern regions of their range), and periodically return to the colony year-round (David and Renner 2003). In the spring months, adults will come ashore and begin setting up nests before breeding, the timing of which varies considerably by latitude and region. The adults remain in the colony through the summer for egg incubation (~35 days), chick guarding (hatching to ~30 days old), and chick crèching (~ 30-80 days old). However, unlike the other *Pygoscelis* species, gentoo penguins return to the breeding colony to provision their chicks even after the chicks have fledged, and stay relatively close to the colony in the winter months (Davis and Renner 2003, Lynch 2013). Gentoo penguins have a relatively high degree of colony fidelity and mate fidelity, though the latter is highly variable (Lynch 2013), meaning that mate bonding and interactions with other penguins in the colony are likely to be important behavioral aspects of colonial life.

Gentoo penguins have become a species of interest, as they have been identified as a “climate change winner” in contrast to Adélie and chinstrap penguins, which have declined along the Antarctic Peninsula where the three species overlap. With warming temperatures, decreasing

sea ice, and altered prey distributions due to climate change on the Western Antarctic Peninsula, gentoo penguin populations are increasing and they have expanded their range southward in recent years (Lynch *et al.* 2012). In part due to this increasing population, they have recently been downgraded from Near Threatened to Least Concern by the IUCN (BirdLife International 2018). Gentoo penguins are also exposed to unique pressures as a focus of the rapidly expanding ecotourism industry along the Antarctica Peninsula, which disproportionately overlaps with gentoo penguin breeding areas (Bender *et al.* 2016).

I chose to focus in part on vocalizations, as they are a key aspect of group behaviors and individual behaviors within a group in a wide variety of species. Vocal communication provides a unique individual signature that can be recognized by mates, neighbors, and offspring (Speirs and Davis 1991, Seddon and van Heezik 1993, Tibbetts and Dale 2007, Jouventin and Dobson 2018) and provides a means to be identified in large noisy colonies (Aubin and Jouventin 2002, Searby and Jouventin 2005). Alarm calls provide an early warning system for the group (Griesser 2008) and agonistic vocalizations aid in territory defense when resources are guarded (Renison *et al.* 2006, Viñuela *et al.* 1995). While many bird species have complex vocalizations with multiple song variants, most penguin vocalizations are relatively simple (Jouventin 1982). However, both the basic form and pattern of gentoo penguin vocalizations are understudied compared to other penguin species. Nest defense is critical for reproductive success, and conspecific territorial interactions can be more numerous and more deterministic of reproductive success than predator defenses (Ashbrook *et al.* 2014).

In noisy environments such as a breeding colony, vocal distinctiveness is essential for mate choice, pair bonding, and nest defense. However, the noisier the environment, the more critical it is for individuals to quickly recognize each other (Lengagne *et al.* 1999, Mathevon *et*

al. 2003, Hase *et al.* 2018). For territorial species, geographic variation in vocalizations is common and often associated with geographic isolation (e.g., Wright 1996, Dalisio *et al.* 2015, Shizuka *et al.* 2016) or range expansion (Xing *et al.* 2013), and may potentially occur on very short time scales in response to changes in the environmental soundscape (Rheindt 2003, Villain *et al.* 2016). These vocal differences may be a result of geographic isolation and cultural drift, or they may be important in conveying group membership to the listener (Miyazaki and Nakagawa 2015, Hamao 2016). In philopatric colonial species such as the gentoo penguin, there is a high degree of genetic differentiation between regions, reinforcing this geographic isolation (Levy *et al.* 2016), and previous studies have alluded to variation in vocalizations on very large geographic scales (Jouventin 1982, de Dinechin *et al.* 2012). In order to examine more fine-scale geographic variation, I analyzed recordings from breeding colonies across broad geographic range from Argentina to the Antarctic Peninsula. I used acoustic parameters of the ecstatic display call to assess the variation between individuals as well as between breeding colonies, as a way to understand the relationships between colonial living and individual distinctiveness (Chapter 2).

While individual recognition has been studied in multiple penguin species, including gentoo penguins (Speirs and Davis 1991, Jouventin and Aubin 2002, Jouventin and Dobson 2018), group vocal behavior has not been studied in detail. Mutual display calls between a breeding pair reinforce mate bonding (Jouventin and Dobson 2018), but little description has been given to communication between non-mates or the ways in which individuals modify their behavior in large noisy environments. Many bird species exhibit group vocalizations, either in duets or choruses that can aid in group bonding, territory definition, and anti-predator behavior (Aubin 2004, Griesser 2008, Baker 2009, Colombelli-Négrel and Evans 2017). In some species

individuals have been observed to take turns vocalizing, or otherwise wait for another individual to finish vocalizing (Okobi *et al.* 2019). To explore other behavioral aspects of the “cocktail party problem”, I examined the temporal pattern of ecstatic display calls and group calls, as a means of assessing whether calling behavior was modified following other calls in the colony to avoid signal jamming in a crowded vocal space. To further examine the vocal behavior of an individual within a crowd, I identified a group calling behavior (defined as including three or more individuals) not previously described, and compared the acoustic measurements of the calls precipitating these group responses to those that did not (Chapter 3).

An important aspect of colonial species is that, unlike other group-living species, they do not have a social structure or cooperative breeding, rearing of offspring, or defense. In a densely populated breeding colony, interactions between conspecifics are often hostile and occur more frequently than interactions with predators. While interactions between adults rarely result in injury, these interactions do have energetic costs to both individuals involved, and may result in damage or predation to the egg if one individual stands up or leaves the nest. Given the heterogeneous spatial layout of a gentoo penguin colony, some areas have a higher nest density than others, even on a very fine scale of several meters. To better understand these conspecific interactions, I studied how frequently conspecific encounters resulted in aggressive reactions, and the escalation of those reactions, and what individual factors may predict the level of reaction from a nesting penguin. In the course of this study I also described the acoustic parameters of the agonistic vocalization used in some of the most aggressive encounters, and examined how it varies from the other vocalizations in the vocal repertoire which was previously thought to be quite limited (Chapter 4).

Gentoo penguins face a unique change to their environment, with the rapidly growing Antarctic tourism industry. Only a few decades old, this industry has rapidly expanded and only a few assessments have been made on the short or long term impact to wildlife. While studies in other systems have examined human disturbance to wildlife, including penguins (Nimon *et al.* 1995, Crosbie 1999, Lynch *et al.* 2010, Coetzee and Chown 2016), results have been decidedly mixed depending on the system and the metric used (e.g., van Heezik and Seddon 1990, Holmes *et al.* 2006, Carlini *et al.* 2007, Ellenberg *et al.* 2007, Barbosa *et al.* 2013). The majority of tourist landings are at gentoo penguin breeding colonies, and it follows that gentoo penguins have the potential to be heavily affected by this industry (Bender *et al.* 2016). To examine whether or not tourism intensity has regional impacts, I examined fecal glucocorticoid metabolite levels from non-invasive guano sampling at breeding colonies with a range of tourist visit frequency, including those that are closed to tourism (Chapter 5).

All of these studies were conducted primarily using field observations of wild, unmarked birds. While there are limitations to this type of work, namely that individual's age, sex, and other traits are not known, the benefits to non-invasive observational work are worth emphasizing. My work, as described in the following chapters, gives insight into differences between individuals within the same colony and between different colonies and thus provides a more nuanced look into gentoo penguin behavior and behavioral ecology even in the absence of individual-level demographic information.

2 – Variation in the ecstatic display call of the gentoo penguin across regional geographic scales

2.1 - Abstract

Geographic variation in bird vocalizations is common, and has been associated with genetic differences and speciation, as well as short term changes in response to anthropogenic noise. As vocalizations are used for individual recognition in many species, geographic variation in these traits may affect mate choice, pair bonding, and territory defense. Anecdotal evidence suggests the existence of geographic variation in vocalizations between isolated populations of gentoo penguins (*Pygoscelis papua*), but to date there is no comprehensive study of gentoo penguin vocalizations across a broad geographic range. Here I addressed two main questions regarding gentoo penguin vocalizations: 1) How do ecstatic display calls vary both within and between individuals, colonies, and regions? and 2) Can ecstatic display calls be used to distinguish subspecies? To address these questions, I used acoustic recordings of ambient colony sound at 22 breeding colonies on the Antarctic Peninsula and South Shetland Islands, South Georgia, the Falkland Islands, and Argentina. I found high levels of variation between individuals and between colonies, but little additional variation between regions or subspecies. I found no trends to suggest a latitudinal gradient in vocal characteristics although I did find that some measures varied with relative distance between colonies. Although I found significant differences at the colony level, unknown calls could not easily be categorized to colony or region by machine learning. I conclude that the vocal soundscape of each colony is driven by variation between individuals within a colony and, developing independently from neighboring colonies,

becomes differentiated from other colonies through a process of drift. While individual calls could, in most cases, be identified to subspecies by machine learning, my analysis suggests that subspecies differences may be driven by variation among colonies and that subspecies identification may be unreliable using acoustics alone.

2.2 - Introduction

Vocal communication has been widely studied among birds and is known to be important for mate choice, pair bonding, and territorial defense. In the noisy environment such as a seabird colony, vocal distinctiveness allows mates to recognize each other within a breeding season as well as across seasons (e.g., Aubin and Jouventin 1998, Leader *et al.* 2002, Tibbetts and Dale 2007) and allows individuals to communicate information such as fitness (de Kort *et al.* 2009) and relatedness (McDonald and Wright 2011). Variation in vocal traits may be associated with geographic isolation (e.g., Wright 1996, Dalisio *et al.* 2015, Shizuka *et al.* 2016), speciation (Mulard *et al.* 2009, Pieplow and Francis 2011, Greig and Webster 2013), and range shifting (Xing *et al.* 2013), and can even occur over short time scales in response to anthropogenic noise (Rheindt 2003, Villain *et al.* 2016).

Vocal characteristics in penguins are relatively understudied compared to other bird taxa, but studies have shown gradual interspecies differentiation over time (Thumser *et al.* 1996, Favaro *et al.* 2016). Penguins that build nests or burrows to incubate eggs and chicks can use geographic cues to guide them to their nest, thus their calls may be less complex than those from king or emperor penguins, who use vocalizations to identify a mate or chick within massive, noisy colonies (Searby and Jouventin 2005). However, despite this reduced complexity,

individual recognition has been observed in rockhopper penguins (Searby and Jouventin 2005), Adélie penguins (Speirs and Davis 1991), gentoo penguins (Speirs and Davis 1991, Jouventin and Aubin 2002), African penguins (Seddon and van Heezik 1993, Favaro *et al.* 2016), Magellanic penguins (Clark *et al.* 2006), and macaroni penguins (Searby *et al.* 2004). These studies all indicate that while there may be differences in complexity between species, vocalizations play an important role in behavior across all penguins.

Gentoo penguins are distributed widely across geographically isolated sub-Antarctic islands in the Atlantic, Indian, and Pacific oceans as well as the Antarctic Peninsula (Lynch 2013). In the Atlantic region of their range, the Polar Front creates a strong ecological boundary between populations in Argentina and the Falkland Islands and those in South Georgia, the South Sandwich Islands, the South Orkney Islands, and the Antarctic Peninsula (Figure 2-1). This geographic and ecological isolation, combined with high mate and colony fidelity (Lynch 2013), results in strong population genetic structure between regions (Levy *et al.* 2016). There are currently two described subspecies, originally based heavily on morphology (Stonehouse 1970) and now confirmed with genetics (de Dinechin *et al.* 2012, Levy *et al.* 2016). *Pygoscelis papua papua* lives above the Polar Front in the Falkland Islands, and *Pygoscelis papua ellsworthii* on the Antarctic Peninsula and sub-Antarctic islands below the Polar Front (Levy *et al.* 2016). Gentoo penguins have recently colonized Isla Martillo in the Beagle Channel in Argentina, though their subspecies designation is not yet known. de Dinechin *et al.* (2012) proposed a third subspecies for the sub-Antarctic islands above the Polar Front in the Indian and Pacific Oceans.

The ecstatic display call is the most common contact call used by gentoo penguins and serves to attract and contact mates, though in some cases it is used in the absence of a mate and without obvious provocation; in these situations, its function remains unknown. Regardless, the

ecstatic display call can be easily distinguished from the calls associated with pair bowing as well as the calls of the other *Pygoscelis* spp. penguins (Jouventin 1982). It is characterized by a series of repeated pairs of syllables, each comprised of a long exhale followed by a short inhale with a highly variable number of syllables (Figure 2-2). Prior to recent genetic evidence, several authors noted differences in gentoo penguins across broad ecoregions and included assessments of vocal similarity. Jouventin (1982) notes that while ecstatic display calls are similar between Macquarie Island, Kerguelen Islands, and Crozet Island, these calls differ from those heard in the Falkland Islands, South Orkney Islands, and South Georgia. Both Jouventin (1982) and de Dinechin *et al.* (2012) suggest that ecstatic display calls might then be used as an indicator of geographic and reproductive isolation.

In order to more fully investigate vocalizations, I undertook a survey of gentoo penguin ecstatic display calls across the Antarctic Peninsula and South Shetland Islands, South Georgia, the Falkland Islands, and Argentina to address two main questions: 1) How do ecstatic display calls vary both within and between individuals, colonies, and regions? and 2) Can ecstatic display calls be used to distinguish subspecies? These questions address a knowledge gap in both our basic understanding of vocalizations of gentoo penguins and how those vocalizations differ in a highly site-faithful bird with a broad geographic range.

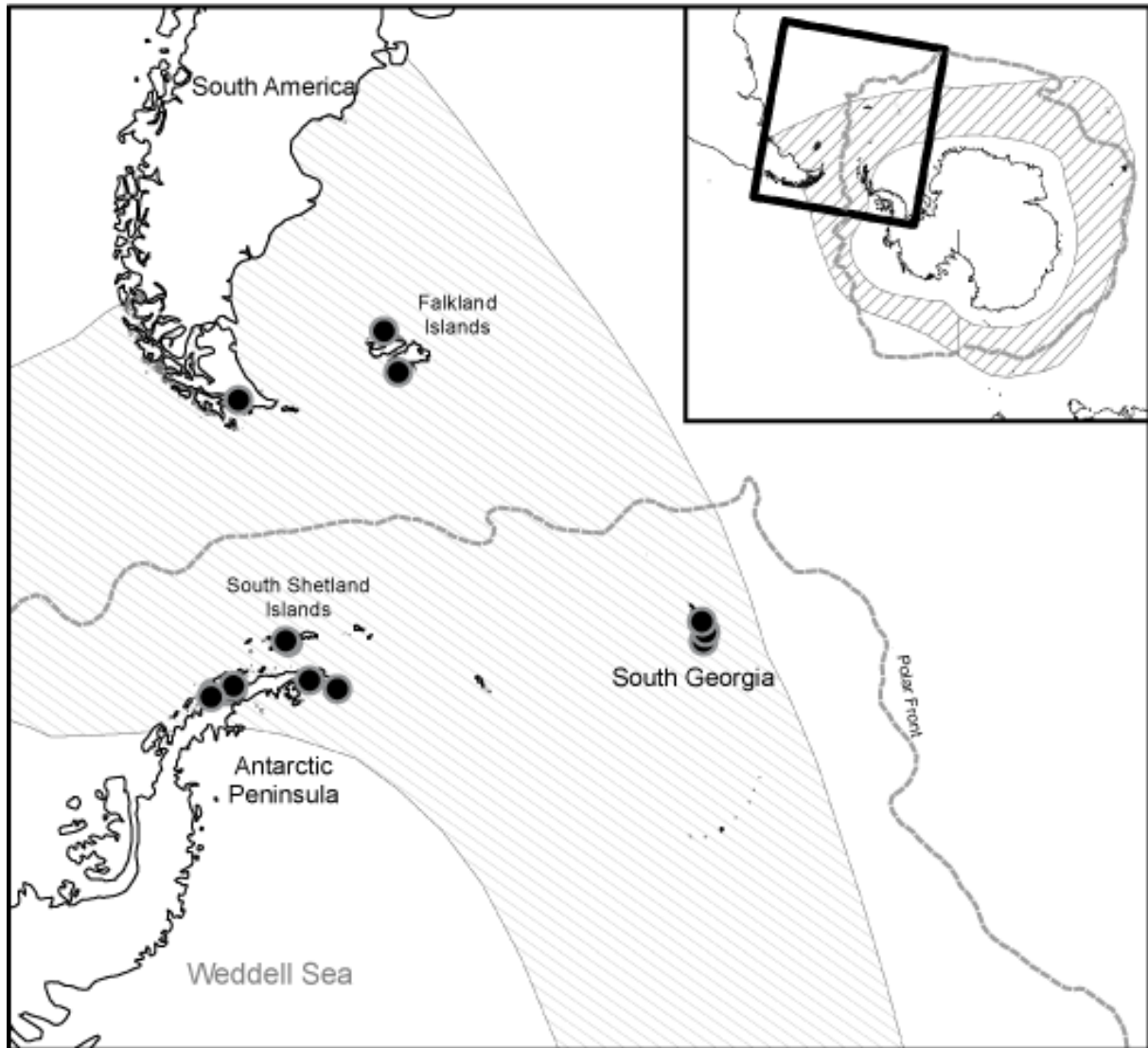


Figure 2-1: A map showing the sampling locations on the Antarctic Peninsula, South Georgia, the Falkland Islands, and Argentina.

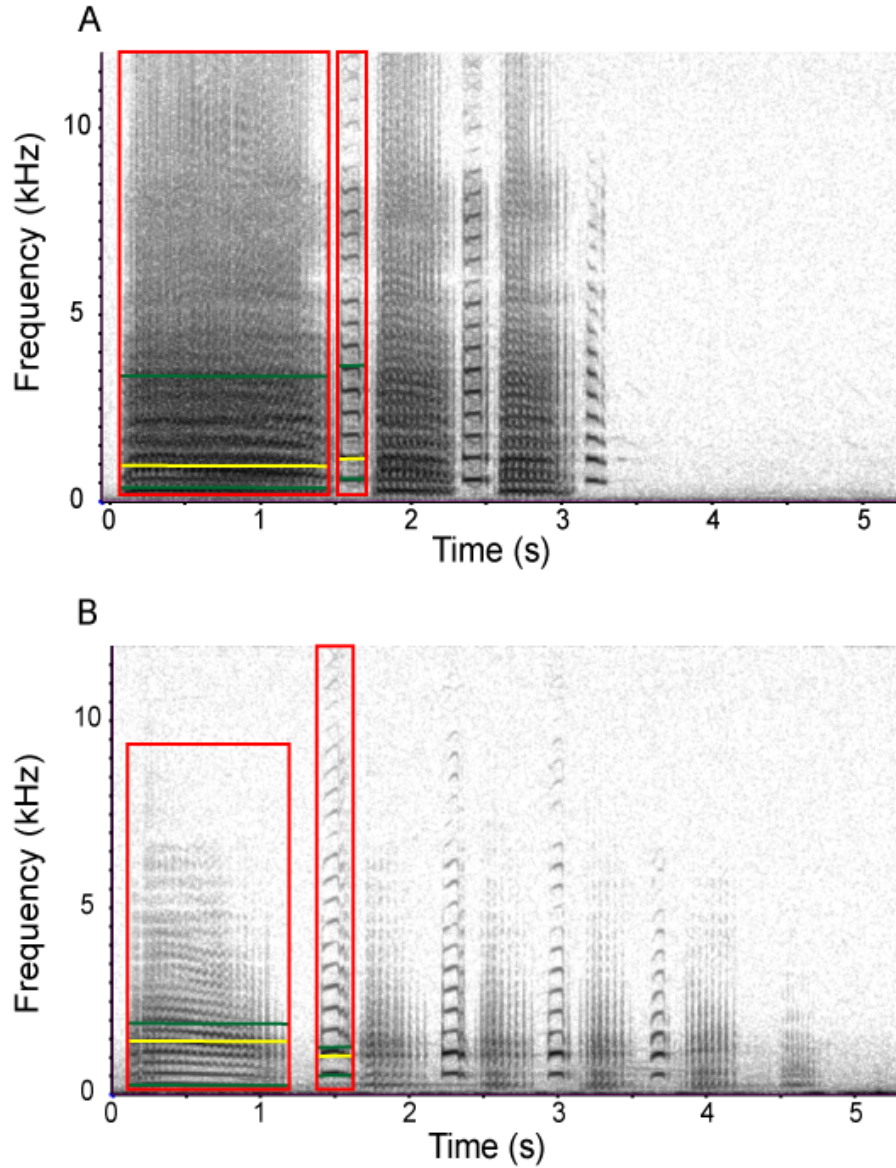


Figure 2-2: Spectrograms of the ecstatic display call of the gentoo penguin showing the repeating series of exhale and inhale syllables from Cape Tuxen (Antarctic Peninsula, *P. papua ellsworthii*) (A) and Carcass Island (Falkland Islands, *P. papua papua*) (B). The x-axis indicates time in minutes:seconds and the y-axis displays frequency in kHz. Red boxes denote the first exhale (left box) and first inhale (right box) syllable of the call. Darker hues indicate more power at that frequency.

2.3 - Methods

Passive soundscape audio recordings were taken during the breeding season at 22 gentoo penguin colonies on the Antarctic Peninsula and South Shetland Islands, South Georgia, the Falkland Islands, and Argentina (Figure 2-1, Table 2-1) using Song Meter SM2+ recorders (24000Hz sampling rate, stereo recordings). Recordings were taken with stationary units and were not targeted at specific penguins, and as such they recorded the ambient soundscape of the colony from which high quality individual calls were selected. Audio recorders were placed 3-5m from one or more small subgroups of nesting gentoo penguins within each colony and paired with either a video recorder (GoPro Hero3+) or a time-lapse camera (Brinno TL200) that were used in subsequent analysis to identify, where possible, the individual penguins associated with each vocalization. Penguins were neither tagged nor marked but were identified by the location of the nest they were incubating. All recordings were from colonies during egg or chick incubation, such that only one parent was attending the nest during recordings and usually remained on the nest for the duration of the recording (approximately 2-4 hours). Because the highest quality audio recordings were frequently from penguins not captured on video (e.g., nearby in the colony but not within the camera frame), not all of the recorded ecstatic display calls could be identified to individual.

Ecstatic display calls were analyzed in Raven Sound Analysis Software (Bioacoustics Research Program 2014) (window size=625 samples, overlap=65%, DFT size=2048 samples). Ecstatic display calls were identified within the recordings using a band limited energy detector, selected based on quality, and manually classified. I defined the ecstatic display call as any call that followed the pattern described in Jouventin (1982) with a repeated series of long, low frequency exhale syllables and short, higher frequency inhale syllables. While the mutual display

call is almost identical to the ecstatic display call (Jouventin 1982), only calls made by a single individual were selected, so it is highly unlikely that any mutual display calls were included in this analysis. Given that recordings were usually taken between mid-morning and late afternoon, pair exchange on the nest was unusual, further decreasing the likelihood of mutual display calls being included in the analysis. Duration, center frequency, 5% frequency, 95% frequency, peak frequency contour, peak frequency contour slope, and peak frequency inflection points were measured for each individual syllable as well as for the entire call (Figure 2-1, Table 2-2). A total of 544 calls were analyzed from 14 colonies on the Antarctic Peninsula and South Shetland Islands ($n=359$ calls), 5 colonies in South Georgia ($n=117$ calls), 2 colonies in the Falkland Islands ($n=41$ calls), and 1 colony in Argentina ($n=27$ calls). Of those calls, 183 were identified to individual (Figure 2-2, Table 2-1). Because the number of syllables was highly variable, I included in the analysis only measurements for the entire call and for the first two syllables (the first exhale and first inhale) of each call.

Ecstatic display calls were only selected if they could be isolated without any interference from other animal vocalizations (e.g. chicks, flying birds, elephant seals) or from other background noise. Given that there may be differences in acoustic environment between sites and especially between regions (e.g. rock and ice habitat on the Antarctic Peninsula, and tussock grass habitat in South Georgia) background noise was filtered out from each selection made in Raven Sound Analysis Software. The low frequency filter was minimized for each site, and ranged from 100-150Hz. After analyzing background noise at select colonies from each region, I found that while the center frequency of background noise was highest in South Georgia and the Antarctic Peninsula, the 95% frequency of background noise was consistently below the 5%

frequency of any ecstatic call measured, minimizing the possibility that background noise interfered with the analysis.

Call measurement data were standardized, and then visualized with Principal Components Analysis (PCA). A nested random effects analysis of variance (ANOVA) on the first principal component was used to partition variation between individuals, colonies, regions, and subspecies. I first ran the nested ANOVA on only those calls (n=183) from individuals that could be identified. I then repeated the analysis on the entire dataset (n=544) using two different assumptions about the identity of unknown individuals (thus covering the range of possible pseudoreplication among unidentified calls). In the first scenario, all unidentified calls within each colony were considered to be from the same individual, and in the second scenario all unidentified calls within each colony were considered to be from unique individuals.

Because the first principal component captured only a portion of the variation among calls, I also used a non-parametric permutation test (n=5000 permutations) on the multivariate analysis of variance (MANOVA) F statistic to quantify the effect of colony and region on the suite of measurements for the entire call, the first syllable, and the second syllable. Permutations at the region level maintained the colony identity of each call but permuted the region associated with each colony. With only two subspecies and four regions, I did not have enough power to detect a statistically significant effect of subspecies through permutation of the subspecies-region relationship, so differences associated with subspecies were examined by permuting the subspecies associated with each colony instead.

As a third approach to investigating differences among calls, I trained a random forest machine learning algorithm (R package 'h2o'; Aiello *et al.* 2016) (sample rate = 0.8, number of

trees =5000) on a known subset of calls using the suite of measurements for the entire call, the first syllable, and the second syllable, and then classified calls to which the algorithm was naïve. To address the disparity in sample sizes between categories, a random subsample of calls (n=82 for each subspecies, n=123 for each region) were used in the random forest analysis.

While the existence of unidentified individuals may raise concerns regarding pseudoreplication for the MANOVA and random forest analyses, I had few repeat calls from the same individuals where individuals could be identified and it is reasonable to assume that repeated calls would occur at a similarly low rate among unidentified penguins. For relevant statistical methods, tests with p -values < 0.05 are considered strong evidence against the null hypothesis and are referred to as statistically significant. Samples of audio recording for each site have been deposited in Dryad (DOI:10.5061/dryad.rm228); videos are available upon request from the authors.

Table 2-1: Sampling locations on the Antarctic Peninsula, South Georgia, the Falkland Islands, and Argentina with colony size at time of sampling (number of breeding pairs), number of ecstatic calls used in the analysis, and number of those ecstatic calls that could be identified to individual.

Region	Colony	Colony Code	Latitude	Longitude	Colony Size	Calls Analyzed (Calls Identified)
Falkland Islands	Carcass Island	CARC	51.280 S	60.563 W	528	20 (4)
	Sea Lion Island	SELI	52.423 S	59.078 W	1265	21 (6)
South Georgia	Stromness	STRO	54.160 S	36.712 W	58	7 (5)
	Whistle Cove	WHIS	54.160 S	36.813 W	114	25 (7)
	Godthul	GODT	54.283 S	36.300 W	1491	17 (2)
	Ocean Harbour	OCEA	54.341 S	36.246 W	201	25 (0)
	Gold Harbour	GOLD	54.619 S	35.946 W	209	43 (1)
Argentina	Isla Martillo	MART	54.906 S	67.375 W	30	27 (22)
South Shetland Islands	Yankee Harbour	YANK	62.526 S	59.768 W	5499	32 (9)
	Fort Point	FORT	62.543 S	59.578 W	837	11 (5)
Antarctic Peninsula	Heroína Island	HERO	63.394 S	54.608 W	215	12 (2)
	Brown Bluff	BROW	63.522 S	56.905 W	676	31(15)
	Selvick Cove	SELV	64.647 S	62.571 W	737	13 (5)
	Georges Point	GEOR	64.669 S	62.670 W	3354	42 (9)
	Cuerverville Island	CUVE	64.684 S	62.623 W	9642	22 (16)
	Neko Harbour	NEKO	64.838 S	62.533 W	1507	40 (10)
	Brown Station	ALMI	64.896 S	62.870 W	204	12 (4)
	Booth Island	BOOT	65.067 S	64.026 W	1805	28 (16)
	Pleneau Island	PLEN	65.103 S	64.052 W	2786	21 (6)
	Petermann Island	PETE	65.172 S	64.142 W	3085	30 (15)
	Moot Point	MOOT	65.204 S	64.074 W	558	20 (7)
	Cape Tuxen	TUXE	65.267 S	64.118 W	342	45 (17)

Table 2-2: Descriptions of spectrogram measurements used in analysis. Measurements were chosen from a suite of measurements in Raven Sound Analysis Software.

Measurement	Description
Duration 90%	The difference in time between two points that contain the lower 5% and upper 5% of time. (i.e. The middle 90% of the time)
Center Frequency	The frequency that divides the selection into two frequency intervals of equal energy (i.e. the 50% frequency). A more robust measure than Peak Frequency alone
5% Frequency	The frequency that divides the selection into two frequency intervals containing 5% and 95% of the energy in the selection
95% Frequency	The frequency that divides the selection into two frequency intervals containing 95% and 5% of the energy in the selection
Peak Frequency Contour	A trace of the peak frequency across the duration of the selection
Peak Frequency Contour Slope	A trace of measurements of slopes between consecutive peak frequency measurements in the peak frequency contour
Peak Frequency Contour Average Slope	An average of peak frequency contour slope across the entire selection
Peak Frequency Contour Inflection Points	The number of inflection points in the trace of peak frequency across the entire selection

2.4 - Results

Ecstatic display calls were characterized by wide variation with respect to several measures of frequency and duration. Calls ranged from 2 - 15 syllables and 0.8 - 5.3 seconds (mean = 2.66 seconds) in duration, and had center frequencies that ranged from 117Hz to 2203Hz (mean = 770Hz) in the first syllable and from 117Hz to 3023Hz (mean = 858Hz) in the second syllable. The 5% frequency (a measure of the 5% quantile of power within the spectrogram) varied between 105Hz and 668Hz (mean = 225Hz) in the first syllable and from 106Hz to 891Hz (mean = 235Hz) in the second syllable, indicating that spectral power was concentrated in the low frequencies for both syllable types.

I found significant variation both within and between colonies, and while comparisons of select colonies within the PCA showed differences in colony- or region-specific ellipse area and location, there was no clear pattern (Figure 2-3, Table 2-3) or linear relationship between single variables and latitude (e.g., 5% frequency $p=0.09$; center frequency $p=0.93$). I did find a slight negative trend for change in center frequency ($p<0.001$), and a slight positive trend for change in 5% frequency ($p<0.001$) when compared to inter-colony distance, though given the considerable variation in these measures of similarity it is not clear whether these trends are biologically significant (Figure 2-4).

Using a three factor random effects nested ANOVA on the first principle component (PC1) for the subset of identified individuals, a large amount of the variation was attributed to differences among colonies (30.20%) and individual penguins within colonies (35.80%) but no significant variation was associated with region or subspecies. When using the entire dataset that includes calls from individuals of unknown identity, the results were robust to the treatment of

these unknown individuals. I found similar results whether I classified all unidentified calls as coming from unique individuals (colonies: 39.18%, individuals: 21.78%) or whether I classified all unidentified calls as coming from the same individual within each site (colonies: 32.13%, individuals: 21.57%), indicating that unknown identifications are unlikely to skew my analyses.

Consistent with the nested ANOVA analysis, the non-parametric permutation test on the MANOVA F statistic for the suite of measurements revealed highly significant differences between colonies ($F=3.72$, $p<0.001$), but no significant difference between regions ($F=3.81$, $p=0.47$) or subspecies ($F=3.81$, $p=0.43$).

The random forest algorithm was able to classify unknown calls into correct colonies better than an untrained random classification (30.0% vs. 5.4% accuracy), consistent with genuine differences between colonies, but error rates in classification remained high. At the regional level, the algorithm correctly classified calls from the Antarctic Peninsula (class error = 14.0%), but performed poorly for other regions (mean per-class error = 40.3%). While the random forest algorithm did correctly classify calls into subspecies (mean per-class error = 20.6%), the ANOVA and MANOVA results suggest this classification may be due to differences between colonies (which are nested within subspecies) rather than true differences between subspecies. All analyses consistently ranked various measures of frequency rather than those related to duration as the most important variables for classification (Table 2-4).

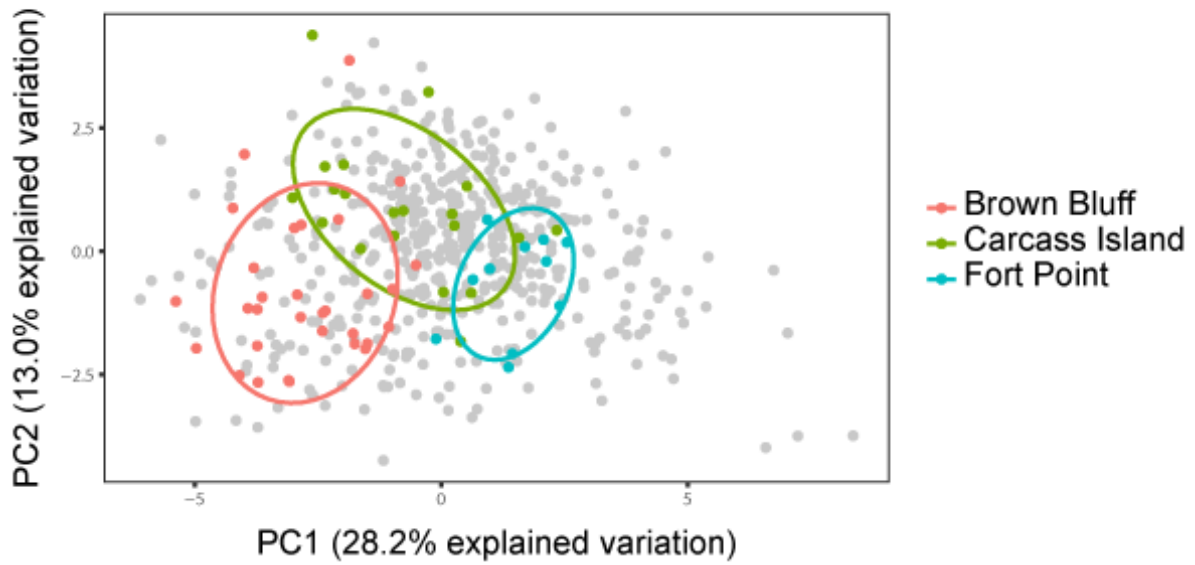


Figure 2-3: Ecstatic display call measurements (entire call, first exhale syllable, and first inhale syllable) along the first two principal components from a Principal Components Analysis (PCA). Three sites are highlighted to illustrate pairwise differences in the parameter space created by the first two PCA axes, but these differences do not follow a discernable pattern between sites.

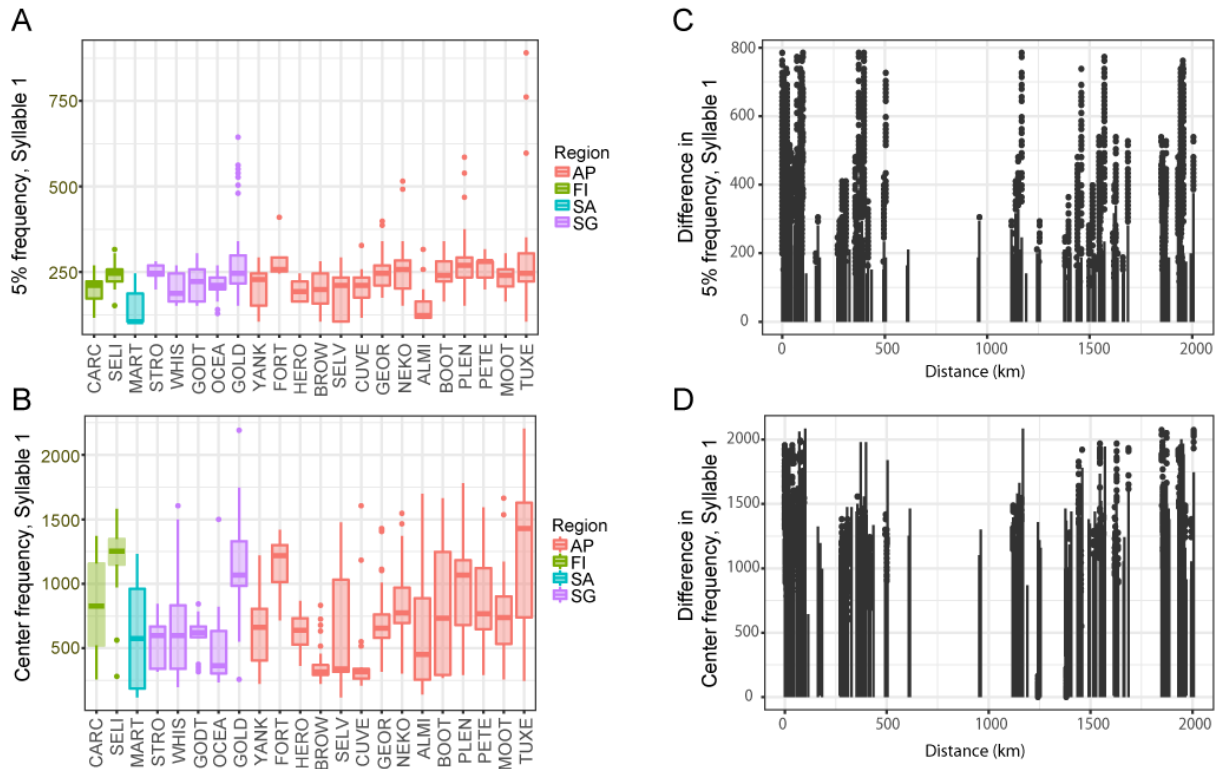


Figure 2-4: Boxplots demonstrating variation at each site in 5% frequency center frequency based on latitude (A and B) and relative distance (C and D). Colony codes are used for brevity; see Table 2-1 for full colony name. For A and B, sites are organized by latitude with lower latitudes on the left and high latitudes on the right. The only exception is Isla Martillo, which was placed next to the Falkland Islands sites as they are believed to be in the subspecies *P. papua papua*. For C and D, pairwise comparisons were made between all individual calls and the difference in call measurement is shown against relative distance (km) between colonies. Individual pairwise comparisons have significant differences, but no clear pattern was found across latitude, and while there were statistically significant trends across distance, the degree of variation shown in this figure make it unlikely that those trends are ecologically significant.

Table 2-3: Variable loadings for each acoustic measurement for the first five principle components. Percentage of variation explained by each principal component is included in parentheses

Measurement	PC1 (28.2%)	PC2 (13.0%)	PC3 (11.0%)	PC4 (9.5%)	PC5 (7.0%)
Center Frequency, entire call	0.36	-0.14	0.03	-0.16	0.02
Duration 90%, entire call	-0.04	0.42	-0.35	-0.14	0.38
5% Frequency, entire call	0.26	-0.29	-0.29	-0.17	0.09
95% Frequency, entire call	0.30	0.13	0.39	-0.03	0.16
PFC Average Slope, entire call	-0.12	0.11	0.20	-0.01	0.35
PFC Inflection Points, entire call	0.08	0.44	-0.36	-0.18	0.28
Center Frequency, 1 st syllable	0.36	-0.05	-0.01	-0.14	-0.06
Duration 90%, 1 st syllable	0.18	0.41	-0.10	0.07	-0.42
5% Frequency, 1 st syllable	0.29	-0.24	-0.27	-0.13	0.02
95% Frequency, 1 st syllable	0.33	0.16	0.34	0.01	0.018
PFC Average Slope, 1 st syllable	0.01	0.04	0.05	0.08	0.42
PFC Inflection Points, 1 st syllable	0.25	0.36	-0.13	0.02	-0.40
Center Frequency, 2 nd syllable	0.28	-0.15	0.09	-0.10	0.15
Duration 90%, 2 nd syllable	-0.18	-0.02	0.14	-0.63	-0.10
5% Frequency, 2 nd syllable	0.23	-0.19	-0.24	0.00	0.17
95% Frequency, 2 nd syllable	0.26	0.19	0.37	-0.01	0.13
PFC Average Slope, 2 nd syllable	-0.12	0.12	0.15	-0.05	0.11
PFC Inflection Points, 2 nd syllable	-0.14	0.04	0.11	-0.66	-0.12

Table 2-4: The five most important variables from Random Forest machine learning for subspecies classification.

Classification	Variable	Scaled Importance	Percentage
Subspecies	5% frequency, 1 st syllable	1.00	15.30%
	Center frequency, 2 nd syllable	0.78	11.94%
	Center frequency, 1 st syllable	0.62	9.46%
	Duration, 1 st syllable	0.53	8.12%
	5% frequency, 2 nd syllable	0.46	7.08%

2.5 - Discussion

I found a high degree of between-individual variation in ecstatic display calls within gentoo penguin breeding colonies. Even with this large within-colony variation, I found significant differences between colonies, which can be attributed primarily to frequency parameters of the ecstatic display call. Long-term geographic and reproductive isolation in this highly site faithful species may have resulted in differentiated vocal traits between breeding colonies. These colony-specific vocalizations may drift over time, and may be mostly independent of the characteristics of other colonies.

Based on the random forest variable importance values, gentoo penguin ecstatic display calls were most easily differentiated on frequency-related variables, even though the duration of calls, both in terms of temporal length and number of syllables, was highly variable. This is consistent with previous work by Jouventin and Aubin (2002) that found frequency to be the key variable for individual recognition between *Pygoscelis* species chicks and their parents, and that changes in pitch of as little as 25Hz may affect the ability of a chick to recognize its parents. As such, the frequency differences of over 100Hz that I observed between colonies are likely to be biologically meaningful in terms of penguin behavior.

In addition to the variation between colonies, I found a large amount of variation between individual penguins within the same colony. It may be beneficial for an individual to be differentiated from others in the colony if this differentiation allows for mate recognition, though high colony fidelity suggests there may be little benefit to differentiated vocalizations beyond the immediate geographic area of the breeding colony. The independent origins of each colony's vocal portfolio results in variation but shows no discernable geographic pattern in ecstatic

display calls across the gentoo penguin range. Geographic variation likely arises by slow drift over time between colonies, while within colony variation is more likely to reflect an active process occurring on faster time scales that exploits what appears to be a relatively distinctive individual trait. Here we see aspects of the colony itself influencing individual characteristics. The nature of the colony as a crowded, noisy environment requires precise identification and recognition between individuals and the philopatry of gentoo penguins allows for cultural drift between isolated colonies.

These findings are important considering the vocal differences described in de Dinechin *et al.* (2012) and Jouventin (1982) who both noted vocal differentiation between the Indo-Pacific Sub-Antarctic Islands and the Atlantic Sub-Antarctic Islands. This is the most comprehensive study of the geographic variation in gentoo penguin ecstatic calls to date, and provides a finer geographic scale at which to examine vocal differentiation. Genetic data from de Dinechin *et al.* (2012) and Levy *et al.* (2016) show the Falkland Islands as divergent clades from the Antarctic Peninsula, South Georgia, and the South Orkney Islands. The Polar Front provides a strong ecological barrier that is likely to maintain this separation and may have led to drift of ecstatic display calls over a long period of geographic isolation. While the random forest was able to successfully classify subspecies, given the nonsignificant findings in both the ANOVA and MANOVA permutation analyses, I suspect that differences between subspecies may be difficult to discern and may stem from inter-colony differences rather than robust differences between the two subspecies. As such, I suggest caution in inferring subspecies based on recorded vocalizations of individuals.

While these results suggest that classification of subspecies is complicated due to colony-level variation, the ability to differentiate subspecies vocal characteristics would have interesting

implications for determining the origin of new colonies. The population at Isla Martillo in Argentina is relatively new and it was suspected that these penguins were related to Falkland Island population (Raya Ray *pers. comm.* 2015). Surprisingly, the random forest algorithm classified them as *P. papua ellsworthii* when it was trained on data that excluded the Argentina population, and those calls were significantly different from all other regions in post-hoc Dunn Tests for frequency variables of the entire call as well as both the first and second syllables. However, given the challenges I have identified in determining subspecies designations through acoustic analyses alone, genetic analyses will be necessary to determine the origin of the Isla Martillo population.

Future investigation into the degree of plasticity and the role of genetics in vocal characteristics may help to disentangle how these processes play out on behavioral, ecological, and evolutionary time scales. Playback experiments may expand our understanding of individual recognition, and also help to determine how individuals become differentiated from their neighbors and if that process happens continuously or during a set phase of development. Understanding vocal characteristics of gentoo penguins and how those traits vary between individuals and regions may give us a better understanding of behavioral ecology and how individual interactions shape ecological processes such as the assembly and establishment of new colonies.

3 – Patterns of vocal behavior in gentoo penguin colonies

3.1 - Abstract

The repertoire for vocal communication among gentoo penguins *Pygoscelis papua* is limited, and most research has focused on calls associated with pair bonding or mate contact. In this study, I analyzed the soundscape of a colony of penguins to understand vocal behavior more inclusively, including calls unrelated to courtship and breeding. I found group calling behavior, in which two or more individuals call at the same time to be relatively frequent, and of a separate category than the previously described mutual display calls between mates. I also found robust evidence for a period of waiting after an individual call, during which time calls from other individuals were less likely to occur than would be expected by random chance. This waiting period is shortened prior to a group call but remains statistically significant. I interpreted this waiting period as an effort to avoid vocal 'jamming' within the colony, with a waiting period commensurate with the time required for a mate to respond to a contact call. I contrasted these results with recordings from captive environments, which were characterized by echoes that may mimic a noisier environment, and found no waiting period and less frequent group calling behavior among captive gentoo penguins. This study provides a more nuanced picture of gentoo penguin vocal behavior that may help illuminate ways in which these seabirds communicate and interact within a colony.

3.2 - Introduction

Many studies have examined the importance of avian vocalizations for territorial defense, mate bonding, social reinforcement, and information sharing. For colonial seabirds such as

penguins, information between neighbors and mates and between adults and chicks must be transmitted effectively even in a crowded noisy environment. The majority of vocal studies on penguins relate to individual identification, such as those in king penguins *Aptenodytes patagonicus* (e.g., Aubin and Jouventin 2002, Lengagne *et al.* 2001), Magellanic penguins *Spheniscus magellanicus* (Clark *et al.* 2006), rockhopper penguins *Eudyptes chrysocome* (e.g. Searby *et al.* 2004), African penguins *Spheniscus demersus* (Favaro *et al.* 2017), little penguins *Eudyptula minor* (Colombelli-Négrel and Smale 2018, Miyazaki and Nakagawa 2015), as well as Adélie *Pygoscelis adeliae* and gentoo penguins *Pygoscelis papua* (Aubin 2004, Jouventin and Aubin 2002). A few studies have also examined the importance of vocalization on facilitating mate bonding and breeding behavior (e.g., Waas *et al.* 2000, Setiawan *et al.* 2007, Jouventin and Dobson 2018).

The gentoo penguin has a more limited repertoire of vocalizations compared its congeners, the Adélie penguin and the chinstrap penguin. The most commonly used gentoo penguin vocalization is the ecstatic display call, which is defined as a series of repeated low frequency exhale syllables separated by short, broadband inhale syllables, with a highly variable number of total syllables (Jouventin 1982). Jouventin and Dobson (2018) describe an ecstatic display call used for individual recognition and territory assertion, as well as a mutual display call, which is almost identical in structure but is performed as a duet by mates for bonding and is also used for individual recognition. Penguin colonies are often characterized as a collection of autonomous breeding pairs, with little social structure or communication within the colony between individuals that are not mates. Jouventin and Dobson (2018) note that the mutual display call may be contagious among individuals within the colony, but to date there has been very little study of vocalizations unrelated to courtship or breeding. Here, I explore vocal

behavior within both wild and captive populations of gentoo penguins, with a specific focus on the timing of vocalizations within the colony and whether vocalizations facilitated other vocalizations throughout the colony. Studying vocal behavior such as this furthers our understanding of the social structure of penguin colonies and within colonial systems more generally.

3.3 - Methods

For this analysis, I considered only those calls that followed the ecstatic display call pattern and had at least three syllables (exhale – inhale - exhale). Ambient colony sound was recorded at four gentoo penguin breeding colonies on the Antarctic Peninsula (D'Hainaut Island, Pleneau Island, Moot Point, Cape Tuxen [Table 3-1]) as well two different captive populations containing gentoo penguins (Detroit Zoo [83 penguins of 4 species, including 23 gentoo penguins], Kansas City Zoo [54 penguins of 3 species, including 36 gentoo penguins]). At each wild colony, a Song Meter 2+ audio recorder was placed near a small subgroup of gentoo penguins for approximately 3-4 hours during the austral summer breeding season. A 90 minute sample was taken from each recording for analysis for a total of 360 minutes of analyzed recordings at wild colonies. For each gentoo penguin vocalization in that 90 minute sample, a time stamp was recorded and the call was categorized as an individual or group call. Individual calls were defined as a vocalization of at least three syllables that did not overlap with another individual's vocalization. Group calls were defined as any call in which two or more individuals overlapped their calls, regardless of degree of synchrony. While it is possible that this categorization of group calls may include mutual display calls between mates, these are unlikely to be more than a small proportion of the dataset, due to a general lack of synchrony in calls

analyzed and the fact that recordings were taken during the late morning or mid-afternoon when usually only one individual was attending the nest.

At each zoo, Song Meter 2+ audio recorders were placed around the interior of the exhibit for a duration of 4-5 days early in the breeding season, recording during the daylight hours in the exhibit, approximately 8am-8pm. Two 90 minute samples were taken from each zoo, one from the morning and one from the afternoon and in a similar time frame as that of the wild recording, for a total of 360 minutes of analyzed recordings in captive populations. For each penguin vocalization in that 90 minute sample, a time stamp was recorded and the call was categorized as a gentoo penguin call or a call from another species. At both zoos, gentoo penguins were housed in an enclosure with multiple other penguin species - king penguins, rockhopper penguins, and macaroni penguins at the Detroit Zoo; king penguins and rockhopper penguins at the Kansas City Zoo.

The time lag between each vocalization and the one preceding it was calculated. The distribution of observed time lags was compared, using a Kolmogorov-Smirnov Test, to the distribution of lags generated by 1000 simulated time series in which calls were placed randomly within the recording interval. The cumulative number of calls following each call was also recorded in time windows of varying length ($t = 1-300$ seconds), from which I calculated an L statistic (a variant of the Ripley's K [Dixon 2002]); this L statistic was also compared to 1000 simulated time series in which calls were randomly placed within the recording interval. The L statistic is calculated for each time bin t following any given call,

$$L_t = K_t - (\lambda * t)$$

where K_t is the total number of calls observed in a window of length t and λ is the average rate of calling across the entire recording. The expected number of calls within any window of length t is $\lambda * t$ and so a negative (positive) L value indicates that vocalizations are less (more) frequent than expected in the t seconds following a vocalization.

A subset of 100 individual calls and 81 gentoo penguin calls from captive populations were used for acoustic analysis in Raven Sound Analysis Software, following the basic methodology of Lynch and Lynch (2017). Spectrogram measurements and descriptive measurements were recorded for the entire call as well as each individual syllable within the call. In captive populations, only the call duration and number of syllables were recorded, as the echoes in the enclosed environment made analysis of acoustic structure inaccurate. When possible, the first syllable of a group calls from wild populations was also analyzed (N=50). The duration available for analysis was determined by when the secondary penguin or penguins joined in the call, and therefore the duration parameter was not included in these measurements (Table 3-2). Because there are correlations among the features of a penguin call, a Principle Components Analysis was used to reduce the dimensionality of each penguin call's measurements and to visualize differences in parameter space between calls.

Table 3-1: Sampling locations for wild gentoo penguin breeding colonies along the Antarctic Peninsula

Colony Name	Latitude	Longitude
Cape Tuxen	-65.267	-64.118
Pleneau Island	-65.104	-64.056
D'Hainaut Island	-63.902	-60.792
Moot Point	-65.206	-64.078

Table 3-2: Descriptions of spectrogram measurements used in analysis. Measurements were chosen from a suite of measurements in Raven Sound Analysis Software (reproduced from Lynch and Lynch 2017).

Measurement	Description
Duration 90%	The difference in time between two points that contain the lower 5% and upper 5% of time. (i.e. The middle 90% of the time)
Center Frequency	The frequency that divides the selection into two frequency intervals of equal energy (i.e. the 50% frequency). A more robust measure than Peak Frequency alone
5% Frequency	The frequency that divides the selection into two frequency intervals containing 5% and 95% of the energy in the selection
95% Frequency	The frequency that divides the selection into two frequency intervals containing 95% and 5% of the energy in the selection
Peak Frequency Contour	A trace of the peak frequency across the duration of the selection
Peak Frequency Contour Slope	A trace of measurements of slopes between consecutive peak frequency measurements in the peak frequency contour
Peak Frequency Contour Average Slope	An average of peak frequency contour slope across the entire selection
Peak Frequency Contour Inflection Points	The number of inflection points in the trace of peak frequency across the entire selection

3.4 - Results

A total of 1355 wild calls (954 individual, 401 group) and 1644 captive calls (119 gentoo penguin, 1525 from other species) were analyzed. Wild environments were similarly noisy compared to captive environments; average call density was 0.063 calls/second in the wild (gentoo penguins only) compared to 0.076 calls/second in captivity (all species), however the vast majority of calls in captivity were made by other species and captive gentoo penguins only averaged 0.006 calls/second. I found no significant difference in the distribution of calls or time lags between colonies, and while the Detroit Zoo was had more calls from other species per second, the density of gentoo penguin calls per second was similar in both the Detroit Zoo and the Kansas City Zoo (0.005 and 0.006 cells/second, respectively). The average time lag between any two calls was 15.8 seconds in the wild and 12.9 seconds in captivity. Group calls were more frequent in wild populations, comprising 29.6% of analyzed calls, while in captivity, only 7 of 119 (5.9%) gentoo penguin calls analyzed were group calls, and that behavior only occurred at the Kansas City Zoo (Figure 3-1A).

In the wild populations, there was a significant time lag (K-S test, $p < 0.001$) following both individual and group calls. Individual calls were significantly less frequent than expected for a period of 8 seconds following an individual call and 12 seconds following a group call, creating a period of relative quiet within the colony that I refer to as a waiting period. After these waiting periods, the timing of calls could not be distinguished from a random (Poisson) process. There was no analogous waiting period for group calls, and there was no reduction in group calling behavior following either individual or group calls (Figure 3-1B).

Acoustic analysis of wild populations revealed a high degree of individual variation among calls, but no clear distinction between calls that precede an extended pause (10-60 s)

compared to those that proceed shorter pauses (1-10 s). While there was no statistically significant difference between acoustic parameters, there is a slight trend towards longer pauses following individual calls with a larger number of syllables. There were however, some differences in the first syllable of a group call compared to the first syllable of an individual call, trending towards a higher peak frequency slope in calls that initiate a group response (Figure 3-2). Multivariate acoustic analysis was not possible on captive populations due to poor sound quality in the captive environment.

There was no analogous pattern of waiting in captivity, and I found no significant time lag in the seconds immediately following a call from either a gentoo penguin or another species. The distribution of calls in time did not differ from expected under a Poisson process, and all L statistics fell within 95% of the random simulations, except for the Detroit Zoo in which calls were actually more closely grouped together than expected (Figure 3-1C). While gentoo penguin ecstatic display calls were significantly less frequent in captivity, their calls, and those from other species appear to be more randomly distributed in time than in wild populations.

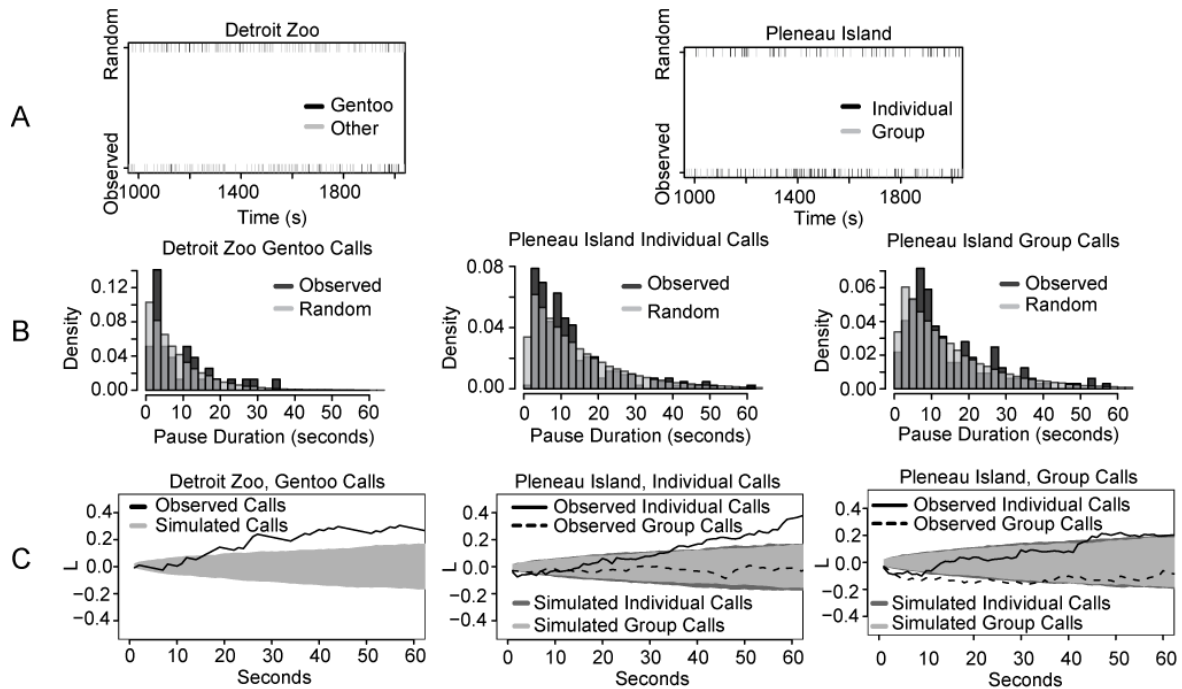


Figure 3-1: Visualization of calls in time (A), histograms of pause duration prior to any given call (B), and the calculated L statistic following any given call (C). Data shown are for one example each of a captive population (Detroit Zoo) and a wild population (Pleneau Island). (A) Strip chart illustrating the timing of calls in a section of audio recording. For the captive population (Detroit Zoo), calls from gentoo penguins are in black, while calls from other species of penguin are in gray. For the wild population (Paulet Island), individual calls are in black while group calls are in gray. (B) Simulated random data are plotted against observed pause durations prior to a gentoo penguin call in captivity prior to an individual gentoo penguin call in the wild and prior to a group gentoo penguin call in the wild. (C) L statistics are plotted against simulated random data for gentoo penguin calls in captivity, individual gentoo penguin calls in the wild, and group gentoo penguin calls in the wild.

Group vs Individual Calls

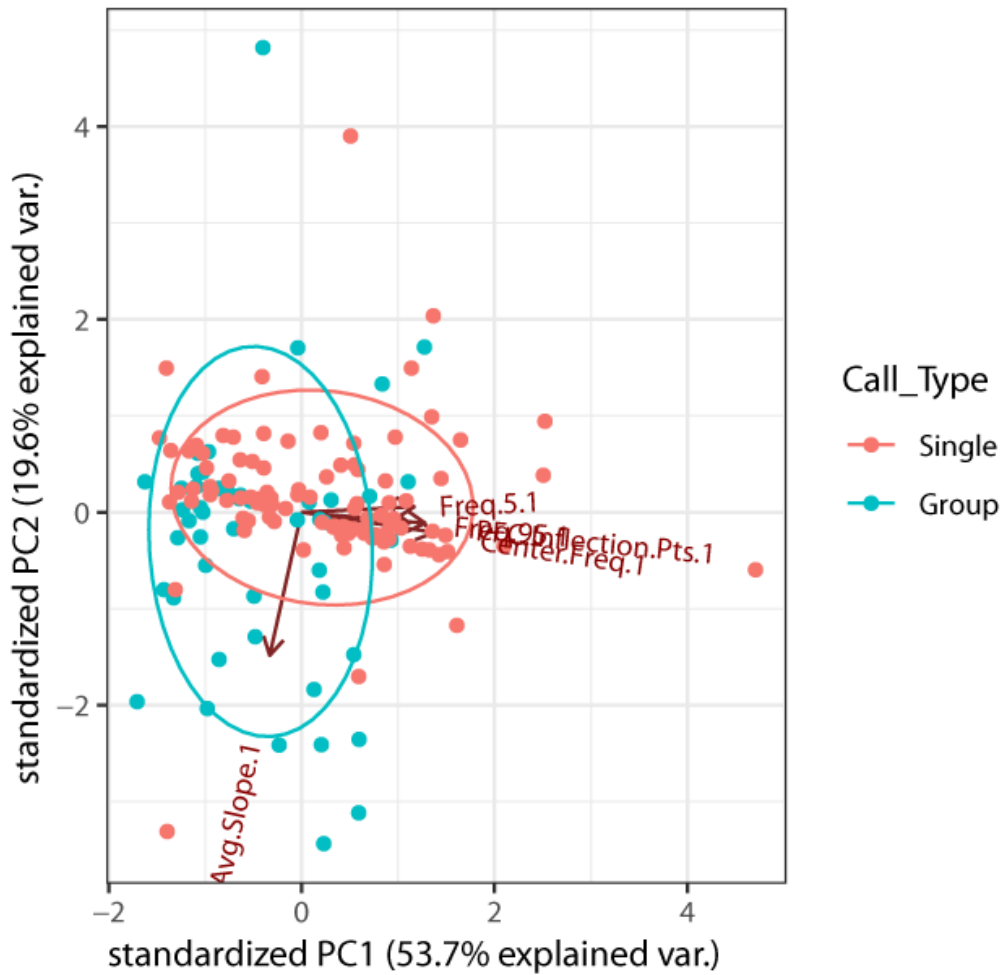


Figure 3-2: Principle Components Analysis of acoustic characteristics of an individual gentoo penguin call (first syllable only) compared to that of a group call. Group calls were primarily differentiated by the second principle component.

3.5 - Discussion

I conclude that group calls, involving three or more individuals calling simultaneously, should be described as a separate vocalization as they are clearly distinct from both individual ecstatic display calls and mutual display calls between mates. These calls represent nearly one third (29%) of all the vocalizations recorded in wild populations, but only 6% in captive populations.

In wild populations, I found that individual calls have a longer than expected waiting period following an individual or a group call. Whereas individual ecstatic display calls were preceded by an extended period of quiet within the colony, there was a shorter pause in calling preceding a group call. In this sense, gentoo penguins seem to wait for silence before producing an individual ecstatic display call, consistent with the ecstatic display call's functions requiring individual recognition and territory assertion. If these calls are contact calls, as described by Jouventin (1982) and Jouventin and Dobson (2018), it is plausible that a gentoo penguin would wait for silence to avoid garbling the message to its mate, or that a penguin would avoid calling immediately after a stranger's contact call. I hypothesize that group calls may serve a different function not requiring individual recognition. PCA results suggest acoustic differences between the beginning of the originating call of a group call and the beginning of an individual ecstatic display call, but it was analytically difficult to further study the individual contributions of each penguin participating in a group call. Because of the speed with which group calls are initiated, it is possible that group calls are actually initiated by visual cues associated with the initial call (e.g., wing flapping, head movements, or external cues) and not by the characteristics of the call itself.

Gentoo penguin vocalizations in wild populations on the Antarctic Peninsula appear to be non-random in their timing. Along with the existence of frequent group calling behavior, these findings suggest that calling may play a more important role in a penguin's colonial environment than has been previously appreciated. The unique aspect of group living in a colony requires the behavioral adjustment of this waiting period in order to avoid signal jamming and allow for individual recognition, and the production of group behaviors whose role is not yet understood.

By contrast, gentoo penguin colonies in captivity display no characteristic waiting period, have a pattern of calling that cannot be distinguished from random, and perform fewer group calls. Ambient noise in a captive enclosure includes other species of penguins, as well as mechanical noise from climate control and operations within the exhibit are potential explanations for these behavioral differences. The acoustic environment itself, with long echoes within the enclosure may preclude any period of silence following a call. Additionally, captive penguins have no predators, food is provided, and potential mates are always nearby, which may limit the importance of the ability to identify individuals using ecstatic display calls.

These findings help us to better understand gentoo penguin behavior and suggest the need for further research into the functional nature of penguin vocalizations. Further work will be required to identify the causes of group calling behavior, as well as the benefits and drawbacks to living in a noisy colony for individual vocalizations.

4 – Conspecific aggression in gentoo penguin colonies

4.1 - Abstract

The population costs and benefits of coloniality in seabirds have been studied in a variety of systems, showing effects on reproductive success at both high and low densities of nesting birds. One common cost of colonial breeding systems is conspecific aggression, either between adults and chicks or between adults in the colony. These small scale interactions within the colony and the behaviors associated with them are relatively understudied, especially in penguin species. In order to understand what factors lead to agonistic behaviors and the escalation of aggression within those behaviors, I examined behavior at gentoo penguin breeding colonies on the Antarctic Peninsula. I observed rates of interaction, frequency of agonistic response, and the level of aggression in the response and found that the most predictive factors for both the presence of a response and the level of aggression in the response were related to how frequently that individual encountered conspecifics and how many times they had encountered the same individual. I described the agonistic call of the gentoo penguin in more detail, showing that it appears to be a distinct call rather than an abbreviation of an ecstatic display call. These findings lead to a better understanding of individual-level behaviors that represent costs of colonial living, and how those costs are unevenly distributed across individuals within the colony.

4.2 - Introduction

Approximately 95% of all seabird species are colonial (Schrieber and Burger 2002), and the cost, benefits, and evolution of coloniality have long been discussed in the literature (e.g.,

Hoogland 1979, Danchin and Wagner 1997, Danchin *et al.* 1998, Dubois *et al.* 1998, Varela *et al.* 2007, Ashbrook *et al.* 2014, Evans *et al.* 2016). Coloniality may provide defense from predators, easy access to mates, and opportunities for group foraging, and social stimuli has been shown to cue and synchronize breeding behaviors. However, breeding in dense aggregations also increases competition for food, territory, and mates, facilitates disease and parasite transmission, and may actually concentrate the effect of predators, particularly introduced species (Schrieber and Burger 2002).

Competition for resources and high nesting density can lead to high rates of agonistic interactions and conspecific aggression within colonial seabirds. These interactions may play out between adults (e.g., Pius and Leberg 1997, Ellis and Good 2006, Viera *et al.* 2011) or as adult aggression toward unrelated chicks (e.g., Ramos 2003, Ashbrook *et al.* 2008, Villanueva-Gomil *et al.* 2009). In many species, agonistic interactions increase with average nesting density (Burger and Gochfeld 1988, Hill *et al.* 1997), which lead to decreased reproductive success at high density in some species (Butler and Trivelpiece 1981, Hill *et al.* 1991, Stokes and Boersma 2000, Ashbrook *et al.* 2008). However, the impact of conspecific interactions within the colony is nuanced, in that aggressive interactions may be reduced in mixed species colonies (Pius and Leberg 1997, Ellis and Good 2006) or may be affected by the location of the individual within the colony (Viñuela *et al.* 1995, Côté 2006). The escalation of agonistic interactions may depend on the value of the disputed resource (Viñuela *et al.* 1995, Renison *et al.* 2006) or the presence of a mate during the interaction (Waas 1991). Breeding colonies are noisy environments and vocalizations play an important role in these agonistic interactions from territory defense to alarm calls (Jouventin 1982, Jouventin and Dobson 2018). Vocalizations can also stimulate

aggressive responses, such as in royal penguins, when artificially increasing colony sound can elevate rates of aggressive reactions (Waas *et al.* 2000).

In this study, I have used the gentoo penguin as a study species for understanding conspecific aggressive behavior. Gentoo penguins breed in relatively small colonies on the Antarctic Peninsula that range from a few hundred to several thousand nests (Humphries *et al.* 2017). They have nearly even parental investment throughout the austral summer breeding season - while one parent guards the nest, the other will forage and they will alternate duties approximately once per day. Thus, at most points in the day, the breeding colony is comprised mostly of adults incubating eggs or guarding chicks on a nest. As with other *Pygoscelis* spp. penguins, gentoo penguins build nests out of loose stones (incorporating other material in the more vegetated areas of their breeding range) in rocky areas that are relatively snow free near the shore. Since stones are often in short supply, penguins will often steal this nesting material from other individuals throughout the breeding season to build and fortify their own nests. However, nests are spaced such that a penguin incubating eggs on one nest cannot easily reach the stones of the neighboring nest. For this reason, there are relatively few agonistic encounters between incubating adults, and conspecific encounters are usually with other individuals walking through the nesting area, either on their way to or from a foraging trip or attempting to steal stones from others nests.

As vocalizations are an important aspect of aggressive interactions (Jouventin 1982, Jouventin and Dobson 2018), I also investigated the specific vocalizations involved in agonistic interactions within the gentoo penguin colony. While other penguin species have differentiated calls for at sea contact calls, mate recognition, mate bonding, and agonistic interactions, gentoo penguins have a relatively simple suite of calls. Previous work has described the ecstatic display

call as being identical to the mutual display call between mates, and the agonistic call as an abbreviated ecstatic display call (Jouventin 1982, Jouventin and Dobson 2018).

Here I examined conspecific interactions within multiple gentoo penguin breeding colonies on the Antarctic Peninsula to determine what factors influence an agonistic reaction and what factors affect the escalation of that reaction. I also examined the acoustic parameters of agonistic vocalizations as well what factors affect the use of vocalizations within a conspecific encounter. By exploring details of conspecific interactions within a colony I hope to further the discussion on conspecific aggression and the pros and cons of coloniality, and illuminate the ways in which these costs and benefits may be unevenly distributed within the colony.

4.3 - Methods

Behavior was observed from audio and video taken at nine gentoo penguin breeding colonies on the Western Antarctic Peninsula during in the 2014-15 and 2018-2019 breeding seasons (Table 4-1). At each colony, a GoPro Hero 3+ was paired with a Song Meter SM2+ audio recording unit and recorded ambient colony sound and colony behavior. Audio and video recordings were used to examine agonistic encounters within the colony and determine whether different vocalizations are made in response to different stimuli. The penguins in this study were not tagged or otherwise marked and I did not therefore have demographic information on age or relationships among individuals. Behavioral observations were made using BORIS Behavioral Observation Software (Friard and Gamba 2016) and acoustic measurements were made using Raven Pro Sound Analysis Software (Bioacoustics Research Program 2014). All statistical analyses were run in R.

Conspecific Interactions

For the interaction analysis, 34 individuals were selected from 5 sites as focal subjects and observed for at least two bouts of 20 minutes each, resulting in 1317 subject-minutes of observation. During each observation bout, encounters were recorded between the focal subject gentoo penguin and any other individual (including other species, e.g. snowy sheathbill) and the level of reaction from the focal subject was recorded. Encounters were defined as another individual approaching the nest of a focal subject, and reactions were categorized according to an ordinal scale representing increasing levels of reaction and aggression (Table 4-2). For every interaction, the focal subject and the stimulus individual were identified and recorded, as were other characters related to the focal subject including the density of nests in close proximity. I recorded the time since last encounter involving the focal subject, as well as the time since the focal subject's last encounter with the specific stimulus individual.

Vocalizations

For the acoustic analysis, 373 gentoo penguin vocalizations from 5 sites were categorized with the behavior associated with the vocalization – whether it was a reaction to a conspecific, a mate, another species, or had no apparent stimulus (Table 4-2). The calls that had no observed behavioral stimulus were assumed to be ecstatic display calls, used for partner identification and bonding. These calls followed the typical pattern of a repeated series of low frequency exhales and broad band higher-pitched inhales, or were calls made during an interaction with another individual. Calls were included in the analysis if they were distinguishable as a single

individual's call, rather than a mutual display call between mates or a group call in which more than one individual calls at the same time (see Chapter 3). Behavioral stimuli were determined by observing any interactions prior to and following each vocalization. A subset of 138 of these calls was selected from two sites (Georges Point n=79, Petermann Island n= 59) for detailed analysis of the acoustic parameters of the first syllable and characters of the entire call. For these parameters, only the first exhale syllable of each call was measured, as many of the conspecific interaction calls had only one syllable (Table 4-3). Acoustic measurements were made in Raven Sound Analysis Software (Bioacoustics Research Program 2014) (window size=625 samples, overlap=65%, DFT size=2048 samples).

Statistical Analyses

Acoustic parameters of the subset of 138 vocalizations were analyzed separately by colony, given the potential for large variation in acoustic parameters between colonies (Lynch and Lynch 2017). A Principal Components Analysis (PCA) was run on the acoustic parameters of the subset of 104 calls to visualize differences in parameter space based on behavioral stimulus. As a way to incorporate all of the variation in the acoustic measurements not captured in the first two principle components, a non-parametric permutation test (n=5000) on a multivariate analysis of variance (MANOVA) F statistic was run to quantify the effect of behavioral stimuli on the suite of acoustic measurements of the first syllable of each call. All acoustic measurements were standardized (mean=0, SD=1) before analysis.

For each encounter, the time (in seconds) since the last encounter and the time since the last encounter with the same stimulus individual were calculated. For each focal subject and each

stimulus penguin, encounter rate was calculated as the number of encounters per minute of observation, and reaction frequency was calculated as the percent of encounters that resulted in a reaction. Since many reactions involved multiple behaviors, reactions were ordered in terms of escalating aggression, from no reaction at the lowest end to the nesting penguin moving off the nest to chase another away at the highest end. As these categories are not mutually exclusive, many encounters involved more than one reaction type of response. The highest escalation of nest density of the focal subject was estimated as the number of penguins nesting within approximately two penguin body lengths from the focal penguin's nest, an estimate of how many nesting penguins the focal subject could potentially interact with. Generalized estimating equations (GEE) with an independent correlation and a binomial distribution were used to test if these variables predicted whether or not a focal penguin would react in any given encounter, and whether or not that reaction would involve a vocalization. GEEs provide a semiparametric estimate of population-averaged effects, and can accommodate repeated sampling of subjects over time and an unknown correlation structure between covariates. While both GEEs and generalized linear mixed models (GLMMs) can model non-independent binary responses, the GEE estimates the population average log odds, while the GLMM estimates a mean-zero effect with random effects for each individual (Liang and Zeger 1986, Hubbard *et al.* 2010). Ordinal logistic regression was used to test if these variables predicted the level of the maximum response in any given encounter. Continuous variables were standardized (mean=0, SD=1) before analysis.

Table 4-1: Names and locations of gentoo penguin breeding colonies, sampling season, and which analyses were performed. A subset of vocalizations from sites with asterisks were used for acoustics analyses.

Colony Name	Latitude	Longitude	Season	Analyses
Cape Tuxen	-65.267	-64.118	2014-15	Interactions
Dorian Bay Beacon	-64.811	-63.511	2014-15	Interactions
Georges Point	-64.669	-62.670	2014-15	Vocalizations*, Interactions
Port Lockroy	-64.825	-63.494	2014-15	Vocalizations
Mikkleson Island	-63.902	-60.792	2014-15	Vocalizations
Petermann Island	-65.173	-64.135	2014-15	Vocalizations*, Interactions
Pleneau Island	-65.104	-64.056	2014-15	Vocalizations
Waterboat Point	-64.824	-62.858	2018-19	Interactions

Table 4-2: Categories of gentoo penguin reactions to conspecific encounters, ordered by increasing aggressiveness. The frequency for each category is calculated as number of that specific reaction category divided by total number of reactions. Each category was not mutually exclusive and many reactions involved multiple reaction categories.

Reaction Category	N	Frequency (total reactions=601)
Craning neck	566	0.942
Open beak/snaps	378	0.630
Vocalizes	135	0.225
Stands/sits upright on the nest	72	0.120
Chases	5	0.008

Table 4-3: Descriptions of spectrogram measurements used in analysis. Measurements were chosen from a suite of measurements in Raven Sound Analysis Software (reproduced from Lynch and Lynch 2017).

Measurement	Description
Duration 90%	The difference in time between two points that contain the lower 5% and upper 5% of time. (i.e. the middle 90% of the time)
Center Frequency	The frequency that divides the selection into two frequency intervals of equal energy (i.e. the 50% frequency). A more robust measure than Peak Frequency alone
5% Frequency	The frequency that divides the selection into two frequency intervals containing 5% and 95% of the energy in the selection
95% Frequency	The frequency that divides the selection into two frequency intervals containing 95% and 5% of the energy in the selection
Peak Frequency Contour Average Slope	An average of peak frequency contour slope across the entire selection
Peak Frequency Contour Inflection Points	The number of inflection points in the trace of peak frequency across the entire selection

4.4 - Results

Conspecific Interactions

Of the 842 conspecific encounters recorded, 71% resulted in an interaction (n=598). The most common reaction was craning neck (94%), followed by open beak and/or snapping beak (63%). Less common were reactions involving vocalizing (22%), standing up (12%) or chasing the stimulus individual away (0.8%). These reactions most commonly scaled in above order, in that it was rare to observe, for example, a focal penguin vocalize without also craning its neck (n=4). There was a large amount of variation in how frequently encounters occurred for focal subjects, ranging from 0-105 total encounters (median=11.5), and 0-1.925 encounters per minute (median=1.0). Reaction frequency ranged from 0 -100%, with the median reaction frequency was 77%. Number of encounters between the same two individuals ranged from 0-71, with a median of 4.

Examining univariate relationships, I found a large amount of variation across all response variables, and a significant positive relationship between the focal subject's encounter rate and their reaction frequency (Figure 4-1). Multivariate model results indicated that the focal subject's overall encounter rate ($p<0.001$), as well as the number of previous times they have encountered the specific stimulus individual ($p=0.02$), were significant predictors of whether or not the focal subject will react in any given encounter. There was a negative relationship between the number of times the focal subject had previously encountered the stimulus individual and the probability of reaction. The number of previous encounters with the same stimulus individual had a significant, negative effect on whether or not focal subject reacted with a vocalization ($p=0.03$), while the number of previous encounters in general had a significant, positive effect ($p<0.001$). The ordinal logistic regression results indicated that the maximum reaction level for

any given interaction was also predicted by number of previous encounters with the same stimulus individual ($p < 0.01$) and overall encounter rate ($p < 0.01$) (Table 4-4).

Vocalizations

Vocalizations resulting from a conspecific interaction made up 62% of all calls in the analysis ($n=232$), while calls with no identified stimulus made up 29% ($n=108$). Vocalizations without any observable stimulus generally followed the pattern of the ecstatic display call, with a repeated series of low frequency exhales (“purrs”) and higher-frequency broadband inhales (“honks”). However, vocalizations from conspecific interactions had fewer syllables (mean number of syllables = 1.9 vs 5.9) and most frequently consisted of 1 or 2 low frequency “purrs” and 0 “honks” (mean number of purrs=1.7 vs 3.5). The initial syllables of the conspecific reaction vocalizations also had lower center frequency than the ecstatic display call (296 Hz vs 866 Hz) and was shorter in duration than the ecstatic display call (0.36 seconds vs 0.77 seconds) (Figure 4-2).

When acoustic parameter space was compared in the PCA, the conspecific reaction calls ($n=54$) differed in parameter space from those with no observed stimulus ($n=50$), indicating generally lower values in all acoustic measures except for center frequency slope (Figure 4-3). The first and second principle components combined accounted for approximately two thirds of the variation in the dataset for each colony. The permutation test of the MANOVA F-statistic results showed that there was significant amount of variation in acoustic parameters associated with behavioral stimulus ($F=2.86$, $p=0.02$; $F=12.12$ $p < 0.001$ for Petermann Island and Georges

Point, respectively), but this result was not significant when examining variation due to individual ($F=1.22$ $p=0.34$; $F=2.06$ $p=0.08$) (Figure 4-4).

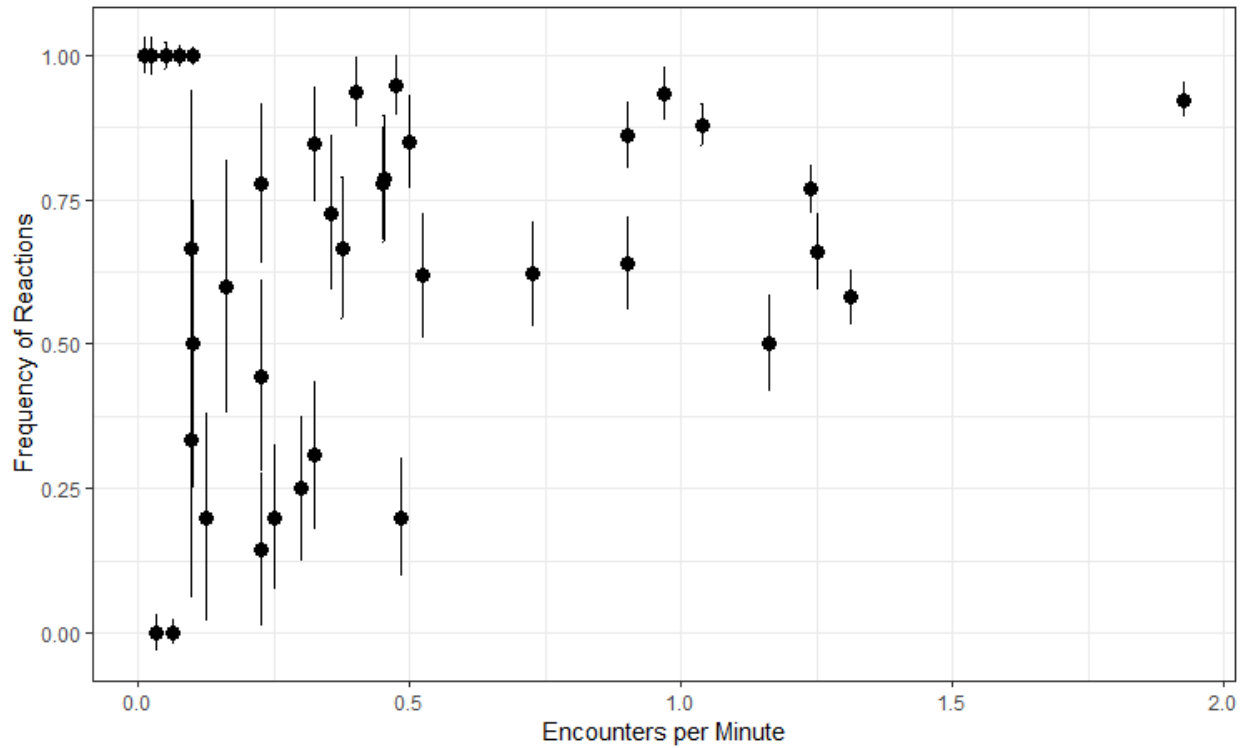


Figure 4-1: Plot of encounter rate and reaction frequency (estimate and 95th percentile confidence interval) for the frequency of reaction as a function of the number of previous encounters.

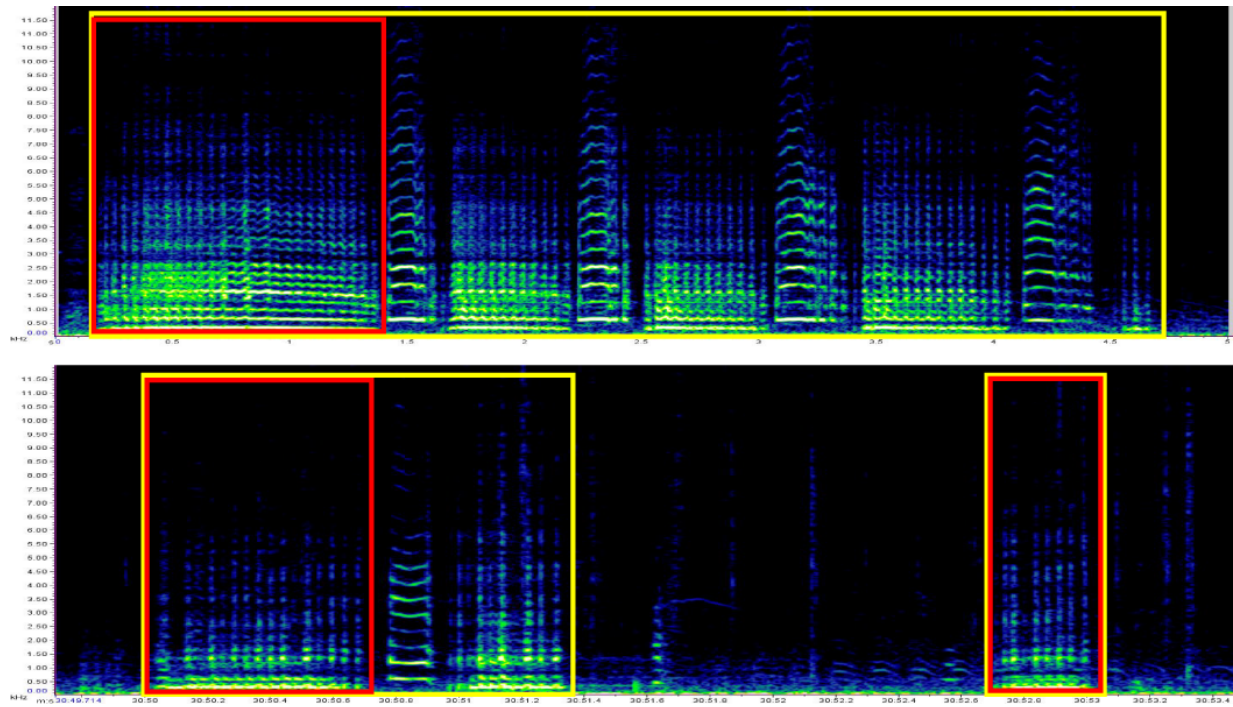


Figure 4-2: Spectrograms of (a) an ecstatic display call and (b) two separate agonistic calls from the same individual, both from Petermann Island. The x-axis indicates time in seconds and the y-axis indicates frequency in kHz. Darker hues indicate more power at a given frequency. The yellow boxes denote the duration of the entire call and the red boxes denote the first syllable of each call. Acoustic measurements of the first syllable were used in statistical analyses.

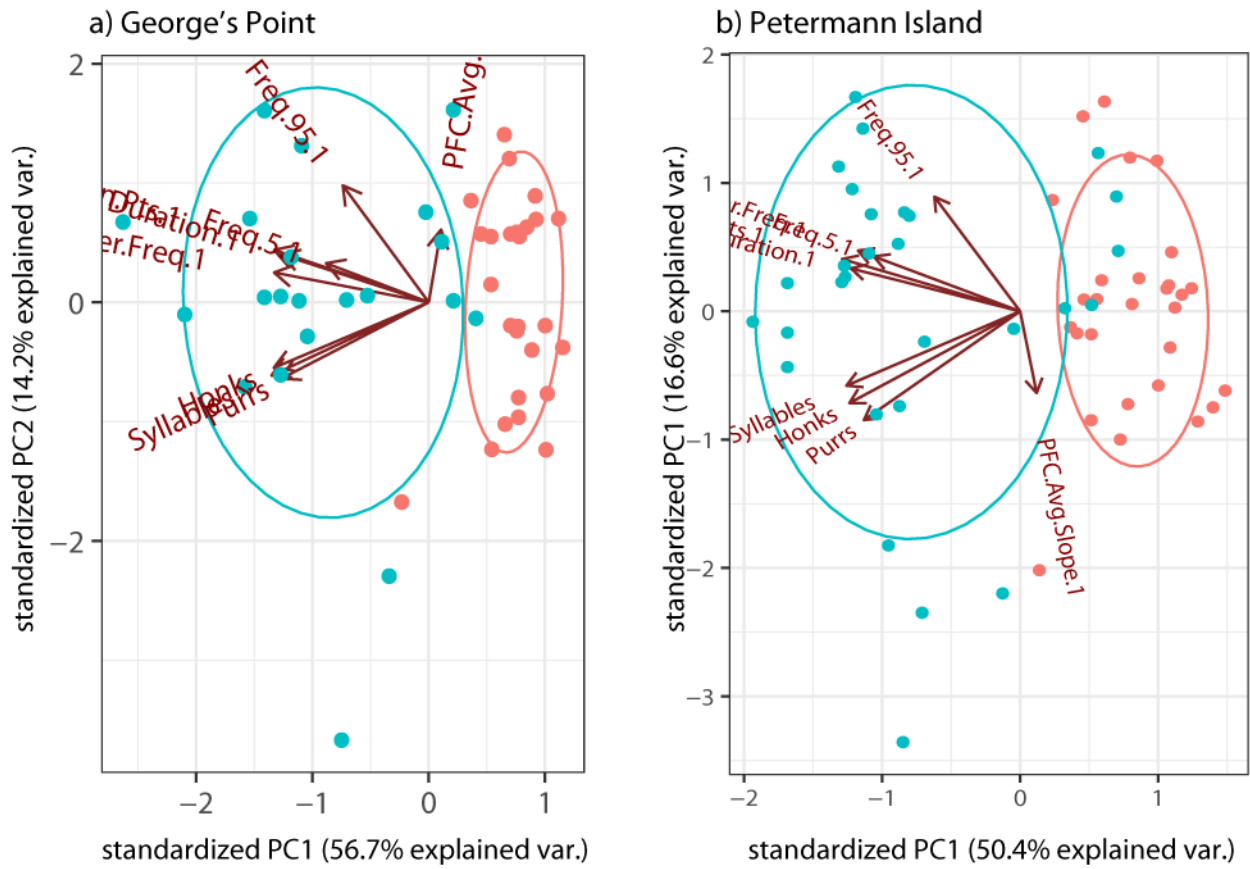


Figure 4-3: Plots of the first and second principle components from the PCA of acoustic measurements of the first syllable of the call from, Georges Point (a) and Petermann Island (b). Teal circles indicate calls made with no apparent behavioral stimulus, while orange circles indicate calls made in conspecific interactions.

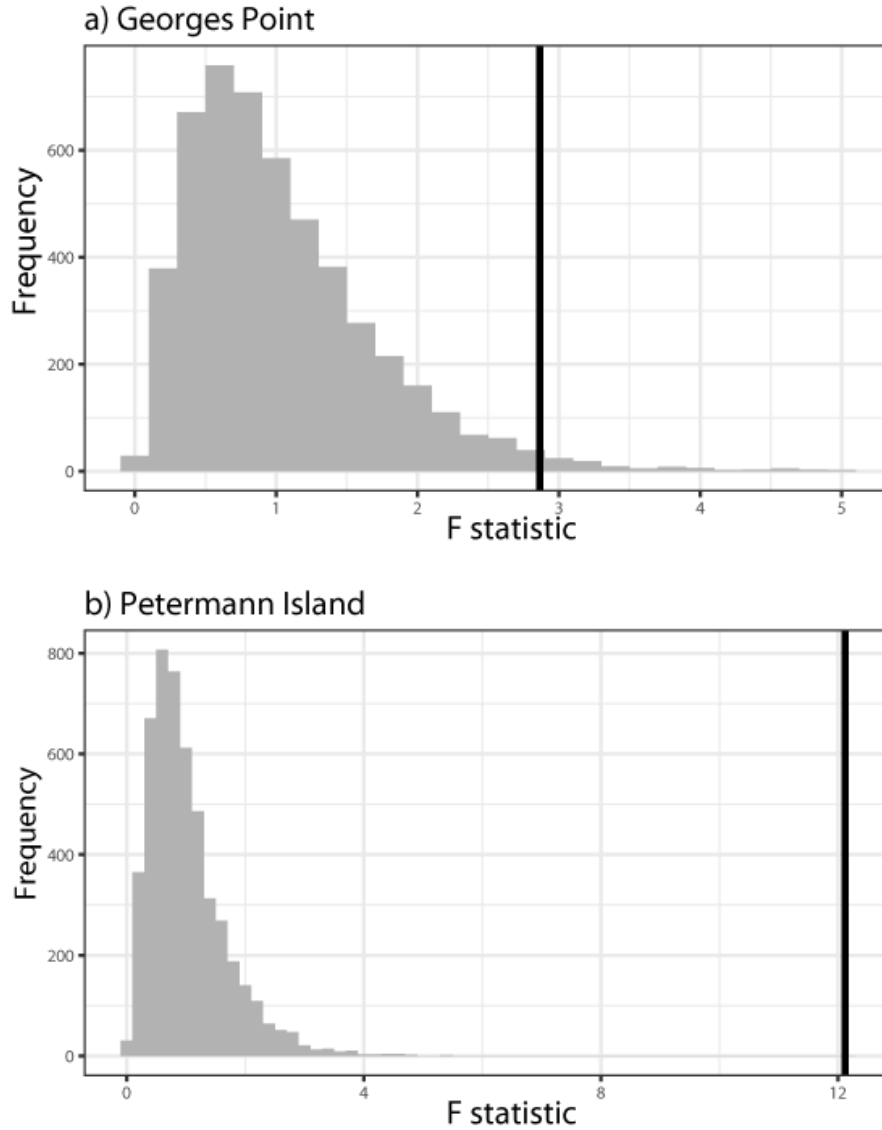


Figure 4-4: Histograms of permuted MANOVA F statistics on a suite of acoustic measurements of the first syllable of each call, compared with the observed F statistic (black line), at two gentoo breeding colonies Georges Point (a) Petermann Island (b). The p value is calculated as the percentage of the permuted F statistics that are greater than the observed F statistic.

Table 4-4: Model results from generalized estimating equations and ordinal logistic regression, showing the coefficient estimate, standard error, test statistic value, and p value for each predictor variable. Asterisks indicate p values <0.05 (*), <0.01 (**), and <0.001(***).

Generalized Estimating Equation				
Reaction (Y/N) ~ Nest Density + Time Since Last Encounter + Time Since Last Encounter with Same + Encounter Rate + Number of Encounters + Number of Encounters with Same				
Variable	Estimate	Robust S.E	Robust Z	p
Intercept	-0.062	0.239	-0.261	0.777
Nest Density	-0.149	0.123	-1.215	0.148
Time Since Last Encounter	-0.149	0.121	-1.751	0.097
Time Since Last Encounter w Same	-0.065	0.119	-0.547	0.538
Encounter Rate	1.274	0.293	4.347	3.543x10 ⁻⁶ ***
Number of Encounters	0.001	0.006	0.223	0.815
Number of Encounters w Same	-0.021	0.012	-1.726	0.020 *

Generalized Estimating Equation				
Vocalization (Y/N) ~ Nest Density + Time Since Last Encounter + Time Since Last Encounter with Same + Encounter Rate + Number of Encounters + Number of Encounters with Same				
Variable	Estimate	Robust S.E	Robust Z	p
Intercept	1.937	0.256	-7.553	9.806 x10 ⁻¹²
Nest Density	-0.190	0.151	-1.261	0.165
Time Since Last Encounter	-0.151	0.250	-0.603	0.469
Time Since Last Encounter w Same	-0.043	0.079	-0.548	0.750
Encounter Rate	-0.008	0.267	-0.028	0.980
Number of Encounters	0.020	0.006	3.428	7.908 x10 ⁻⁴ ***
Number of Encounters w Same	-0.020	0.010	-2.058	0.034 *

Ordinal Logistic Regression				
Reaction Category ~ Nest Density + Time Since Last Encounter + Time Since Last Encounter with Same + Encounter Rate + Number of Encounters + Number of Encounters with Same				
Variable	Estimate	S. E.	t	p
Nest Density	-0.151	0.085	-1.776	0.076
Time Since Last Encounter	-0.002	0.001	-1.753	0.080
Time Since Last Encounter w Same	-0.000	0.000	-0.723	0.470
Encounter Rate	0.520	0.175	-0.723	0.003 **
Number of Encounters	0.003	0.004	2.967	0.400
Number of Encounters w Same	-0.017	0.006	0.842	0.004 **

4.5 - Discussion

Conspecific Interactions

Conspecific encounters are frequent in gentoo penguin colonies, as individuals frequently walk near occupied nests while moving about the colony, or actively attempt to steal nesting material from occupied nests. The overwhelming proportion of these encounters result in an agonistic response from the nesting penguin, but almost one quarter of encounters do not elicit a response. These encounters are also not spread evenly among the individuals within the colony, with some penguins experiencing much higher encounter rates than others. In this study, I had 7 focal subjects with encounter rates lower than 0.05 encounters per minute, and 6 focal subjects with more than one interaction per minute. Adding to the variability, these encounters are not evenly spaced in time, with focal subjects experiencing bouts of encounters, often from the same individual. These agonistic interactions were observed much more frequently than interactions with predators ($n=0$) or other non-predator species (e.g. snowy sheathbill, $n=5$). These interactions therefore play an important role in the daily life of gentoo penguins during the breeding season and represent an important element (and potential cost) in colonial life.

I find that gentoo penguins with higher encounter rates also have a higher frequency of agonistic reaction, which indicates that perhaps there is a cumulative effect of the disturbance from past encounters on any given individual. However, I also find a large amount of variation in response frequency for individuals with lower encounter rates. This may be the result of some individuals being overly reactive compared to their counterparts, or it could be a relic of earlier encounter history that was not recorded.

The importance of past history of an individual is also seen in the multivariate model results, in which both the encounter rate and the number of previous encounters with the same stimulus individual had a significant effect on the binary outcome of whether or not the focal subject would react, as well as the ordinal level of reaction. However, when I limit these data to only reaction, reaction frequency is the only significant predictive factor of the level of the reaction. There is a positive relationship between reaction frequency and level of reaction, indicating that these individuals react more frequently and in a more aggressive manner than their counterparts. Similar variables significantly predict whether or not an individual will vocalize in an encounter. Considering all encounters, the number of previous encounters for that focal subject as well as the number of previous encounters with the stimulus individual are both significant predictors of whether or not the focal penguin will vocalize in the encounter. However, if look only at encounters resulting in a reaction, only the number of previous encounters remains significant.

All of these results indicate that past encounter history influences when and how an individual gentoo penguin may react to an encounter with a conspecific in the colony. Individuals that experience heightened rates of encounters are likely to react more frequently and more aggressively, and are more likely to vocalize during the encounter. There are individuals with low encounter rates that react frequently and strongly, but this may be a result of previous, unobserved encounters or may indicate that some individuals are simply more reactive than others. It is also notable that nest density does not affect whether an individual will react or to what degree. In most cases, individuals with a higher nest density had lower encounter rates, as did individuals without any other nests nearby. This is likely influenced by local small scale topography and colony microhabitat, both of which help determine the location of common

walkways through the area. Presumably in part due to the aggressive reactions of the nesting penguins, it is easier for a penguin to walk around densely packed groups than walk through them. Penguins nesting by themselves are also less likely to have a penguin pass in close proximity in unless it is explicitly trying to steal nesting material.

Vocalizations

Given the frequency of agonistic vocalizations in the colony, it is important to note the novel characters of the agonistic vocalization. I see distinct differences in acoustic measurements when compared to the first syllable of the ecstatic display call, leading us to conclude that the agonistic call is not simply an abbreviated version of the ecstatic display call. Further investigation via playback experiments may help us better understand the specifics of their use and how other penguins react to them.

These results illuminate an important aspect of gentoo penguin breeding behavior. Behaviors related to mate choice, mate bonding, and parental care have been examined in multiple penguin species and are critical elements to understanding penguin life history. Coloniality in seabirds such as gentoo penguins provides many benefits, including increased protection from predators, as well as being a practical reaction for a central place forager with heterogeneous food sources. However, another often underappreciated element of group living is the time and energy spent on conspecific agonistic interactions. Both physical posturing and vocal behavior are energy intensive in a harsh physical environment, and risk harm when physical postures expose eggs or chicks. While these interactions are unlikely to result in a direct decrease in health or reproductive success, the uneven pattern of these agonistic behaviors puts a

higher burden on some individuals within the colony. Further work can be done to examine more long-term trends in agonistic behavior and any adverse consequences of heightened rates of encounters and reactions.

5 – Tourism and stress hormone measures in gentoo penguins on the Antarctic Peninsula

5.1 - Abstract

The impacts of tourism on wildlife have long been a concern in areas where ecotourism is a major industry. The issue is especially pressing in Antarctica, which has a rapidly growing tourism industry largely concentrated around penguin colonies on the Antarctic Peninsula. Guidelines developed by both the Committee for Environmental Protection and the International Association of Antarctica Tour Operators include measures to minimize wildlife impacts. In this study, I examined the relationship between physiologic stress in gentoo penguins (*Pygoscelis papua*) and tourism. Corticosterone is an adrenal glucocorticoid that has been shown in previous studies to increase in response to stressors such as low food availability, environmental conditions, as well as human visitation and proximity. Fecal glucocorticoids (FGM; primarily corticosterone and metabolites) were measured in gentoo penguin guano collected at 19 breeding colonies (n=108, 3-10 samples per site) on the Antarctic Peninsula and the South Shetland Islands, representing a wide range of tourism visitation. I found a large degree of variation in FGM concentrations, and no relationship between FGM concentrations and number of tourists landed at that site. These results suggest that current tourism management guidelines on the Antarctic Peninsula are effective at preventing endocrine indication of increased and chronic stress in gentoo penguins, and demonstrate the use of guano as a non-invasive, low-impact methodology for monitoring gentoo penguin stress.

5.2 - Introduction

Tourism in the Antarctic has grown rapidly over the last five decades, from the first trip in 1969 to the more than 58,000 tourists who visited Antarctica in the austral summer of 2017/18 (IAATO 2018). Given this rapid increase, there is a concern that human activities may have a negative impact on wildlife, particularly on the Antarctic Peninsula where the overwhelming majority of all tourism activity takes place (Lynch *et al.* 2010, Bender *et al.* 2016). Due to the difficulty of landing tourists in the Antarctic, activity on the Antarctic Peninsula is highly spatially concentrated, with more than 76% of landings occurring on a total of 200 hectares of land (Bender *et al.* 2016). Adding to concerns about wildlife impacts, the most heavily visited sites usually have penguins that are either incubating eggs or guarding chicks at the time of visitation.

In the context of human-wildlife interactions during tourism visits, the Antarctic Treaty has adopted general guidelines for tour ship operations and a series of site-specific visitor guidelines for key tourism locations. This was done in consultation with the International Association of Antarctica Tour Operators (IAATO), with all proposed guidelines reviewed by the Treaty's Committee for Environmental Protection (CEP). The first Visitor Guidelines were adopted at the 1994 Antarctic Treaty Meeting, following the 1991 Protocol on Environmental Protection to the Antarctic Treaty, which designated Antarctica as a natural reserve, and set out environmental obligations for all tourist or scientific operations. These guidelines include site-specific regulations as well as general practice guidelines, the latter of which include limiting the number of landed visitors at a location to 100 at any one time and enforcing a 5 meter minimum distance from nesting penguins and greater distances from other nesting seabirds (e.g., Southern Giant Petrels) and mammals (e.g., elephant seal wallows). These guidelines also require

maintaining a 1:20 guide: tourist ratio, strict prohibitions against depositing garbage on land or at sea, and against removing any biological or non-biological material from visitor sites. All visitor sites share these guidelines while certain sites of historical or ecological sensitivity have even tighter restrictions placed on the number of landed visitors at any given time, or are closed during certain parts of the season. However, even with these restrictions in place, some popular sites receive more than 21,000 visitors each year (IAATO 2018), and as such, understanding both acute and cumulative wildlife impacts remain an area of active research (e.g., Coetzee and Chown 2016).

Previous work on the effects of human activities on penguins (in the Antarctic and elsewhere) has shown mixed results depending on the species and the response metric used (Coetzee and Chown 2016). Adélie penguins (*Pygoscelis adeliae*) display increased heart rates when approached by humans (Culik *et al.* 1990), whereas heart rates in gentoo penguins (*Pygoscelis papua*) are not elevated when humans approach slowly and remain at a distance of at least 3 m (Nimon *et al.* 1996). Gentoo penguins do, however, show increased threat displays towards humans and increased vigilance behaviors even after a human stimulus has been removed (Holmes 2007). Human disturbance increased nest desertion, resulting in increased nest predation, in African penguins (*Spheniscus demersus*) (Hockey and Hallinan 1981). Conversely, no effect was seen on reproductive success and fledging success due to tourism in Adélie penguins (Carlini *et al.* 2007) or gentoo penguins (Cobley and Shears 1999, Holmes *et al.* 2006). Elevated concentrations of stress hormones have been observed in areas with heavy tourism impact in Magellanic penguins (*Spheniscus magellanicus*) (Walker *et al.* 2005) and Yellow-eyed penguins (*Megadyptes antipodes*), resulting in decreased fledging weights and breeding success

(Ellenberg *et al.* 2007). The indirect effect of gentoo penguin nest predation by skuas (*Catharacta* spp.) was found to be unaffected by tourist presence (Crosbie 1999).

Another group of studies suggests habituation, in which repeated exposure eventually decreases the magnitude of the response to that stimulus, may play a role in mediating responses to human activity. Previous work has shown this effect with decreased flight responses in areas of heavy tourism compared to those with low levels of human interaction, in African (van Heezik and Seddon 1990) and Magellanic penguins (Walker *et al.* 2005) and decreased threat displays and vigilance in gentoo penguins near a scientific station compared to those breeding farther from the station (Holmes *et al.* 2006). While Yellow-eyed penguins showed increased heart rate in response to human approach, short and consistent approaches allowed them to habituate and diminished the elevated heart rate response (Ellenberg *et al.* 2009). Habituation has also been seen in stress hormone response, with lower and more consistent corticosterone concentrations in heavily visited areas in Magellanic penguins (Fowler 1999, Walker *et al.* 2006) and in gentoo penguins (Barbosa *et al.* 2013). In the case of such habituation, I would expect a quadratic relationship between stressor and metric (Busch and Hayward 2009).

In this study, I examined the impacts of tourism on the Antarctic Peninsula on gentoo penguins using measurement of fecal corticosterone and its metabolites (FGM) as a measure of adrenal glucocorticoid stress response. When short-term stress is experienced, elevated concentrations of corticosterone promote physiological coping mechanisms (e.g., creation of glucose from energy stores, increased cerebral blood flow, immune system regulation, etc.). However, continued exposure to stressors accompanied by chronically heightened levels of corticosterone has been shown to be detrimental to reproductive success, including increased nest abandonment, decreased chick provisioning, or impaired reproductive system function

(Groscolas *et al.* 2008, Busch and Hayward 2009, Spée *et al.* 2010, Thierry *et al.* 2013a, 2013b). As with other response metrics, chronic exposure to a stressor may result in habituation, resulting in the down-regulation of the adrenal system in which both circulating and levels of corticosterone are suppressed (Rich and Romero 2005, Busch and Hayward 2009).

Measuring FGM in penguin guano allows for a minimally-invasive approach that does not require handling birds or taking biological samples from them, thus minimizing researcher impact. Sampling from guano (fecal samples) also provides a longer-term more cumulative measure of stress than sampling from blood, generally reflecting less than 24 hours without containing the pulsatile and short-term stress release patterns occurring with measures from blood samples. Guano is therefore a useful metric, especially for examining seabirds on a colony or regional scale (Cavigelli *et al.* 2005, Möstl *et al.* 2005, Young and Hallford 2013). I examined FGM concentrations in penguin guano at 19 breeding colonies along the Antarctic Peninsula, representing the largest geographic range of a study of physiologic stress in penguins (Figure 1). I also used detailed tourism data on the number of tourists visiting each breeding colony to examine the relationship between visitation and stress hormone concentration.

5.3 - Methods

Guano was collected from 19 gentoo penguin breeding colonies along the Antarctic Peninsula and the South Shetland Islands in 2017-18 and 2018-19 (Figure 5-1, Table 5-1). In each season, samples were collected within one hour of each other on a single day for each colony. Guano samples (~10g, n = 108) were collected non-invasively from freshly excreted samples on the ground within the breeding colony. Given that samples were collected on the

ground, and given the density of gentoo penguin nests within a colony, each sample has a possibility of including more than one excretion, potentially from more than one individual, and cannot be identified to an individual of known sex or age. Though corticosterone measures have been shown to vary by sex, age, reproductive status, and other individual factors (Cockrem 2013), my sampling was opportunistic and, though not randomized, is unlikely to be significantly different in terms of age or sex composition between colonies, which at any point contain both sexes and a range of ages. Samples were dried in a food dehydrator set on low (approximately 110°) for 8-12 hours and stored at room temperature until processing (adapted from Galama *et al.* 2004).

Dried guano samples were crushed, and 0.2 grams were weighed out and placed into a 16mL borosilicate glass vial. 5mL of 100% ethanol was added to each tube and then briefly vortexed, before being placed on a plate shaker for 1hr at 650 rpm and finally centrifuged at 650-G for 20 minutes. Supernatant was carefully removed to avoid collecting any fecal sample and placed into a clean borosilicate vial. Guano tubes were rinsed with another 5mL of 100% ethanol, briefly vortex and spun down (650-G for 20 minutes) and supernatant was again collected and combined into appropriate vials and evaporated under air. Dried extracts were reconstituted with 2mL of assay buffer and stored at -80C until analysis. 100uL of each sample was analyzed using a commercially available kit (Corticosterone ELISA kit, Enzo Life Sciences Inc. USA). Spike recovery was performed using a known standard, recovery was consistently above 90%, and therefore no further adjustment of concentrations was made as there was low variability among samples. Assay characteristics included parallelism of standard curve with a serial dilution of extract; this was also used to determine the dilution of extract measurable on the standard curve. Assay sensitivity was 27 pg/ml. Antibody cross reactivity was corticosterone

(100%), deoxycorticosterone (21.3%), desoxycorticosterone (21.0%), progesterone (0.46%), testosterone (0.31%), tetrahydrocorticosterone (0.28%), aldosterone (0.18%), cortisol (0.046%) and <0.03%: pregnenolone, estradiol, cortisone, 11-dehydrocorticosterone acetate. As such, the assay measured primarily corticosterone and two primary metabolites. Intra-assay variability was CV=3.56% and inter-assay variability was C=2.09%. The final concentration of FGM was expressed as micrograms of FGM per gram of guano.

Several population-level variables were recorded for each sampled colony, including gentoo penguin abundance and annual growth rate for the colony (extracted from www.penguinmap.com; Humphries *et al.* 2017), as well as tourism visitation on time scales ranging from one day prior to sampling to the total landings for the season (Stanwell-Smith, IAATO, personal communication). The measure of tourist landings was “small boat landings” as defined by IAATO, in which passengers were physically on shore at the colony. Wild birds were not identified to individual so no individual-level parameters were recorded.

I fit a linear regression model with both linear and quadratic terms to examine the relationships between log-FGM (in sample j at site i in year t) and the annual growth rate of the colony (averaged over 6-10 years, depending on data availability) (Growth Rate_i), the abundance of gentoo penguin breeding pairs (averaged over both years of sampling if applicable) (Abundance_i), the number of landed passengers in the 24-hour period prior to sampling ($\text{1Day Landings}_{it}$), and a linear and quadratic interaction term to capture the potentially non-linear influence of longer-term visitation (the total number of landed passengers that season [$\text{Season Landings}_{it}$]). Abundance and growth rate are included as proxies for unmeasured demographic factors such as reproductive success in the colony. The interaction terms are included because ongoing exposure to landed passengers may habituate penguins and

thereby affect the influence of visitation as captured in 1Day Landings_{it}. Specifically, mean log-FGM for colony *i* in year *t* (μ_{it}) was modeled as:

$$\begin{aligned} \mu_{it} = & \alpha + (\gamma_1 * \text{Growth Rate}_i) + (\gamma_2 * \text{Abundance}_i) + (\beta_1 * \text{1Day Landings}_{it}) \\ & + (\beta_2 * \text{1Day Landings}_{it} * \text{Season Landings}_{it}) \\ & + (\beta_3 * \text{1Day Landings}_{it} * \text{Season Landings}_{it}^2) \end{aligned}$$

I assumed normally distributed residuals, so that log-FGM for sample *j* at site *i* in year *t* (Y_{itj}) is given by

$$Y_{itj} \sim N(\mu_{it}, \sigma^2)$$

where μ_{it} is the mean log-FGM at colony *i* in year *t*, and σ^2 represents intra-sample variation within the colony. I estimated model parameters in a Bayesian framework, using broad prior distributions for all parameters, i.e. a normal distribution for the α , γ , and β parameters [$N(\mu=0, \sigma=0.001)$] and a Gamma distribution for the variance parameter σ^2 [$\text{Gamma}(k=0.001, \theta=0.001)$]. Models were run for 100,000 iterations with a burn-in of 1,000 iterations.

While the above model is the one that best captures the key covariates of interest for my analysis, I used a series of t-tests to explore the inclusion of additional covariates that might have explained some of the residual site-level variation, including the presence/absence of a research station at the breeding colony, sampling year, time of day, and whether the colony was a mixed-species breeding colony.

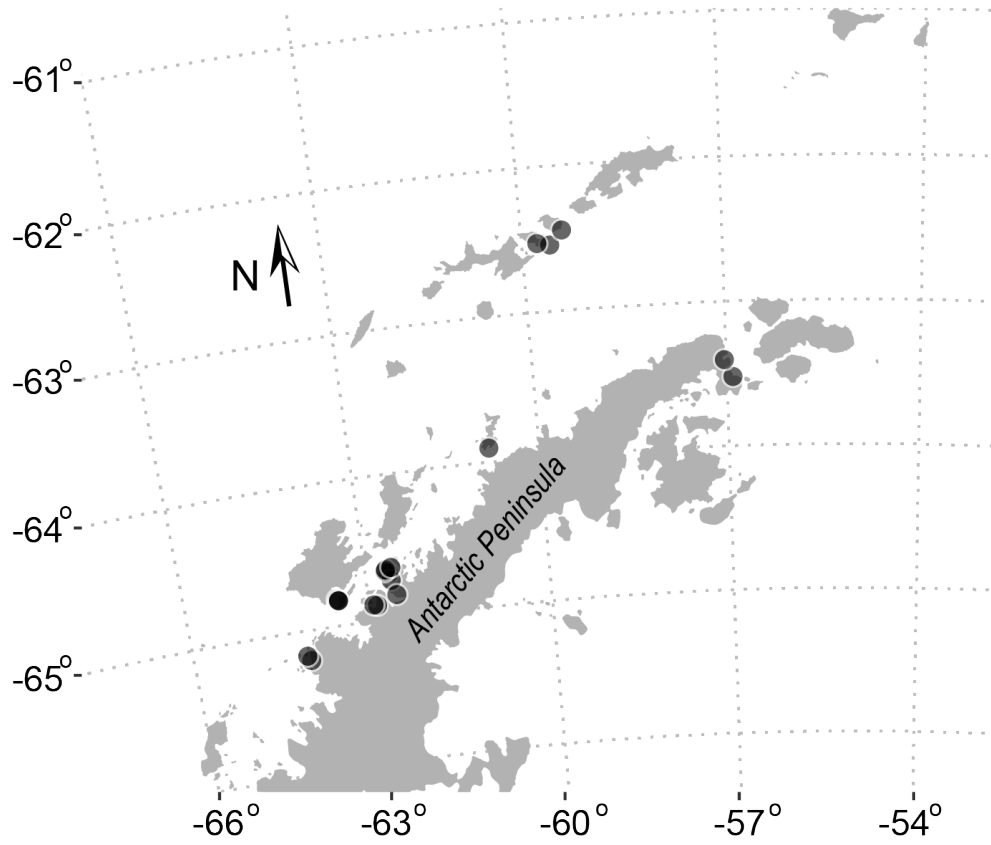


Figure 5-1: Sample locations on the Antarctic Peninsula and the South Shetland Islands.

Table 5-1: Full names and location of sampled gentoo penguin breeding colonies, with number of samples, mean, and standard error of FGM concentration at each colony as well as IAATO landings per season and gentoo penguin abundance. For colonies with two years of sampling, the mean of both years' season landings and gentoo penguin abundance are reported here with an asterisk, however data specific to each sampling year were used for analysis.

Code	Colony Name	Latitude	Longitude	Samples	Mean (Standard Error) FGM (µg/g)	IAATO Season Landings	Abundance (breeding pairs)
ALMI	Brown Station	-64.896	-62.870	8	0.6958 (0.1285)	10,078*	177*
BROW	Brown Bluff	-63.522	-56.905	3	0.5161 (0.0122)	11,229	711
BRYE	Bryde Island East	-64.890	-62.927	8	0.6346 (0.0369)	0*	520*
CUVE	Cuverville Island	-64.682	-62.621	9	0.4488 (0.0913)	16,311	6903
DAMO	Damoy Point	-64.816	-63.510	6	0.9212 (0.2309)	8432*	2129*
DANC	Danco Island	-64.734	-62.594	8	0.3028 (0.0329)	10,520*	2732*
FORT	Fort Point	-62.543	-59.579	6	0.4002 (0.0670)	1060	1006
GEOR	Georges Point	-64.669	-62.670	3	0.4684 (0.1203)	1074	3949
HOPE	Hope Bay/Esperanza Station	-63.404	-57.026	6	0.8870 (0.1520)	769	519
JOUG	Jougla Point	-64.828	-63.492	6	0.5368 (0.1350)	8200*	1111*
LOCK	Port Lockroy/Goudier Island	-64.825	-63.494	6	0.6615 (0.1660)	16,653*	548*
MIKK	Mikkleson Island	-63.902	-60.792	3	0.7329 (0.2591)	8820	1262
MOOT	Moot Point	-65.206	-64.078	6	0.7243 (0.0496)	0	693
NEKO	Neko Harbour	-64.838	-62.533	10	0.8105 (0.0591)	21,504	973
ORNE	Orne Islands	-64.659	-62.663	3	0.6575 (0.2370)	793	483
PETE	Petermann Island	-65.173	-64.135	5	0.8429 (0.1664)	11,584	3516
ROBE	Robert Point	-62.448	-59.386	3	0.6197 (0.0568)	2496	917
SELV	Selvick Cove	-64.647	-62.571	6	0.6522 (0.1473)	133*	616*
YANK	Yankee Harbour	-62.526	-59.769	3	0.6900 (0.1212)	5233	5466

5.4 - Results

FGM concentrations in this study ranged from 0.127-1.959 $\mu\text{g/g}$. I found large within-colony variation in log-FGM concentrations ($\sigma^2 = 4.006$), which was larger than that between colonies ($\text{var}(\mu_{it}) = 0.017$). There was also large variation in annual tourism visitation at sampled colonies, ranging from 0 (Bryde Island, Moot Point, Selvick Cove) to 21,504 landings per year (Neko Harbour), with a median of 8,420 landings per year (Jougla Point) (Figure 5-2). I found no significant relationships between log-FGM and the presence of a research station ($t=1.303, p=0.445$), the sampling year ($t=0.785, p=0.434$), the time of day ($t=-0.026, p=0.980$), or whether the colony was a mix-species breeding colony ($t=0.441, p=0.241$). At colonies where samples were taken in a variety of location, there was no clear pattern in FGM concentrations between areas more frequently used by tourists than those farther away from common tourist areas, although it should be noted that most areas of those colonies are potentially visited by tourists.

Model results indicated that FGM concentrations are not affected by tourism, including the interaction between landings one day prior to sampling and landings throughout the entire season (Table 5-2). I summarized my parameter estimates using the 95th percentile credible intervals (bounded by the 2.5th and 97.5th quantiles of the posterior distribution), which are analogous to 95th percentile confidence intervals but with a more direct interpretation in terms of the probability distribution of the model parameter. Although a 24 hour period is a priori the most physiologically-meaningful time period to consider given the guano evacuation rates of gentoo penguins, FGM concentrations were also unrelated to visitation over the previous 7 or 14 day windows or the entire season. These results are robust to removing the six colonies with the

lowest sample sizes from the model. The only parameter whose credible interval did not contain 0 was that related to colony abundance.

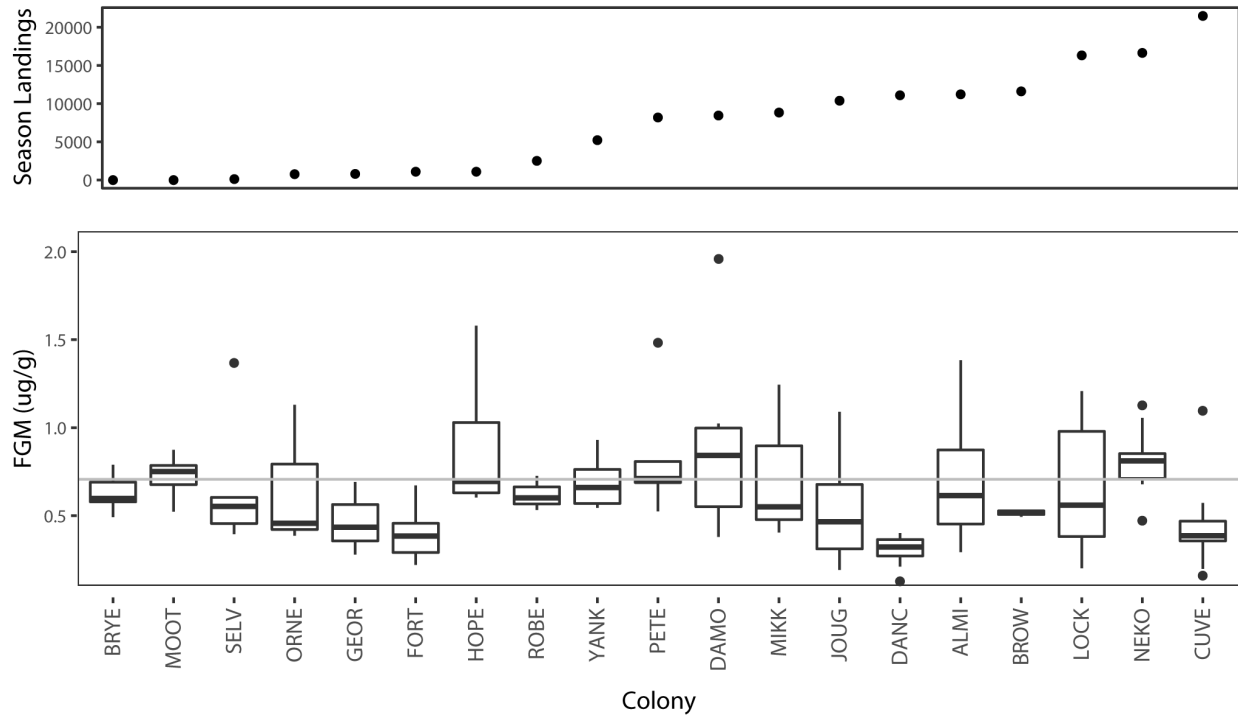


Figure 5-2: Scatterplot showing season landings for each site (top) paired with boxplots demonstrating the variation in FGM concentrations in guano samples at each colony (bottom). The gray line indicates the median FGM concentration for all samples. For colonies with two years of sampling, the mean of both years' IAATO small boat landings per season is reported here, as in Table 5-1. Full colony names are listed in Table 5-1.

Table 5-2: Parameter estimates and credible intervals from a Bayesian model relating log-FGM concentrations to tourism, population growth, and population abundance.

Parameter	Mean Estimate	95% Credible Interval	Description of parameter
α	-0.597	(-0.707, -0.487)	Mean FGM concentration for colony i in year t given mean values for all covariates
γ_1	0.046	(-0.052, 0.144)	Slope parameter for colony growth rate for colony i
γ_2	-0.112	(-0.210, -0.014)	Slope parameter for colony abundance for colony i
β_1	0.034	(-0.225, 0.157)	Slope parameter for 1 day landings for colony i in year t
β_2	0.014	(-0.113, 0.140)	Slope parameter for the interaction between 1 day landings and season landings for colony i in year t
β_3	0.041	(-0.070, 0.153)	Slope parameter for the interaction between 1 day landings and the square of season landings for colony i in year t
σ^2	3.978	(2.952, 5.154)	Variance between samples within colony i in year t

5.5 - Discussion

Given the continuing increase in tourism and the unique nature of environmental management in Antarctica, understanding the impact of tourism on wildlife is an area of substantial concern. Prior studies in a variety of environments have provided a mixed picture of human impacts on penguin populations. Here I use FGM, glucocorticoid stress hormones, in penguin guano to assess whether tourism activities are affecting physiological stress in gentoo penguins, whose colonies are a major draw for the industry.

I found no relationship between FGM concentration and the number of passengers recently landed at a site, nor did I find any indication of an interaction between recent visitors and season total visitors. This suggests that either gentoo penguins do not experience stress related to tourism presence at current levels and under current visitor management regimes, or that other sources of individual and colony variation in hormonal stress are greater than those due to tourism landings alone. However, another possible explanation is that gentoo penguins have become habituated to human presence in and around their colonies and accordingly do not have elevated FGM concentrations in response to tourism operations. While I cannot definitively rule out the effect of habituation given this sampling design, it is worth noting that gentoo penguins at sites with little or no tourism activity do not have higher FGM concentrations than those with heavy tourism, and I found no quadratic relationship between tourism and FGM concentration. Both of these findings are therefore inconsistent with the hypothesis of hormonal habituation at heavily visited sites. Rather, these data suggest that the current IAATO Visitor Guidelines are sufficient to minimize tourism impacts on gentoo penguins compared to other sources in variation, to the extent that impacts are reflected in stress hormone markers.

These results are specific to gentoo penguins and species with overlapping breeding ranges, including chinstrap penguins (*P. antarcticus*) and Adélie penguins, may display different responses to tourism in the same region. These results agree with other studies that indicate no impact of tourism on reproductive success (e.g., Stonehouse 1970, Cobley and Shears 1999) but contrast with the findings of Barbosa *et al.* (2013), who found significantly higher FGM levels in a gentoo penguin colony with high tourist visitation as compared to a nearby colony with little to no tourism impact. However, the study by Barbosa and colleagues (2013) measured corticosterone in feathers, which could reflect a different time scale than that represented in guano. It is also worth noting that this study over a large number of sites finds high levels of inter-site variation that is not explained by patterns of visitation, which suggests that differences between a single pair of sites may be difficult to interpret.

Dunn and colleagues (2018) recently found a negative relationship between trends in gentoo penguin abundance and tourism at Port Lockroy (one of the sites sampled in this study), and cited stress as one potential mechanism for that relationship. While all of my samples from Port Lockroy were from the tourist visited (rather than the closed) area and I cannot directly assess whether the penguins on the visited side of the island are more or less stressed than those in the closed area, I find no evidence for a link between visitation and gentoo penguin FGM levels excreted in guano. The relationship that I found between FGM and colony abundance, though not strong, does suggest that stress responses may be driven by population-level processes, and I concur with Dunn and colleagues (2018) that these processes are likely affected by complex interactions between both intrinsic and extrinsic factors.

I focused this study specifically on tourist visitation from IAATO member companies, yet there are likely to be many factors that contribute to hormonal stress measures. I have also

examined the effect of some of these variables such as time of day, year, and presence of a research base as an indication of increased exposure to human activity, as well as those related to colony abundance and growth which can be related to food availability, habitat quality, or predator interactions. However, there are multiple intrinsic and extrinsic factors not measured here for any given individual at any colony that may affect FGM concentration. My results do not attempt to explain the ultimate drivers of the variation I see between colonies, but do emphasize that this variation does not appear to be driven by tourism or the other factors that I have explored here. It is likely that other individual factors (e.g. sex, age, body condition, frequency of aggression from predators and conspecifics) and colony factors (e.g. food availability, seasonal weather changes, distance to foraging grounds) affect FGM levels to a larger degree than tourism visitation.

Given the large amount of variation found both within and between colonies, continued monitoring is important as Antarctic wildlife management moves forward. Guano sampling, being non-invasive, provides a relatively easy and inexpensive method for doing so regularly. Repeated measures from the same group of individuals may help minimize the inter-sample variation and control for changing demographics over time, while increased sample size within a colony as well increased number of colonies sampled will help clarify these relationships. Adding such physiological markers to current work on population monitoring will help us to better understand both the short- and long-term effects of tourism on gentoo penguins in the Antarctic.

6 – Conclusion

Through this dissertation, I have studied gentoo penguin behavior and its relationship to aspects of group living. By examining behavior at the regional scale as well as at the individual scale within a colony, I have explored ways in which individuals cope with the costs of coloniality and how those mechanisms may lead to geographic variation. These findings not only help us to better understand the behaviors themselves, but also illuminate ways in which these behaviors scale to regional patterns.

Individual recognition is critical for mate bonding and fledgling success in gentoo penguin colonies. By comparing ecstatic display calls from a broad geographic range, I have shown that there is large degree of variation within colonies as well as between colonies. However, that variation does not correlate with distance or geography and is likely the result of a cultural drift of vocal characters within the colony (Chapter 2). This indicates that the geographic differences between any two colonies (or within any small number of samples per colony) may reflect random between-site variation, and thus interpreting these differences as related to some causal driver may lead to flawed conclusions. These finding suggest that vocal differentiation and individuality are limited to a local scale and any larger geographic differences are likely the result of chance rather than direct pressure.

Group vocal behavior in gentoo penguins has not been previously described in detail, either in the acoustic parameters or the frequency of its use in the colony. By examining the temporal patterns of individual ecstatic display and group calls within colonies, I found that group calls make up almost one third of calling behavior, and that although they follow the same basic structure, they appear acoustically distinct from ecstatic display calls. Results also indicate that gentoo penguins display a pause following any other vocalization (which I interpret as a

means to avoid signal jamming) while group calls do not follow this pattern. This pattern was significant in field studies but was not observed in captive penguins, which may have fewer pressures for individual recognition within the colony (Chapter 3). These results illuminate the differences in acoustic parameters between the two different types of vocalizations, and again point to the importance of individual recognition in the ecstatic display call. The finding is further explored by noting the difference in behavior between captive and wild penguins, which both face noisy, crowded environments but with a markedly different set of pressures. This work represents the most detailed natural history descriptions to date of two different vocalizations – those used in group behavior and those used in aggressive scenarios.

Conspecific aggression is common in group living species, and has been studied in many colonial seabirds. These interactions require vigilance from the nesting individual and energy expenditure in order to defend the nest. While it had been previously described as an abbreviated ecstatic display call, I showed that the acoustic measurements of the agonistic call differ significantly from the ecstatic display call. I also found that conspecific encounters and subsequent aggressive interactions are much more common than interactions with predators or other species, and are a significant element of territory defense among gentoo penguins. However, this aggression was markedly heterogeneous across the colony with some penguins experiencing heightened rates of aggressive interactions while others had very few. I found that the most predictive factor for the level of response from an individual was its personal encounter history – how many previous encounters it had experienced and how many encounters with the same individual rather than factors such as nest density (Chapter 4). This is in contrast to other species, in which defensive actions are positively correlated with nest density (Burger and Gochfeld 1988, Hill *et al.* 1997, Côté 2000).

Over a large portion of their range, gentoo penguin breeding colonies have become ecotourism destinations. There has been a lot of attention paid to the impacts of human disturbance on penguin populations, and regulations such as minimum approach distances have been developed for the Antarctic. I measured corticosterone levels in guano as a non-invasive way to assess stress responses in multiple colonies that experience a range of ecotourism intensities. The results indicate no clear signal from tourist activities, but rather illuminate the large degree of variation in stress hormone concentration within a colony (Chapter 5). This variation is likely due to individual factors and cannot be attributed to tourism, in line with other studies indicating that gentoo penguins acclimate to human activities relatively well (Cobley and Shears 1999, Barbosa *et al.* 2013). In this context, however, it is worth noting that the level and type of acclimation may be relatively complex and colony-specific (Dunn *et al.* 2019); to that end, these findings should help inform future monitoring studies focused on gentoo penguin disturbance.

These results provide a more nuanced picture of gentoo penguin behavior within the colony, with important implications for other colonial seabird species. While gentoo penguins are not currently considered at risk, many colonial seabirds face a myriad of conservation threats, and understanding the many ways in which coloniality affects individuals and groups may help us better evaluate those threats. Increasing our understanding of behavioral responses, communication, and stressors can help us to better understand behavior not just at individual scales, but also at the colony, region, and population scale.

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