ACOUSTIC COMMUNICATION IN SMOOTH-BILLED ANIS

ACOUSTIC COMMUNICATION IN THE JOINT-NESTING SMOOTH-BILLED ANI, *CROTOPHAGA ANI*

By

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Preface

This thesis consists of an abstract, general introduction, three chapters, a synthesis with suggestions for future research, and appendices outlining working lab procedures, nest monitoring guidelines, and pilot research. Chapters I—III have been written as manuscripts for publication in peer-reviewed scientific journals. Chapter II has been published in *Animal Behaviour*, chapter I has been submitted to *Ibis*, and chapter III is in preparation for submission to *Behavioral Ecology and Sociobiology*. Information about each chapter is detailed below:

Chapter I: Vocal repertoire of the cooperatively breeding Smooth-billed Ani, *Crotophaga ani*.

Authors: L.A. Grieves, D.M. Logue, and J.S. Quinn.
Contribution: Field work was performed by the candidate under the guidance of J.S. Quinn and D.M. Logue. The statistical analyses were conducted by the candidate and D.M. Logue and manuscript writing was conducted by the candidate with guidance and suggestions from the co-authors.

Chapter II: Joint-nesting Smooth-billed Anis, *Crotophaga ani*, use a functionally referential alarm call system.

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Chapter III: Ready to fight: reliable signals of aggression in a joint-nesting non-passerine, the Smooth-billed Ani.

Authors: L.A. Grieves, D.M. Logue, and J.S. Quinn.
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Last, but certainly not least, I thank my supervisor, Dr. James Quinn, for allowing me to take an idea and run with it long enough to explore communication in anis in a way that had not been done before. Thank you, Jim, for your continual support, advice, and generosity.

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Abstract

I studied acoustic and visual communication in the Smooth-billed Ani, a joint-nesting, cooperatively breeding cuckoo. In chapter I, I describe the vocal repertoire of this species using both qualitative and quantitative methods. This is the first formal description of the Smooth-billed Ani's vocal repertoire. I provide verbal descriptions of each call type, the contexts in which each call is produced, spectrograms, and acoustic measurements for each call type. I used multivariate statistics to show that call types can be correctly classified based on acoustic measurements alone. In chapters II and III, I show that Smooth-billed Anis are capable of complex communication, including the use of functionally referential alarms (chapter II) and signals of aggression that reliably predict attack (chapter III). Functionally referential signals are produced in response to a specific set of stimuli and elicit predictable, appropriate responses in signal receivers, even in the absence of any other cues. In chapter II, I show that anis produce two distinct signal types, *chlurps* and *ahnee alarms*, in response to two different predator classes, aerial and terrestrial, respectively. I also show that receiver responses to playback of these alarm signals are distinct and appropriate to evade predation from aerial and terrestrial attackers. Aggressive signals should increase in aggressive contexts, predict subsequent aggression and elicit responses from signal receivers. In chapter III, I show that *hoots*, an acoustic signal, and throat inflation, a visual signal, both increase in aggressive contexts and reliably predict aggressive escalation in the form of direct attacks on a mount. The receiver response to *hoots* and throat inflation remains to be tested. In the synthesis, I provide suggestions for future research. The appendix contains information on lab procedures, nest monitoring protocols, and pilot research.

Introduction

Animals regularly face a variety of challenges and obstacles which they must overcome to survive and reproduce successfully. Animals must locate food, water, shelter, and mates, and avoid predators. It is also necessary to interact with conspecifics, including family, helpers, competitors, and rivals, in ways appropriate for survival. This is particularly true for animals with complex social systems, where individuals interact frequently and in different contexts with many different individuals, often the same individuals over time (Freeberg et al. 2012). Social animals also spend a greater proportion of their time engaged with conspecifics than do solitary species. As a result, many social species have developed complex communication systems, those that contain many structurally and functionally distinct elements (Freeberg et al. 2012). Of course, solitary species also communicate, and both solitary and social animals communicate with heterospecifics as well as conspecifics.

Communication involves the transfer of information between one or more individuals. This information transfer has an effect on the current or future behaviour of the individual receiving that information. Communication thus requires both a sender (signaler) and a receiver. A signal can be defined as "an act or structure that alters the behaviour of another organism, which evolved because of that effect, and which is effective because the receiver's response has also evolved" (Maynard Smith & Harper 2003, p. 3).

Signals can include vocalizations, body movements, and colour patterns that evolved because they transmit information that is beneficial to the individual exhibiting those traits (Otte 1974; Searcy & Nowicki 2005). Animals communicate using a variety of senses or modalities, including chemical, vibrational, visual, and acoustic signals. Acoustic and visual communication is particularly widespread in birds. My thesis focuses on acoustic and, to a lesser extent, visual communication in a highly social non-passerine bird, the Smooth-billed Ani, *Crotophaga ani*.

In chapter I, I describe the vocal repertoire of Smooth-billed Anis using both qualitative and quantitative methods. By assigning a standardized name to each call, describing the context in which calls are made, reporting acoustic measurements of call features, and presenting spectrograms of the calls, I hope that future researchers will find it easier to pursue acoustic research in this species.

In chapter II, I focus on alarm signaling in Smooth-billed Anis. Alarm signals have garnered significant research attention (reviewed in Bradbury & Vehrencamp 2011). Alarm signalling systems can be complex, with species producing distinct signal-types for different classes of predatory threat. These ‘functionally referential’ alarms are defined by stimulus-specific signalling, in which each signal-type is elicited by a narrow set of stimuli, and context-independent responses in receivers, in which the signal alone is sufficient to elicit an appropriate response from signal receivers (Macedonia & Evans 1993). I present two experiments demonstrating functionally referential alarm communication in Smooth-billed Anis.

In chapter III, I focus on communication in staged aggressive encounters. Aggressive signals increase in aggressive contexts, predict subsequent aggressive escalation, and elicit a response in signal receivers (Searcy & Beecher 2009). Smooth-billed Anis produce at least one potentially aggressive acoustic signal, the *hoot* call. However, aggressive signals are not restricted to the acoustic modality. Visual signals of aggression are common in many taxa, including birds, and may be displayed permanently, as is often the case with badges of status, or discretely; that is, the signal can be turned ‘on’ or ‘off’. Discrete signals often include postural displays and ritualized body movements (Maynard Smith & Harper 2003; Searcy & Nowicki 2005; Bradbury & Vehrencamp 2011). I tested whether the *hoot* call, an acoustic signal, and the throat inflation display, a visual signal, predicted aggressive escalation in the form of direct attacks on a conspecific taxidermic mount.

Smooth-billed Anis are a member of the cuckoo family (Family: Cuculidae, Subfamily: Crotophaginae). Their range extends from Southern Florida, throughout the Caribbean, and into parts of South and Central America and the Bahamas. They are joint-nesting cooperative breeders; multiple females lay eggs in a single, shared nest. Groups consist of one or more socially monogamous breeding pairs that all contribute parental care and defend all-purpose territories (Quinn & Startek-Foote 2000). Unlike many cooperatively breeding species, group members are typically unrelated (Blanchard 2000; Vehrencamp & Quinn 2004). Joint-nesting is a rare breeding system limited to approximately 15 avian species worldwide (Vehrencamp & Quinn 2004). While the majority of research on this species has focused on their unique breeding system (Davis 1940; Loflin 1983; Blanchard & Quinn 2001; Schmaltz et al. 2008a,b; Quinn et al. 2010), my thesis takes a different approach.

I studied communication in this highly social, group-living species at the Cabo Rojo and Laguna Cartagena National Wildlife Refuges in southwestern Puerto Rico over three consecutive field seasons spanning 2011 – 2013. I show that Smooth-billed Anis are capable of complex acoustic and visual communication, including the use of referential alarms and signals of aggressive motivation that predict attack. Given the paucity of data on communication in anis, my thesis also raises unanswered questions and novel research directions. In the synthesis section, I outline some of the questions arising from my research and suggest future research directions. In the appendices, I outline the current working lab protocols for molecular work (A1), the nest monitoring protocol I have developed specifically for nestling monitoring and collection of provisioning data (A2), and summarize some of the pilot research I have undertaken (A3, A4).

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Chapter I:

Vocal repertoire of the cooperatively breeding Smooth-billed Ani, *Crotophaga ani*

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Abstract

The call-based acoustic communication systems of non-passerines have received relatively little research attention in comparison to the song systems of passerine birds. Here, we provide the first complete, quantitative description of the vocal repertoire of the Smooth-billed Ani, a joint-nesting, cooperatively breeding cuckoo. We recorded naturally occurring calls from 22 groups of anis over two breeding seasons. We visually classified 13 call types and one group vocalization. We provide spectrograms, describe the acoustic structure of each call type, the context in which calls are given, and suggest potential functions for several common calls. After visual classification, we used an automated sound measurement program to quantify the calls' acoustic features. Discriminant function analysis (DFA) correctly classified 74.5% of calls based on these features. Our results demonstrate that a quantitative approach can support qualitative call classification, identify the most important acoustic variables that distinguish call types, and clarify structural relationships among call types. This description of the Smooth-billed Ani vocal repertoire will standardize the call nomenclature, aiding future research on this species.

Introduction

The majority of avian vocal communication research focuses on passerine birds (Marler 2006; Benedict & Krakauer 2013). By contrast, the vocal systems of non-passerines remain little studied. Non-passerines do not produce songs like passerine birds, but they do produce and respond to a tremendous diversity of vocal signals, collectively referred to as 'calls'. Although there is no single set of parameters that can be used to distinguish a song from a call, songs tend to be longer and more complex, often containing a variety of notes and syllables delivered in a reliable sequence. Calls tend to be short, monosyllabic vocalizations, but there are some notable exceptions (e.g., the *chick-a-dee* call of black-capped chickadees, *Poecile atricapillus*). Birds use calls to facilitate the coordination of reproductive activities, maintain spatial proximity with group members, locate and exchange food, and warn social companions of approaching predators (Evans et al. 1993; Bretagnolle 1996; Evans 1997; Lefevre et al. 2001; Seddon et al. 2002; Radford 2003; Radford 2004a,b; Baker 2004; Wilson & Evans 2012). Although historically understudied, calls are functionally diverse, phylogenetically widespread signals worthy of additional research (Marler 2004; Benedict & Krakauer 2013).

Many studies that describe acoustic signal repertoires use qualitative assessments of spectrographs and behavioural context to distinguish among call or song types, but fewer studies provide quantitative analyses when discriminating among call or song types in avian or other taxa (for examples, see Staicer 1996; Hammerschmidt & Fischer 1998; Sharp & Hatchwell 2005; Gamba & Giacoma 2007). We follow the commonly used qualitative approach, but then take the additional steps of measuring many acoustic parameters of each call, and attempting to validate our categorization scheme with discriminant function analysis (DFA). Multivariate analyses like DFA are powerful tools for assessing avian vocalizations (Sparling & Williams 1978; Martindale 1980). Our approach provides detailed acoustic information about each call type, characterizes the acoustic similarity between call types, and reveals the most important acoustic parameters for distinguishing among call types.

We provide the first quantitative analysis of the vocal repertoire of the Smooth-billed Ani, *Crotophaga ani* (family Cuculidae, subfamily Crotophaginae), a highly social non-passerine. Anis are joint-nesting, cooperatively breeding birds. Joint-nesting is rare, limited to approximately 15 avian species worldwide (Vehrencamp & Quinn 2004). Smooth-billed Anis breed as socially monogamous pairs in groups of two to 17 adults of which multiple females typically lay eggs in a single nest, and all group members care for eggs and young (Quinn and & Startek-Foote 2000). Anis can breed as single pairs, but the average group size in our study population is 6 (Quinn et al., unpublished data). Groups roost communally and defend all-purpose territories from both intruding groups and solitary anis (Quinn & Startek-Foote 2000). Unlike most other cooperative breeders that form full or partial kin groups (Riehl 2013), adult group members in this species are typically unrelated (Blanchard 2000).

In the first experimental study of communication in this species, we demonstrated that Smooth-billed Anis use a functionally referential alarm call system, producing two distinct alarm calls in response to two different classes of predatory threat (Grieves et al. 2014). Acoustic communication appears to be important for anis, suggesting that this is a promising research direction.

The goal of our repertoire analysis was to promote consistency across studies and facilitate future research on acoustic communication in Smooth-billed Anis. We extend the qualitative descriptions of Smooth-billed Ani vocalizations made by Davis (1940), expand on the basic measurements provided by Quinn & Startek-Foote (2000), and describe the contexts in which ani calls are produced.

Methods

Study Area

Field recordings were made at the Cabo Rojo (17º59'N, 67º10'W) and Laguna Cartagena (18º00'N, 67º06'W) National Wildlife Refuges in southwestern Puerto Rico during the rainy season from October 1 to December 15 2011 and September 8 2012 to January 5 2013, spanning the peak breeding season for Smooth-billed Anis (Quinn & Startek-Foote 2000). Smooth-billed Ani research has been ongoing at these sites since 1998. Groups are highly territorial and group composition tends to be stable during the breeding season (Quinn & Startek-Foote 2000). We identified the groups we recorded based on territory location, group composition, and, where possible, the presence of colour-banded birds.

Recording Protocol

Calls were recorded with two portable Marantz Solid State recording units (sampling rate = 44.1 kHz, bitrate = 16 bits); a Marantz PMD660 equipped with a Sennheiser ME66 microphone capsule (short shotgun) and a Marantz PMD661 equipped with a Sennheiser ME67 microphone capsule (long shotgun). Both microphones used a Sennheiser K6 power module.

Recordings were made *ad libitum* throughout the study period before and after groups left their roost trees (from 0600–0700 hours), throughout the anis' main activity periods of the day (0700–1200 and 1500–1715 hours), and as groups were re-entering their roosts for the night (1715–1845 hours). We did not record from 1200–1500 hours, the hottest portion of the day when the anis show reduced activity, often retreating to roost trees within their territory. We documented the following variables for each recording session: group ID, recording duration, behavioural context, intraspecific interactions (within or between groups), and interspecific interactions, including interactions with researchers. Multiple recordings were taken from as many groups as possible, but we were not able to obtain samples of all call types from all 22 groups used in this study. Field methodologies were approved by the McMaster University Animal Research Ethics Board (Animal Utilization Protocol number 09-27-25).

Repertoire Analysis

Calls were visualized as spectrograms in Syrinx v2.6f (John Burt, www.syrinxpc.com; FFT window = 4 ms, FFT window type = Blackman, transform size = 0.0116 seconds). One of us (L.A.G.) classified calls according to their appearance on spectrograms and the context in which they were given. For each breeding group, the first high quality (high signal-to-noise ratio) recording of each call type was selected for further analysis. These calls were high-pass filtered at 350 Hz in Syrinx. We then analysed their characteristics using Sound Analysis Pro 2011 (SAP) software (Ofer Tchernichovski, http://soundanalysispro.com; FFT window = 9.1 ms, frequency range = 11025 Hz, advance window = 1.00 ms, contour threshold = 10).

Calls were scored with the “feature statistics across interval” option within the “explore & score” function in SAP, which calculates the average value of each measured variable across the length of the call. We measured duration (ms); the mean, minimum, maximum, and variance of: mean frequency (Hz), frequency modulation (FM, degrees), and Weiner entropy; the mean, minimum, and maximum values of peak frequency (Hz) and duration of acoustic state (DAS, ms); the mean and maximum values of continuity over frequency (Hz) and continuity over time (ms); and the mean and variance of pitch (Hz), goodness of pitch, and amplitude modulation (AM, 1/t). Tchernichovski (2012) and Feher et al. (2009) describe these features in detail.

We ran a discriminant function analysis (DFA) with qualitatively determined call type as the dependent (grouping) variable and the acoustic parameters measured in SAP as the independent variables. This analysis serves two purposes. First, it tests the validity of our qualitative classification of call types. Second, it quantifies the acoustic differences among call types, allowing us to see which variables are most useful for separating call types and to visualize the structural similarities and differences among call types.

To control for group of origin, only one call of each type from each group was analysed. We guarded against overparameterization by 1) excluding poorly sampled call types from the analysis, and 2) using a stepwise model selection procedure to reduce the number of acoustic variables in the model. We excluded the following call types due to low sample size: *ee-oo-ee* (n = 2), *growl* (n = 2), *shout* (n = 3), and *whistle* (n = 2). The lowest sample size from the remaining songs was the *chlurr* with n = 7, so we tuned the stepwise model parameters to generate a model with seven predictor variables (final parameters: F to enter = 10, F to remove = 5). Our remaining sample sizes were: *ahnee* (n = 15), *ahnee alarm* (n = 11), *chlurp* (n = 9), *flight* (n = 13), *grunt* (n = 15), *hoot* (n = 9), *pre-flight* (n = 13), and *whine* (n = 10). We classified calls using 'leave one out' cross-validation. The DFA was conducted in SPSS v17.0 (SPSS, Chicago).

We identified one group vocalization, the *ahnee chorus*. To analyze it, we selected the first high quality (high signal-to-noise ratio) recording available from each group (n = 7) and high-pass filtered the chorus at 350 Hz in Syrinx. We then used the cursors in Syrinx to measure the chorus duration. We only measured call duration (s) of the *ahnee chorus* because this call consists of a bout of overlapping vocalizations and we therefore could not accurately measure other call features. We defined the *ahnee chorus* as all repetitious morning calling in the roost tree made by two or more group members. All vocalizations with less than 5 s of silence between calls were considered part of a single chorus. Ani groups often chorus several times before leaving the roost, and chorusing bouts can vary in duration and in the number of call types made, but we do not know whether differences in bout duration or structure are biologically meaningful. Thus, for recordings with multiple choruses (n = 5), we calculated the average chorus duration for that group. We then calculated the grand mean *ahnee chorus* duration from all groups measured.

In an effort to consolidate previously published data on Smooth-billed Ani vocalizations, we compared the calls we identified with the descriptions provided by Davis (1940), and spectrograms and call descriptions presented in Quinn and Startek-Foote (2000).

Results

We identified thirteen call types: *ahnee, ahnee alarm, chlurp, chlurr, ee-oo-ee, growl, grunt, hoot, flight, pre-flight, shout, whine,* and *whistle* (Fig. 1). The mean values (± SD) for each measured variable are summarized in Table 1. Additional descriptive statistics can be found in the supplementary materials (Tables S1-S2). We also identified a group vocalization, the *ahnee chorus* (Fig. 2). The average duration of the *ahnee chorus* was 14.5 s (range = 6.9 to 27 s, n = 7). Audio clips of each call type and the *ahnee chorus* are available online (xeno-canto, http://www.xeno-canto.org/contributor/JPAYUABGRO).

Overall, Smooth-billed Ani calls are short (< 7 s) and frequency-rich. All call types comprise a single note (i.e. they make a continuous trace on a spectrogram), with strong harmonic structure, strong frequency modulation (often rising in frequency over the duration of the call), formants (concentrations of acoustic energy around a particular frequency), and non-linear and chaotic (high-entropy) elements. Although we were able to classify calls into discrete types, we also noted substantial variation in structure within each call type.

Call Comparisons and Descriptions

We were able to match all four calls presented in Quinn and Startek-Foote (2000) and eight of the thirteen calls described by Davis (1940) with the thirteen single calls and one group vocalization we identified in this study (Table 2). Below, we describe the structure of each call, contexts in which calls were made, and the behaviours that were associated with each call. Context and behavioural descriptions are based on field observations.

Common Calls

Ahnee

The *ahnee* call (Fig. 1a) is a loud harmonic stack that rises smoothly in frequency and then terminates with a rapid drop in frequency. Energy is concentrated in the lower harmonics and in formants. Some *ahnee* calls include an abrupt shift from a lower fundamental frequency to the main frequency sweep after ~ 60 ms, and some include subharmonics (visible as traces midway between harmonics in Fig.1a). *Ahnee* calls are structurally similar to several other ani vocalizations, including the *ahnee alarm*, *flight*, *pre-flight*, and *shout* calls (Fig. 1, Fig. 3, Table 1). The *ahnee* call is the most commonly produced call in this species. It is given at dawn before birds leave the roost, as part of the *ahnee chorus*. *Ahnee* calls are also produced throughout the day, during foraging, and when defending the territory from intruding or neighbouring groups and individuals.

Chlurr

Chlurrs (Fig. 1d) are high-entropy, low-amplitude calls, with rapid amplitude modulation (~ 5 amplitude pulses) and no apparent harmonic structure. The *chlurr* is a common call, typically associated with movement or a change in activity. *Chlurrs* are often produced by multiple group members when two or more individuals are perched together, but are also produced by anis perched alone.

Flight-associated Calls

Pre-flight

Pre-flight calls (Fig. 1j) are harmonically rich with rapid, irregular, low-amplitude frequency modulation, and a terminal burst of higher-amplitude frequency modulation. Some have subharmonics. This call is often produced one or more times just prior to taking flight, at which point anis switch to the *flight* call. *Pre-flight* calls have only been recorded before the individual producing the call takes flight, but anis may also initiate flight without calling.

Flight

Like *ahnee* calls, *flight* calls (Fig. 1i) are harmonically rich vocalizations that gradually ascend in frequency and terminate with a rapid drop in frequency. Unlike *ahnees*, however, *flight* calls also include rapid, irregular frequency modulation. They often include subharmonics and non-linear frequency shifts. Individuals may produce only one or two *flight* calls at the start of a flight, but they often produce *flight* calls throughout the entire duration of a flight. Anis are also capable of flying without delivering this call.

Close-range Calls

Grunt

Grunts (Fig. 1g) are short, low-frequency, highly variable calls. Some have fairly strong harmonic structure, some are very noisy (high entropy), and some include both harmonics and noise. *Grunts* are often produced in the morning before the anis leave their roost. *Grunts* can be made singly or interspersed among *ahnees* or *whines* produced by other individuals. Two or more individuals sometimes *grunt* back and forth. *Grunts* are often produced by adults at the nest, particularly when chicks are present, and can be directed at either chicks or other adult group members. *Grunts* are also heard during chasing, described below.

Hoot

Hoots (Fig. 1h) are short, harmonically rich calls that ascend and then descend in frequency. The beginning and end of each *hoot* contains chaotic (entropic) elements. Some of these calls include subharmonics. *Hoots* are primarily associated with chases. Chasing is an aggressive behaviour that occurs when a group or a lone bird enters another group's territory. Chasing is accompanied by a predictable suite of vocalizations including *hoots*, *grunts*, and *whines* (see below). Typically, one to several birds chase a single bird. While resting between bouts of chasing, the chased bird and the chasing bird will often *hoot* back and forth. *Hooting* typically ends when one bird, typically the bird being chased, retreats. *Hooting* bouts can also end in attacks and fighting between the *hooting* birds. *Hoots* are also sometimes made in the roost tree, both at dawn and dusk, and may involve *hooting* back and forth between group members, or a single bird *hooting* with other group members *whining* or *grunting* in response.

Whine

The *whine* call (Fig. 1l) is a harmonically rich call with a highly variable pattern of frequency modulation. It ends with a rapid increase and then decrease in frequency. *Whines* are often produced during the morning before anis leave the communal roost. Frequently, one individual produces *grunts* or *hoots* while a bird *whines* in response. This calling behaviour occurs between pairs of anis outside of the roost as well. During chasing, *whines* are often made by non-chasing group members and appear to be directed at the group member(s) involved in chasing. *Whines* are also produced by the chased bird after landing, and are apparently directed toward the chasing bird.

Alarm Calls

Ahnee alarm

The *ahnee alarm* call (Fig. 1b) is structurally similar to the *ahnee* call (Fig. 1, Table 1). *Ahnee alarms,* however, include a near-instantaneous jump in frequency near the end of the call (visible in Fig. 1b). The *ahnee alarm* is given in the presence of terrestrial predators such as West Indian mongooses, *Herpestes auropunctatus*, domestic dogs, *Canus familiaris,* and cats, *Felis catus,* and also in the presence of humans, *Homo sapiens*. *Ahnee alarms* stimulate anis perched low or on the ground to fly up to a higher perch.

Chlurp

Chlurps (Fig. 1c) are short calls comprising a rapid increase and then decrease in frequency, followed by a high entropy section that often contains subharmonics, and ending with a harmonic stack that increases and then rapidly decreases in frequency. *Chlurps* are typically produced in response to flying raptors such as Red-tailed Hawks, *Buteo jamaicensis*, Peregrine Falcons, *Falco peregrinus*, and Merlins, *Falco columbarius*. Rarely, anis *chlurp* in response to fast flying doves, American Kestrels, *Falco sparverius*, and Turkey Vultures, *Cathartes aura*, especially in low light conditions. *Chlurps* typically cause individuals to dive down or fly rapidly into vegetative cover.

Shout

The *shout* (Fig. 1k) is shorter and lower in frequency than the *ahnee alarm*, but otherwise the two call types have a very similar structure. The *shout* call is given during periods of intense disturbance from predators or humans. We documented the *shout* call when perched Red-tailed Hawks, *Buteo jamaicensis*, were detected by the group and after hawks passed a group. In cases where a hawk flew over or past the group, *chlurps* were produced (see above). *Shouts* were also produced by some ani groups during researchers' nest visits.

Nest-associated Calls

Growl

Growl calls (Fig. 1f) are low-amplitude, high-entropy vocalizations with some visible harmonics and strong formants. Their fundamental frequency first increases and then decreases (Fig. 1f). *Growls* sound similar to the snarl of a domestic cat. The *growl* call is produced by adults during nest defense. *Growls* were documented in response to researchers visiting the nest and during a mongoose attack on nestlings. During nest visits, the *growling* individual would hop and make short flights around the vicinity of the nest, sometimes approaching to less than 0.5 m from the intruding researcher. *Ahnees*, *ahnee alarms*, and *shouts* were made in conjunction with *growls* (see above). *Growls* are relatively rare calls.

Ee-oo-ee

As its name suggests, the *ee-oo-ee* (Fig. 1e) has three parts. The first and third "*ee*" elements are frequency-modulated such that they look like arches on a spectrogram. In between the arches (the "*oo*") there is an unmodulated low frequency element. The transitions between the sections are very abrupt. The entire call has harmonic structure. The *ee-oo-ee* is an uncommon call. All of our observations of *ee-oo-ees* occurred at the nest and in the nest tree when chicks were present.

Whistle

Whistles (Fig. 1m) increase, plateau, and then decrease in frequency. Like the *ee-oo-ee*, *whistles* are uncommon and have only been recorded at nests or in the nest tree, usually when chicks were present.

Group Vocalizations

Ahnee chorus

The *ahnee chorus* (Fig. 2) is a string of vocalizations produced by groups before leaving their communal roost in the morning. The chorus typically begins with a repeated *ahnee* call from one individual and continues as the other group members join in with overlapping *ahnees*. The *ahnee chorus* may also contain *grunt*, *whine*, and *hoot* calls.

Discriminant Function Analysis

The seven parameters chosen by the stepwise model procedure were duration, mean pitch, mean FM, mean frequency, mean DAS, variance in pitch, and variance in AM. The DFA procedure produces linear combinations of predictors, creating a new latent variable for each function—the discriminant functions. The first two functions explained 79.6% of the total variance (Function 1: 67.9%, eigenvalue = 9.15; Function 2: 12.6%, eigenvalue = 1.72). The first standardized canonical discriminant function included high positive coefficients for duration (0.70), mean frequency (0.56), and mean pitch (0.50), and a high negative coefficient for mean FM (-0.55). The second function included high positive coefficients for mean frequency (0.91), mean DAS (0.85), and mean pitch (0.63), and high negative coefficients for duration (-1.03) and pitch variance (-0.81; Table S3). The distribution of call types in a plot of Function 1 against Function 2 reflects the pattern of structural similarity among call types (Fig. 3).

Using cross-validation to compare different predictive modeling procedures, the DFA classified 74.5% of calls correctly, significantly exceeding chance-level classification (Wilks' λ = 0.005, df = 56, P < 0.001, Fig. 3). In cross-validation, a sample of the data is partitioned into subsets. Analyses are then performed on one subset (the training set), and validation of the analysis is performed on the other subset (the testing set). In leave-one-out cross-validation, one observation from the original sample is used as the testing set, and the remaining observations are used as the training set. This process is repeated so that each observation in the sample is used as the training set once.

Based on our qualitative classification scheme, the DFA correctly classified 86.7% (13/15) of *ahnees*, 81.8% (9/11) of *ahnee alarms*, 77.8% (7/9) of *chlurps*, 100% (7/7) of *chlurrs*, 53.8% (7/13) of *flights*, 66.7% (10/15) of *grunts*, 77.8% (7/9) of *hoots*, 69.2% (9/13) of *pre-flights*, and 70% (7/10) of *whines* (Table S4, appendix).

Discussion

Like previous work on the call repertoires of non-passerines (Clapperton 1987; Collias 1987; Evans et al. 1993, Bretagnolle 1996; Seddon et al. 2002; Miller et al. 2003; Wilson & Evans 2012), our study has revealed a variety of structurally and functionally distinct vocalizations. We identified thirteen Smooth-billed Ani calls, including three alarm calls, two nest-specific vocalizations, and one potentially aggressive call. We also identified a group vocalization, the *ahnee chorus*. Thus, this highly social species has a complex vocal communication system, as predicted by a recent variant of the 'social complexity hypothesis' that addresses communication systems (Freeberg et al. 2012).

Our catalog of call types overlaps partially, but not completely, with previous efforts by Davis (1940) and Quinn and Startek-Foote (2000; Table 2). A multivariate analysis largely supported our categorization scheme, and provided a visual representation of the relationships among call structures in two-dimensional space (Fig. 3). Below, we comment on the structure of each call type, the putative function of the most common calls, discuss our classification scheme in light of previous efforts, and evaluate the results of our quantitative analysis.

Ahnees are the most common Smooth-billed Ani call. This is the species' main contact call and is produced during the morning chorus, throughout the day, and before entering roost at dusk. With their rich harmonic structure and strong formants, this call would be the logical starting point for research on individual vocal recognition in this species (Clapperton 1987; Sharp & Hatchwell 2005; Lefevre et al. 2008). The *ahnee chorus*, which consists primarily of multiple repetitions of the *ahnee* call made by the whole group, may serve as a group-level territorial signal (Radford 2005). Future research on the *ahnee chorus* could test hypotheses of group-specific vocal structure, honest signaling of group size, vocal rallying, and territory defense (e.g. Radford 2003; Radford 2004a,b; Radford 2005).

Ahnee alarms and *chlurps* are the two most common alarm calls produced by Smooth-billed Anis. The *shout* seems to represent a third, possibly intermediate, alarm call, although its structure is so similar to the *ahnee alarm* that it may in fact be a variant of that call. We have shown that *ahnee alarms* and *chlurps* are functionally referential alarm signals produced in response to terrestrial and aerial predators, respectively (Grieves et al. 2014; chapter II). No research has been done on the *shout* call, but our field observations suggest this call may be produced in response to intermediate levels of threat, such as after a perched hawk has been detected but before it flies near a group.

Chlurrs, *grunts*, and *hoots* are relatively quiet calls that are usually given in close proximity to receivers, suggesting that they are close-range signals. Our contextual data offer few additional clues about the functions of *chlurrs* and *grunts*. *Hoots*, however, appear to signal aggressive intent (Grieves et al. in review; chapter III).

Whines are made between individuals at close range and are typically produced by one individual in response to another individual *grunting* or *hooting*. *Whines* were often directed at a bird that was behaving aggressively, so we hypothesize that they communicate submission or appeasement.

Our *growl* call most closely matched Vehrencamp's "high intensity mobbing screams" (S.L. Vehrencamp *in* Quinn & Startek-Foote 2000, p. 5). *Growls* were recorded rarely, limiting our ability to assess them, but were typically produced by an individual group member actively engaged in nest defense. Although this is a context in which one would expect to see mobbing, we are reluctant to label the *growl* as a mobbing call because we never observed other group members approaching the *growling* individual. In fact, we have never observed mobbing behaviour, *per se*, in this population.

Pre-flight and *flight* calls are loud calls with strong harmonic structure and non-linearities. Calls with this kind of structure are thought to be difficult to ignore (Fitch et al. 2002). Based on their structure and strong association with flight, we hypothesize that these calls are involved in the coordination of group movements.

The *ee-oo-ee* and *whistle* calls are infrequently uttered, structurally distinct vocalizations, delivered in the vicinity of the nest. Given the dearth of information about these calls, we will refrain from speculating about their functions.

This report is not the first to attempt to classify the Smooth-billed Ani's vocal repertoire. Davis (1940) identified thirteen Smooth-billed Ani calls. His descriptions were qualitative and no spectrograms or acoustic measurements were included. Quinn and Startek-Foote (2000) provided spectrograms and basic measurements for four Smooth-billed Ani calls, attempting to link these calls to those described by Davis. Both papers contain discrepancies with our research, which we address below and in Table 2. We hope that our inclusion of spectrograms, acoustic measurements, and quantitative analysis alongside verbal and contextual descriptions of calls will enable future researchers to more easily classify and compare Smooth-billed Ani vocalizations.

We were unable to match five of Davis' (1940) thirteen call types with the calls we identified in this study. We do not have any examples of Davis' *chuckle*, produced by mated pairs in the nest tree. We recorded two nest-specific calls, *ee-oo-ees* and *whistles*, but we could not determine from Davis' description whether either of these calls matched the *chuckle*. Davis' *guttural* call, produced when anis are "perplexed" (Davis 1940, p. 188) does not fit with any of the calls we identified. We were also unable to match Davis' *complaint* and *objecting* calls, described as variants of the *ahnee* (also referred to as the *judio*), with our data. Finally, we did not identify Davis' *whew* call, which is apparently a variant of the *quack* (our *chlurp*).

Our DFA was statistically significant, correctly classifying 74.5% of calls with cross-validation, a conservative method of classifying calls. We conclude that this quantitative analysis supports our visual classification scheme. Nevertheless, the incorrect classification of approximately one fourth of the calls raises two questions. First, why were some calls classified incorrectly? We think that the most likely explanation is that our method of extracting measures from whole calls ignores the dynamics of parameters over the course of each call. For example, a call that modulates linearly from 500 Hz to 1000 Hz would have the same mean pitch, maximum pitch, and minimum pitch as a call that modulates linearly from 1000 Hz to 500 Hz. Spectrogram cross-correlation accounts for variation over the course of the signal, but that technique does not offer descriptive statistics of call structure (Baker & Logue 2003).

The second question raised by the misclassification of calls in our study is whether listening anis can distinguish among the call types. Presumably, anis are better at classifying sounds than our DFA because they are sensitive to dynamic variation in call structure (discussed above), and can discern more acoustic variables than the seven used in our analysis. In addition, anis would have contextual cues to help them discern call types. For example, *flight* calls were most often misclassified by DFA as *pre-flights* and vice versa. Birds make *pre-flight* calls just prior to flight and *flight* calls when they are flying, so anis would presumably have enough additional contextual information to differeniate between these calls. *Chlurrs*, *grunts*, and *hoots* are structurally similar (Fig. 3). *Grunts* were most often misclassified as *chlurrs* and the spectrograms of these calls appear similar. Indeed, *chlurrs* may be a series of *grunts* repeated in a short trill (Fig. 1). *Whines* were most often misclassified by DFA as *flight* calls, but *whine* and *flight* calls appear visually distinct (Fig. 1) and are produced in very different contexts. It therefore seems likely that anis can distinguish these calls.

Previous research on Smooth-billed Anis demonstrated that egg loss increases and per capita reproductive success decreases with increasing group size, suggesting that group living incurs substantial reproductive costs (Schmaltz et al. 2008). We suggest that this species' complex vocal communication system mediates the benefits of social living, mitigating some of the costs of sociality.

For example, the birds' alarm system enhances individuals' ability to detect and avoid predators (Grieves et al. 2014). Benefits of sociality may also accrue from the exchange of other types of calls. The ability to alert group members about a food source (Collias 1987, Cocroft 2001) or an intruder posing a threat to resources held within the group territory (Baker 2004) are examples of benefits that can be gained through group living. We hope that our characterization of the Smooth-billed Ani vocal repertoire will enable researchers to more systematically explore potential benefits of group living in this species.

In summary, our discriminant function analysis alone did not classify call types with perfect accuracy, but it supported our visual and contextual classification scheme, and will provide future researchers with a quantitative scheme to assign call types.

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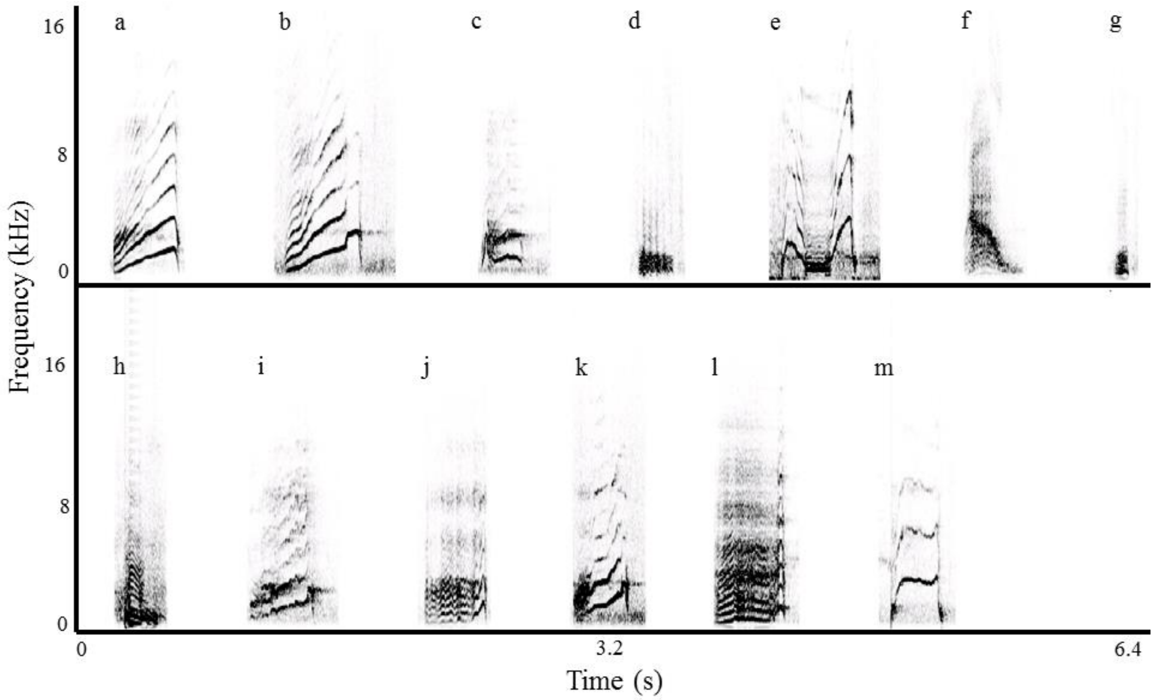


Figure 1. Spectrogram showing representative samples of the 13 Smooth-billed Ani calls identified in this study: a) *ahnee*, b) *ahnee alarm*, c) *chlurp*, d) *chlurr*, e*) ee-oo-ee*, f) *growl*, g) *grunt*, h) *hoot*, i) *flight*, j) *pre-flight*, k) *shout*, l) *whine*, and m) *whistle*.

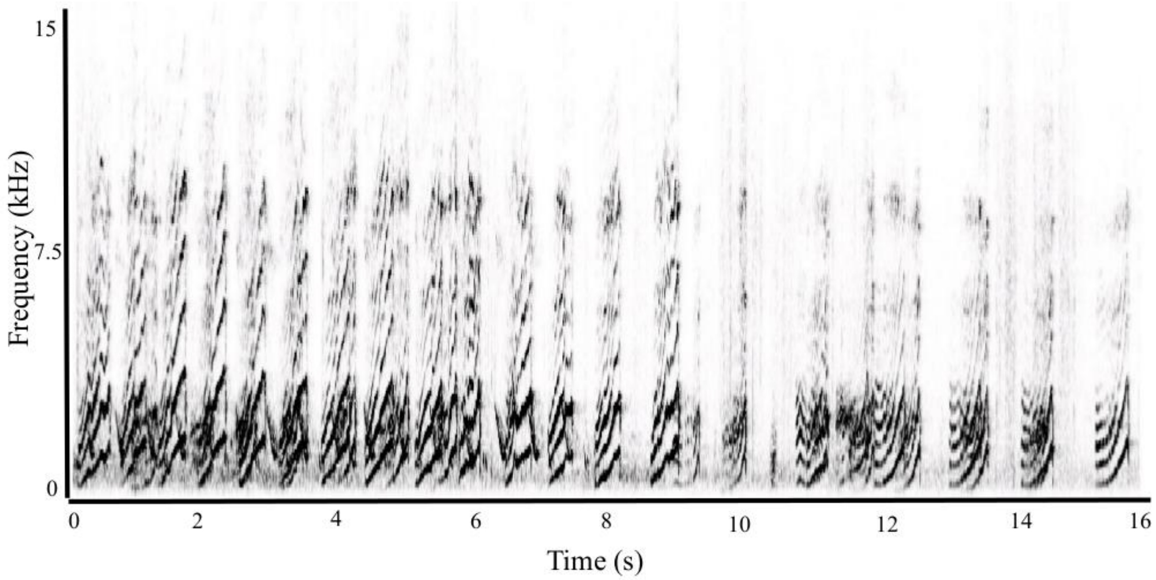


Figure 2. Spectrogram showing a representative sample of the *ahnee chorus* ($\bar{X}$ duration = 18.6 s). Note that the scale of the X-axis differs between figs. 1 and 2.

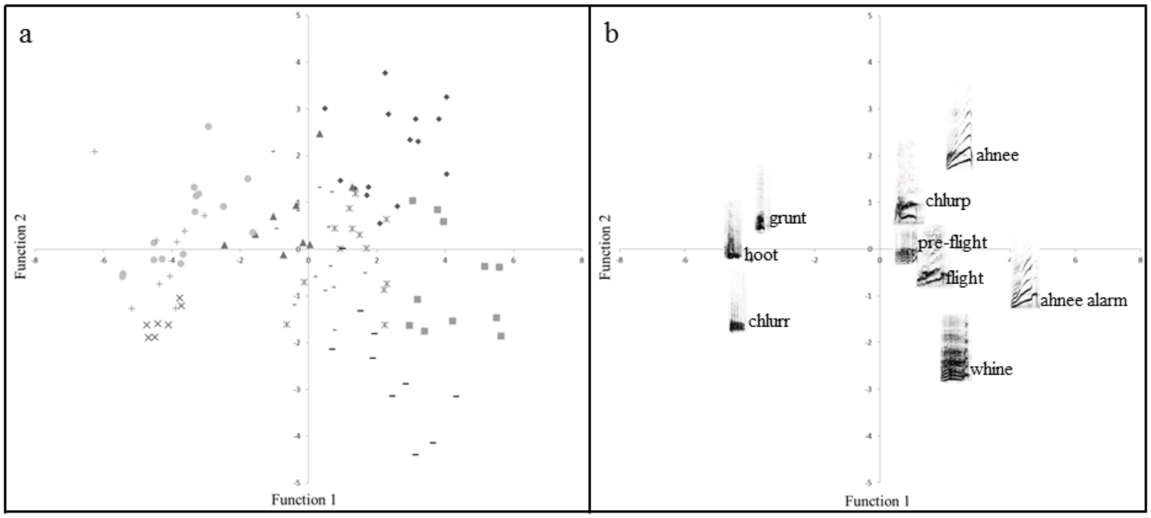
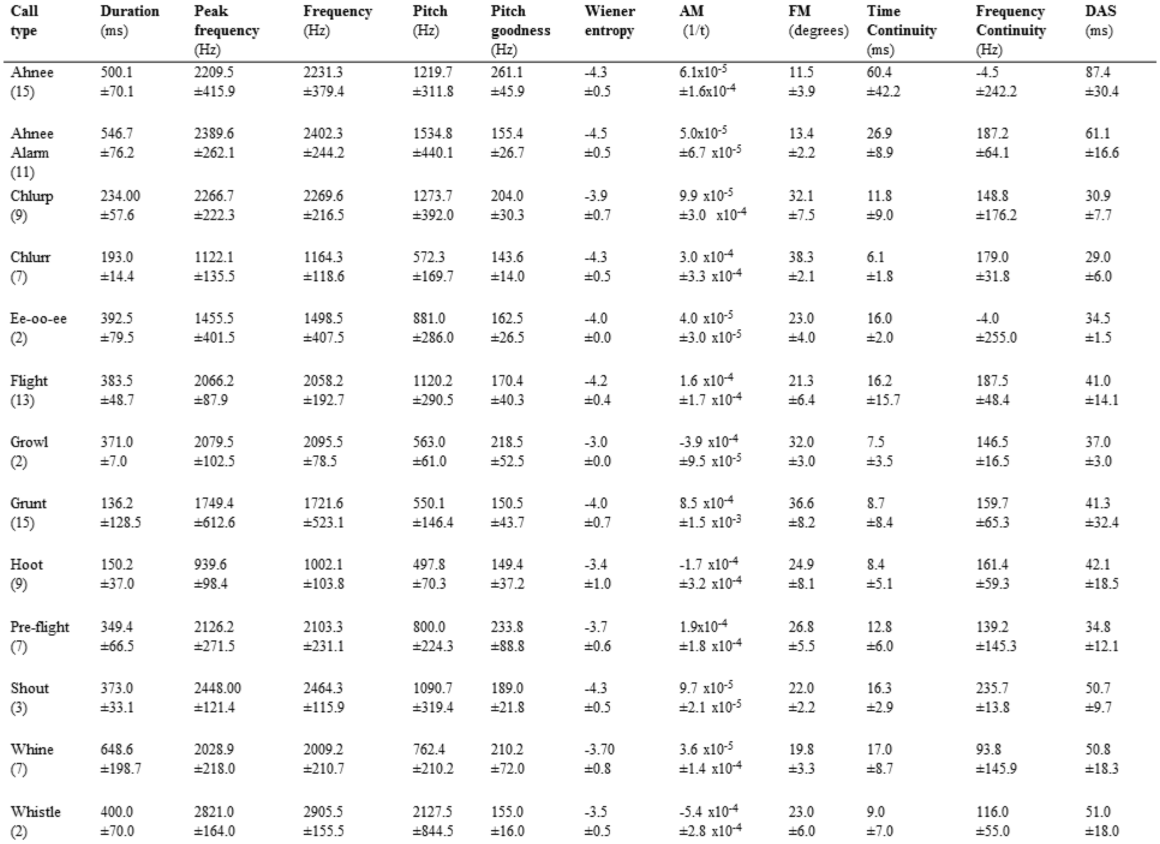


Figure 3. Results of a discriminant function analysis that categorizes nine types of well-sampled Smooth-billed Ani calls according to seven acoustic parameters. a) Scatter plot of discriminant function scores. Prior to analysis, the authors categorized calls as *ahnees* (black diamonds), *ahnee alarms* (grey squares), *chlurps* (dark grey triangles), *chlurrs* (dark grey ‘X’s), *flights* (grey asterisks), *grunts* (light grey circles), *hoots* (grey pluses), *pre-flights* (short black bars), or *whines* (long black bars). b) Group centroids for each call type are plotted as a spectrogram.

Table 1. Mean (± SD) values of 10 acoustic variables measured for the 13 Smooth-billed Ani call types analysed. The numbers in brackets below each call type represent the number of groups sampled. One high quality (high signal-to-noise ratio) example of each available call was chosen from each group for analysis.



Call type	Duration (ms)	Peak frequency (Hz)	Frequency (Hz)	Pitch (Hz)	Pitch goodness (Hz)	Wiener entropy	AM (1/t)	FM (degrees)	Time Continuity (ms)	Frequency Continuity (Hz)	DAS (ms)
Ahnee (15)	500.1 ±70.1	2209.5 ±415.9	2231.3 ±379.4	1219.7 ±311.8	261.1 ±45.9	-4.3 ±0.5	$6.1x10^{-5}$ $\pm 1.6x10^{-4}$	11.5 ±3.9	60.4 ±42.2	-4.5 ±242.2	87.4 ±30.4
Ahnee Alarm (11)	546.7 ±76.2	2389.6 ±262.1	2402.3 ±244.2	1534.8 ±440.1	155.4 ±26.7	-4.5 ±0.5	$5.0x10^{-5}$ $\pm 6.7\ x10^{-5}$	13.4 ±2.2	26.9 ±8.9	187.2 ±64.1	61.1 ±16.6
Chlurp (9)	234.00 ±57.6	2266.7 ±222.3	2269.6 ±216.5	1273.7 ±392.0	204.0 ±30.3	-3.9 ±0.7	$9.9\ x10^{-5}$ $\pm 3.0\ x10^{-4}$	32.1 ±7.5	11.8 ±9.0	148.8 ±176.2	30.9 ±7.7
Chlurr (7)	193.0 ±14.4	1122.1 ±135.5	1164.3 ±118.6	572.3 ±169.7	143.6 ±14.0	-4.3 ±0.5	$3.0\ x10^{-4}$ $\pm 3.3\ x10^{-4}$	38.3 ±2.1	6.1 ±1.8	179.0 ±31.8	29.0 ±6.0
Ee-oo-ee (2)	392.5 ±79.5	1455.5 ±401.5	1498.5 ±407.5	881.0 ±286.0	162.5 ±26.5	-4.0 ±0.0	$4.0\ x10^{-5}$ $\pm 3.0\ x10^{-5}$	23.0 ±4.0	16.0 ±2.0	-4.0 ±255.0	34.5 ±1.5
Flight (13)	383.5 ±48.7	2066.2 ±87.9	2058.2 ±192.7	1120.2 ±290.5	170.4 ±40.3	-4.2 ±0.4	$1.6\ x10^{-4}$ $\pm 1.7\ x10^{-4}$	21.3 ±6.4	16.2 ±15.7	187.5 ±48.4	41.0 ±14.1
Growl (2)	371.0 ±7.0	2079.5 ±102.5	2095.5 ±78.5	563.0 ±61.0	218.5 ±52.5	-3.0 ±0.0	$-3.9\ x10^{-4}$ $\pm 9.5\ x10^{-5}$	32.0 ±3.0	7.5 ±3.5	146.5 ±16.5	37.0 ±3.0
Grunt (15)	136.2 ±128.5	1749.4 ±612.6	1721.6 ±523.1	550.1 ±146.4	150.5 ±43.7	-4.0 ±0.7	$8.5\ x10^{-4}$ $\pm 1.5\ x10^{-3}$	36.6 ±8.2	8.7 ±8.4	159.7 ±65.3	41.3 ±32.4
Hoot (9)	150.2 ±37.0	939.6 ±98.4	1002.1 ±103.8	497.8 ±70.3	149.4 ±37.2	-3.4 ±1.0	$-1.7\ x10^{-4}$ $\pm 3.2\ x10^{-4}$	24.9 ±8.1	8.4 ±5.1	161.4 ±59.3	42.1 ±18.5
Pre-flight (7)	349.4 ±66.5	2126.2 ±271.5	2103.3 ±231.1	800.0 ±224.3	233.8 ±88.8	-3.7 ±0.6	$1.9x10^{-4}$ $\pm 1.8\ x10^{-4}$	26.8 ±5.5	12.8 ±6.0	139.2 ±145.3	34.8 ±12.1
Shout (3)	373.0 ±33.1	2448.00 ±121.4	2464.3 ±115.9	1090.7 ±319.4	189.0 ±21.8	-4.3 ±0.5	$9.7\ x10^{-5}$ $\pm 2.1\ x10^{-5}$	22.0 ±2.2	16.3 ±2.9	235.7 ±13.8	50.7 ±9.7
Whine (7)	648.6 ±198.7	2028.9 ±218.0	2009.2 ±210.7	762.4 ±210.2	210.2 ±72.0	-3.70 ±0.8	$3.6\ x10^{-5}$ $\pm 1.4\ x10^{-4}$	19.8 ±3.3	17.0 ±8.7	93.8 ±145.9	50.8 ±18.3
Whistle (2)	400.0 ±70.0	2821.0 ±164.0	2905.5 ±155.5	2127.5 ±844.5	155.0 ±16.0	-3.5 ±0.5	$-5.4\ x10^{-4}$ $\pm 2.8\ x10^{-4}$	23.0 ±6.0	9.0 ±7.0	116.0 ±55.0	51.0 ±18.0

Table 2. Comparison of Smooth-billed Ani (*Crotophaga ani*) call types identified in this study (Grieves et al. 2014) with those previously published (Davis 1940; Quinn & Startek-Foote 2000). Calls in the same row are presumed to be of the same type and two dashes (--) represent calls for which we could not assign a match.

Grieves et al. (2014)	**Quinn & Startek-Foote (2000)**	**Davis (1940)**
Ahnee	Ahnee, Fig. 2a	Judio (flock)
Ahnee alarm	Fig. 2b	Alarm
Ahnee chorus	--	Get up
Chlurp	--	Quack (danger)
Chlurr	--	--
Ee-oo-ee	--	--
Flight	--	--
Growl	"High intensity mobbing screams"*	--
Grunt	Fig. 2c	Chuck[a]
Hoot	--	Conk[b]
Preflight	--	--
Shout	--	Shout
Whine	--	Whine[c]
Whistle	--	--
--	--	Complaint
--	--	Objecting
--	--	Chuckle
--	--	Gutteral
--	--	Whew

*S.L. Vehrencamp in Quinn & Startek-Foote (2000, p. 5).

[a] Davis' *chuck* may be equivalent to our *grunt*, but we documented a wider usage for this call. Davis describes the *chuck* as a call made only by a chased bird, while we found the *grunt* was used by multiple birds involved in chasing and between group members in roost.

[b] Davis' *conk*, produced by an attacking bird during fighting and in territory defense, most closely matches our *hoot*, as the *hoot* tended to be produced in aggressive contexts, including chases; however, we also documented *hoots* in roost and between group members.

[c] Davis' *whine* may be the same as the *whine* we identified, although this call was not made exclusively during mating as in Davis (1940), but was also produced by birds in roost, during chasing, and between group members out of roost.

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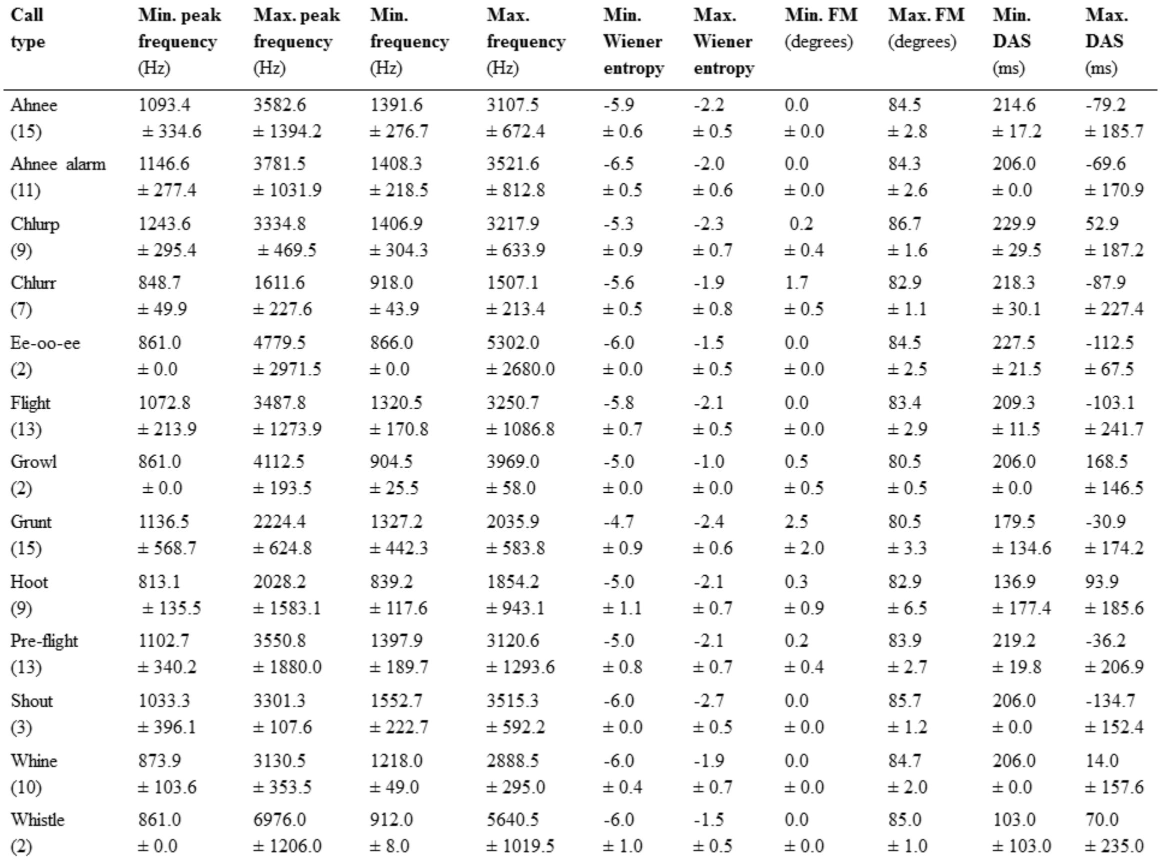
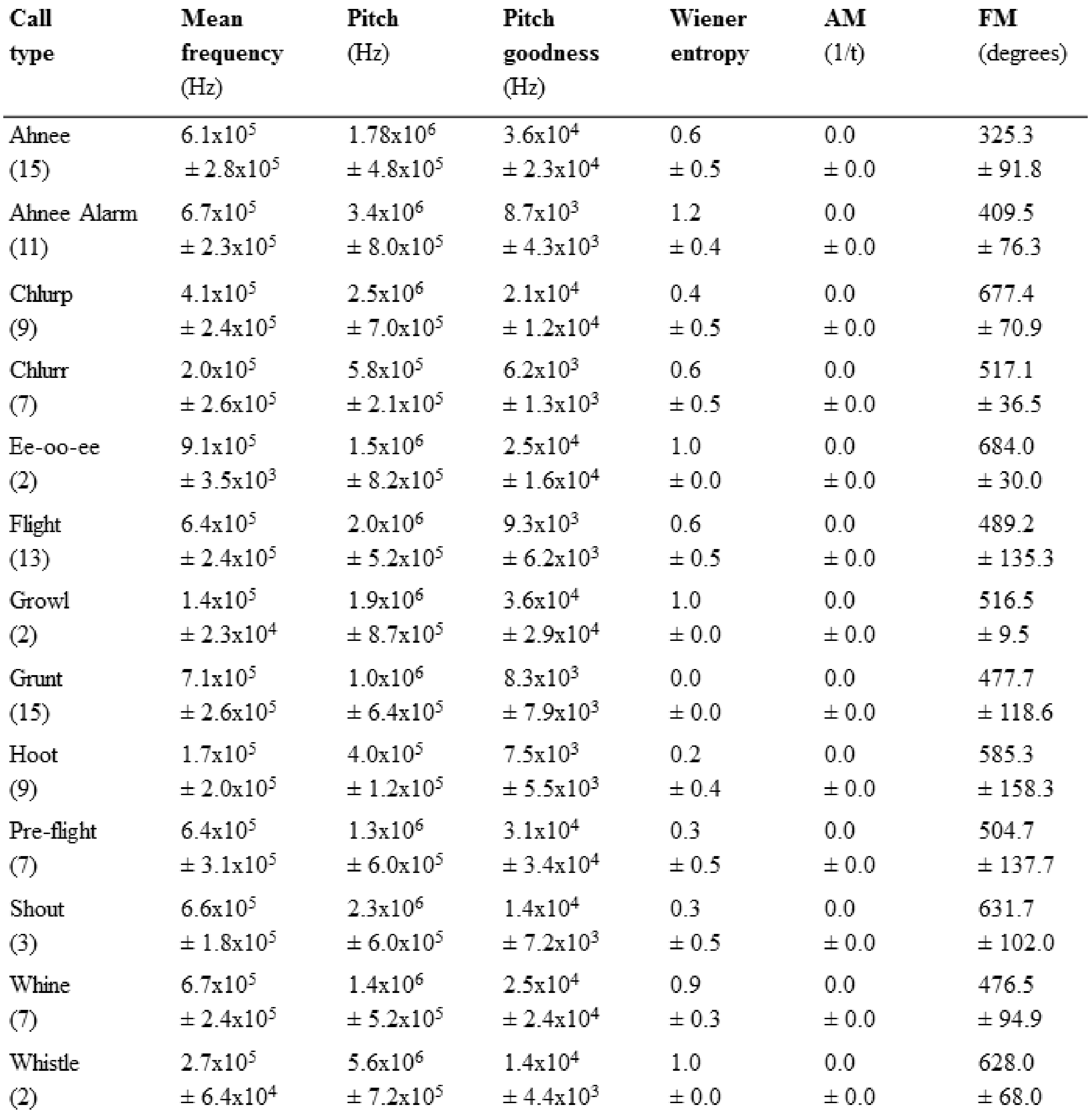
Appendix

Table S1. Mean (± SD) minimum and maximum values of acoustic variables for 13 Smooth-billed Ani calls types. The numbers in brackets below each call type represent the number of groups sampled. One high quality (high signal-to-noise-ratio) example of each available call was chosen from each group for analysis.



Call type	**Min. peak frequency** (Hz)	**Max. peak frequency** (Hz)	**Min. frequency** (Hz)	**Max. frequency** (Hz)	**Min. Wiener entropy**	**Max. Wiener entropy**	**Min. FM** (degrees)	**Max. FM** (degrees)	**Min. DAS** (ms)	**Max. DAS** (ms)
Ahnee (15)	1093.4 ± 334.6	3582.6 ± 1394.2	1391.6 ± 276.7	3107.5 ± 672.4	-5.9 ± 0.6	-2.2 ± 0.5	0.0 ± 0.0	84.5 ± 2.8	214.6 ± 17.2	-79.2 ± 185.7
Ahnee alarm (11)	1146.6 ± 277.4	3781.5 ± 1031.9	1408.3 ± 218.5	3521.6 ± 812.8	-6.5 ± 0.5	-2.0 ± 0.6	0.0 ± 0.0	84.3 ± 2.6	206.0 ± 0.0	-69.6 ± 170.9
Chlurp (9)	1243.6 ± 295.4	3334.8 ± 469.5	1406.9 ± 304.3	3217.9 ± 633.9	-5.3 ± 0.9	-2.3 ± 0.7	0.2 ± 0.4	86.7 ± 1.6	229.9 ± 29.5	52.9 ± 187.2
Chlurr (7)	848.7 ± 49.9	1611.6 ± 227.6	918.0 ± 43.9	1507.1 ± 213.4	-5.6 ± 0.5	-1.9 ± 0.8	1.7 ± 0.5	82.9 ± 1.1	218.3 ± 30.1	-87.9 ± 227.4
Ee-oo-ee (2)	861.0 ± 0.0	4779.5 ± 2971.5	866.0 ± 0.0	5302.0 ± 2680.0	-6.0 ± 0.0	-1.5 ± 0.5	0.0 ± 0.0	84.5 ± 2.5	227.5 ± 21.5	-112.5 ± 67.5
Flight (13)	1072.8 ± 213.9	3487.8 ± 1273.9	1320.5 ± 170.8	3250.7 ± 1086.8	-5.8 ± 0.7	-2.1 ± 0.5	0.0 ± 0.0	83.4 ± 2.9	209.3 ± 11.5	-103.1 ± 241.7
Growl (2)	861.0 ± 0.0	4112.5 ± 193.5	904.5 ± 25.5	3969.0 ± 58.0	-5.0 ± 0.0	-1.0 ± 0.0	0.5 ± 0.5	80.5 ± 0.5	206.0 ± 0.0	168.5 ± 146.5
Grunt (15)	1136.5 ± 568.7	2224.4 ± 624.8	1327.2 ± 442.3	2035.9 ± 583.8	-4.7 ± 0.9	-2.4 ± 0.6	2.5 ± 2.0	80.5 ± 3.3	179.5 ± 134.6	-30.9 ± 174.2
Hoot (9)	813.1 ± 135.5	2028.2 ± 1583.1	839.2 ± 117.6	1854.2 ± 943.1	-5.0 ± 1.1	-2.1 ± 0.7	0.3 ± 0.9	82.9 ± 6.5	136.9 ± 177.4	93.9 ± 185.6
Pre-flight (13)	1102.7 ± 340.2	3550.8 ± 1880.0	1397.9 ± 189.7	3120.6 ± 1293.6	-5.0 ± 0.8	-2.1 ± 0.7	0.2 ± 0.4	83.9 ± 2.7	219.2 ± 19.8	-36.2 ± 206.9
Shout (3)	1033.3 ± 396.1	3301.3 ± 107.6	1552.7 ± 222.7	3515.3 ± 592.2	-6.0 ± 0.0	-2.7 ± 0.5	0.0 ± 0.0	85.7 ± 1.2	206.0 ± 0.0	-134.7 ± 152.4
Whine (10)	873.9 ± 103.6	3130.5 ± 353.5	1218.0 ± 49.0	2888.5 ± 295.0	-6.0 ± 0.4	-1.9 ± 0.7	0.0 ± 0.0	84.7 ± 2.0	206.0 ± 0.0	14.0 ± 157.6
Whistle (2)	861.0 ± 0.0	6976.0 ± 1206.0	912.0 ± 8.0	5640.5 ± 1019.5	-6.0 ± 1.0	-1.5 ± 0.5	0.0 ± 0.0	85.0 ± 1.0	103.0 ± 103.0	70.0 ± 235.0

Table S2. Mean (± SD) variance of the acoustic variables measured for the 13 Smooth-billed Ani calls types analysed. The numbers in brackets below each call type represent the number of groups sampled. One high quality (high signal-to-noise-ratio) example of each available call was chosen from each group for analysis.

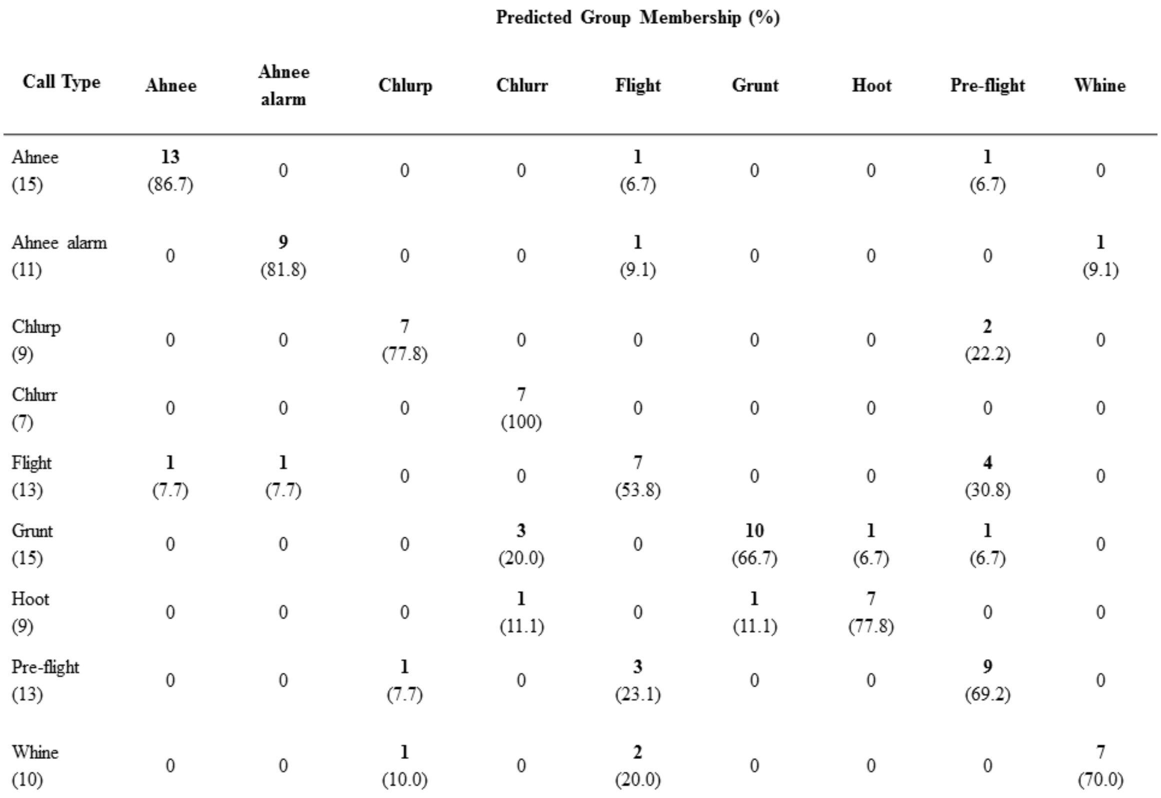


Call type	**Mean frequency** (Hz)	**Pitch** (Hz)	**Pitch goodness** (Hz)	**Wiener entropy**	**AM** (1/t)	**FM** (degrees)
Ahnee (15)	6.1x10^{5} ± 2.8x10^{5}	1.78x10^{6} ± 4.8x10^{5}	3.6x10^{4} ± 2.3x10^{4}	0.6 ± 0.5	0.0 ± 0.0	325.3 ± 91.8
Ahnee Alarm (11)	6.7x10^{5} ± 2.3x10^{5}	3.4x10^{6} ± 8.0x10^{5}	8.7x10^{3} ± 4.3x10^{3}	1.2 ± 0.4	0.0 ± 0.0	409.5 ± 76.3
Chhurp (9)	4.1x10^{5} ± 2.4x10^{5}	2.5x10^{6} ± 7.0x10^{5}	2.1x10^{4} ± 1.2x10^{4}	0.4 ± 0.5	0.0 ± 0.0	677.4 ± 70.9
Chhurr (7)	2.0x10^{5} ± 2.6x10^{5}	5.8x10^{5} ± 2.1x10^{5}	6.2x10^{3} ± 1.3x10^{3}	0.6 ± 0.5	0.0 ± 0.0	517.1 ± 36.5
Ee-oo-ee (2)	9.1x10^{5} ± 3.5x10^{3}	1.5x10^{6} ± 8.2x10^{5}	2.5x10^{4} ± 1.6x10^{4}	1.0 ± 0.0	0.0 ± 0.0	684.0 ± 30.0
Flight (13)	6.4x10^{5} ± 2.4x10^{5}	2.0x10^{6} ± 5.2x10^{5}	9.3x10^{3} ± 6.2x10^{3}	0.6 ± 0.5	0.0 ± 0.0	489.2 ± 135.3
Growl (2)	1.4x10^{5} ± 2.3x10^{4}	1.9x10^{6} ± 8.7x10^{5}	3.6x10^{4} ± 2.9x10^{4}	1.0 ± 0.0	0.0 ± 0.0	516.5 ± 9.5
Grunt (15)	7.1x10^{5} ± 2.6x10^{5}	1.0x10^{6} ± 6.4x10^{5}	8.3x10^{3} ± 7.9x10^{3}	0.0 ± 0.0	0.0 ± 0.0	477.7 ± 118.6
Hoot (9)	1.7x10^{5} ± 2.0x10^{5}	4.0x10^{5} ± 1.2x10^{5}	7.5x10^{3} ± 5.5x10^{3}	0.2 ± 0.4	0.0 ± 0.0	585.3 ± 158.3
Pre-flight (7)	6.4x10^{5} ± 3.1x10^{5}	1.3x10^{6} ± 6.0x10^{5}	3.1x10^{4} ± 3.4x10^{4}	0.3 ± 0.5	0.0 ± 0.0	504.7 ± 137.7
Shout (3)	6.6x10^{5} ± 1.8x10^{5}	2.3x10^{6} ± 6.0x10^{5}	1.4x10^{4} ± 7.2x10^{3}	0.3 ± 0.5	0.0 ± 0.0	631.7 ± 102.0
Whine (7)	6.7x10^{5} ± 2.4x10^{5}	1.4x10^{6} ± 5.2x10^{5}	2.5x10^{4} ± 2.4x10^{4}	0.9 ± 0.3	0.0 ± 0.0	476.5 ± 94.9
Whistle (2)	2.7x10^{5} ± 6.4x10^{4}	5.6x10^{6} ± 7.2x10^{5}	1.4x10^{4} ± 4.4x10^{3}	1.0 ± 0.0	0.0 ± 0.0	628.0 ± 68.0

Table S3. Standardized canonical discriminant function coefficients of the seven acoustic parameters chosen by the stepwise model selection procedure used for discriminant function analysis of nine Smooth-billed Ani calls.

Parameter	Function						
	1	2	3	4	5	6	7
Duration	.699	-1.034	.275	.037	-.254	.325	.151
Mean pitch	.503	.630	.378	.126	-.682	-.404	.988
Mean FM	-.553	-.398	.302	.748	-.042	.569	.405
Mean mean frequency	.564	.912	.665	.459	-.356	-.111	-.346
Mean DAS	-.452	.854	.114	.092	.465	.901	.111
Variance pitch	.008	-.809	-1.306	.071	.738	.409	-.451
Variance AM	.281	-.068	.409	.214	.734	-.349	.269

Table S4. Cross-validation results from discriminant function analysis (DFA). Numbers in bold indicate the number of calls assigned to each category, while numbers in brackets represent the percentage of calls assigned to each category. The sample size of each call is in brackets in the call type column. Overall, the DFA correctly classified 74.5% of calls using cross validation.



	Predicted Group Membership (%)								
Call Type	**Ahnee**	**Ahnee alarm**	**Chlurp**	**Chlurr**	**Flight**	**Grunt**	**Hoot**	**Pre-flight**	**Whine**
Ahnee (15)	**13** (86.7)	0	0	0	**1** (6.7)	0	0	**1** (6.7)	0
Ahnee alarm (11)	0	**9** (81.8)	0	0	**1** (9.1)	0	0	0	**1** (9.1)
Chlurp (9)	0	0	**7** (77.8)	0	0	0	0	**2** (22.2)	0
Chlurr (7)	0	0	0	**7** (100)	0	0	0	0	0
Flight (13)	**1** (7.7)	**1** (7.7)	0	0	**7** (53.8)	0	0	**4** (30.8)	0
Grunt (15)	0	0	0	**3** (20.0)	0	**10** (66.7)	**1** (6.7)	**1** (6.7)	0
Hoot (9)	0	0	0	**1** (11.1)	0	**1** (11.1)	**7** (77.8)	0	0
Pre-flight (13)	0	0	**1** (7.7)	0	**3** (23.1)	0	0	**9** (69.2)	0
Whine (10)	0	0	**1** (10.0)	0	**2** (20.0)	0	0	0	**7** (70.0)

Chapter II:

Joint-nesting Smooth-billed Anis, *Crotophaga ani*, use a functionally referential alarm call system

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Abstract

Accurate, threat-specific alarm systems might play a role in the evolution of joint-nesting systems by offsetting the high costs associated with joint-nesting. The Smooth-billed Ani, a joint-nesting cooperatively breeding bird, produces at least two distinct classes of vocal alarms. Anis give *chlurp* calls in response to flying raptorial birds and they give *ahnee alarms* in response to terrestrial threats. We used acoustic playback to test free-living anis' responses to these alarm calls. Anis responded to *chlurp* playback by diving into vegetative cover or flying low along the grass and into cover. These behaviours are appropriate defensive responses to an aerial threat. Anis that perched in low vegetation or on the ground responded to *ahnee alarms* by flying up to higher perches, an appropriate response to a terrestrial threat. Thus, both calls elicited responses that were appropriate to evade predation in the contexts under which the alarms are typically given. We conclude that Smooth-billed Anis respond to both *chlurp* and *ahnee alarm* calls by taking evasive action consistent with aerial and terrestrial threats respectively. Even in the absence of an actual threat, the calls alone are sufficient to elicit these responses. To our knowledge, this is the first report of a cooperatively breeding, joint-nesting species that uses a functionally referential alarm calling system. We hypothesize that direct benefits garnered from this sophisticated alarm system offset some of the fitness costs incurred by joint-nesting.

Introduction

Animals that detect a predator may emit an alarm signal that warns other potential victims. Alarms are structurally diverse, phylogenetically widespread signals that have garnered significant research attention (reviewed in Bradbury & Vehrencamp 2011). Of particular interest are signalling systems with distinct signal-types for different classes of predatory threat. These so-called "functionally referential" alarm call systems are characterized by stimulus-specific signalling in which each signal-type is elicited by a limited set of stimuli and produces a context-independent response in receivers. Critically, the signal alone is sufficient to elicit an appropriate, specific response from signal receivers (Macedonia & Evans 1993). Functional referentiality need not imply that signals have representational meaning (Macedonia & Evans 1993; Seyfarth & Cheney 2003; Rendall et al. 2009; Owren et al. 2010; Scott-Phillips 2010; Wheeler & Fischer 2012).

Functionally referential acoustic alarm calls have been identified in several mammals (e.g. Seyfarth et al. 1980; Pereira & Macedonia 1991; Manser et al. 2002; Kiriazis & Slobodchikoff 2006; Zuberbühler & Arnold 2006; Murphy et al. 2013) but to our knowledge, empirical evidence for functionally referential alarm calls has been documented in only six bird fspecies (domestic chickens, *Gallus gallus*, Evans et al. 1993; Yellow Warblers, *Dendroica petechia*, Gill & Sealy 2003; White-browed Scrubwrens, *Sericornis frontalis*, Leavesley & Magrath 2005; Platzen & Magrath 2005; Siberian Jays, *Perisoreus infaustus*, Griesser 2008; American Robins, *Turdus migratorius*, Vanderhoff & Eason 2009; Japanese Great Tits, *Parus major minor*, Suzuki 2012 and reviewed by Bradbury & Vehrencamp 2011; Gill & Bierema 2013; Townsend & Manser 2013). Many, but not all, of these systems include one class of signals for aerial predators and another class of signals for terrestrial predators (Bradbury & Vehrencamp 2011).

Our study species, the Smooth-billed Ani, is a joint-nesting, cooperatively breeding bird. Territorial breeding groups comprise one to nine socially monogamous pairs and their immature offspring (Brown 1987; Quinn & Startek-Foote 2000). Females lay eggs in a shared nest, adult group members are typically unrelated, and groups in southwestern Puerto Rico dissolve into large foraging flocks during the dry season and re-form breeding groups at the start of each breeding season (Quinn & Startek-Foote 2000). Group membership often changes from year to year.

There appear to be significant costs to group membership in this species, resulting in decreased per capita reproduction as group size increases (Schmaltz et al. 2008). Anis are visually conspicuous and poor flyers, which suggests that they are particularly vulnerable to predation. A sophisticated alarm system that helps birds detect and avoid predators may offset, in part or in whole, the reproductive costs of group membership.

Davis (1940) identified several call types in this species, and reported the contexts in which they were given. For the purposes of this study, we focused on two types of alarm calls that we named *chlurps* (Davis' '*quack*' or '*danger'* call) and *ahnee alarms* (Davis' '*alarm'* call). Anis produce *chlurps* in response to flying raptor species including Red-tailed Hawks, *Buteo jamaicensis*, Peregrine Falcons, *Falco peregrinus*, and Merlins, *Falco columbarius*. All of these species have been observed attacking anis at our study site (J. Quinn, L. Grieves, and D. Logue pers. obs.). *Chlurps* are usually followed by a "dive" response: the caller and other nearby anis dive down into the vegetation so that they are no longer accessible or visible to flying predators (Davis 1940; this study). In contrast, *ahnee alarms* are given in the presence of generalist terrestrial predators such as West Indian mongooses *Herpestes auropunctatus*, domestic dogs *Canus familiaris*, and cats *Felis catus*, as well as humans, *Homo sapiens*, who may be perceived as threatening when approaching groups closely (J. Quinn & L. Grieves, pers. obs.; this study).

We have observed cats stalking ani groups and capturing adults, mongooses raiding nests and stalking adults, and dogs attacking groups (J. Quinn & L. Grieves pers. obs). *Ahnee alarms* stimulate low-perching signal receivers to fly up to a higher perch while remaining visible (Davis 1940; this study).

Here we report our investigation of functionally referential alarm calling in Smooth-billed Anis. Our goals were to test the hypotheses that (1) *chlurps* and *ahnee alarms* are acoustically distinct, (2) group members produce *chlurps* in response to aerial threats and *ahnee alarms* in response to terrestrial threats, and (3) playbacks of *chlurps* and *ahnee alarms* elicit responses that are appropriate to aerial and terrestrial threats, respectively.

Methods

All research was conducted at Cabo Rojo National Wildlife Refuge in Puerto Rico (17˚59'N, 67˚10'W). This refuge is predominantly second growth dry forest mixed with open grassy areas (see Schmaltz et al. 2008 for a site description).

Context specificity

Macedonia and Evans (1993) write that functionally referential signals must (1) be produced in specific contexts, and (2) elicit specific responses, independent of context. We used field observations to test the first criterion. During regular observation periods (0600 – 1200 hours and 1500 – 1845 hours daily from 6 October to 6 December 2011 and from 10 September 2012 to 3 January 2013), we documented all visible aerial and terrestrial threats (including humans), and all instances of *chlurp* and *ahnee alarm* call production. Because anis produce *ahnee alarms* in response to humans, we were careful not to disturb the birds during observations. Occasionally, we were unaware of the location of all group members being observed and unintentionally elicited *ahnee alarms* by approaching too closely. We included these disturbances as a human terrestrial threat (*n* = 15). We also included visitors to the study site passing through a territory as human terrestrial threats.

We conducted randomized *G* tests on the observational dataset to determine whether call type (*chlurp*, *ahnee alarm*, or neither *chlurp* nor *ahnee alarm*) was associated with context (*aerial threat*, *terrestrial threat*, *no threat*). We ran separate analyses for data collected in 2011 and 2012. We omitted observations that included more than one call type (*n* = 3 in 2011; *n* = 3 in 2012), more than one context (*n* = 3 in 2012), or both (*n* = 2 in 2012). After eliminating one more 2012 observation due to uncertainty about the context, we were left with *n* = 116 observations in 2011 and *n* = 187 observation in 2012. We generated three matrices for each year. The 'observed' matrix was populated with observed counts, the 'expected' matrix was populated with expected counts (used to calculate *G* scores), and the 'randomized' matrix was populated with counts generated by randomly shuffling call type within group. Because call types are shuffled within group, the randomization procedure preserves the number of observations, and the identity of contexts and call types for each group. Thus, shuffling within group accounts for the possibility that groups vary with respect to the types of calls they tend to give and the contexts they tend to experience. We shuffled the data 10 000 times to generate a null distribution of *G* scores, which we compared to the observed *G* score to generate a *P* value. The *P* value represents the realized probability of observing a *G* score as high as or higher than the observed *G* score if calls were randomly associated with contexts. Note that this hypothesis test does not attempt to make inferences about a population, but rather addresses the likelihood of the observed data given the null hypothesis. When overall *G* tests were significant at an $\alpha = 0.05$ level, we re-ran the simulations using the partial G scores for "*chlurp* + aerial threat" and "*ahnee alarm* + ground threat" to test the hypothesis that these associations occurred at rates that exceeded expectations.

Field recordings

Recordings were taken *ad libitum* during field work. We recorded calls (sampling rate = 44.1 kHz, 16 bit) with two portable Marantz solid state recording units: a Marantz PMD660 equipped with a Sennheiser ME66 microphone capsule (short shotgun) and a Marantz PMD661 equipped with a Sennheiser ME67 microphone capsule (long shotgun). Both microphones used a Sennheiser K6 power module. *Ahnee alarms* and *chlurps* are structurally distinct when viewed on a spectrogram, and can be distinguished by human listeners in the field (Fig. 1).

Call Analysis

Initially, we classified *chlurps* and *ahnee alarms* by ear. We then quantified the structure of these calls with Sound Analysis Pro 2011 software (SAP, Tchernichovski et al. 2000) and used Discriminant Function Analysis (DFA) to test whether these features were sufficient to distinguish among these call types and the common *ahnee* group cohesion call. We selected 18 recordings of each of the following call types: *ahnee alarms*, *chlurps,* and *ahnee* calls. Recordings of *chlurps* and *ahnee alarms* from six different groups and *ahnee* calls from 13 different groups were chosen for their high signal-to-noise ratio. The *ahnee alarms* that we used for playback were given in response to an approaching human, and the *chlurps* were given in response to flying Red-tailed Hawks or Merlins. Recordings were high-pass filtered at 350 Hz in Syrinx v2.6f (John Burt, www.syrinxpc.com). We then used the custom-filtering function in Goldwave v5.58 (Goldwave, Inc.) to remove sample-specific noise from each stimulus (Baker & Logue 2007). SAP measured the mean, minimum, and maximum values of each of the following acoustic variables for each sample: pitch (Hz), peak frequency (Hz), frequency (Hz), frequency modulation (degrees), and Weiner entropy. We also measured the mean values of goodness of pitch, amplitude modulation

(1/t), continuity over frequency (Hz), and continuity over time (ms), as well as the duration (ms) of each sample. Tchernichovski (2012) describes these features in detail.

We used a discriminant function analysis (DFA) to test whether the three call types are acoustically distinct, and to identify the features that best separate them in acoustic space. It is likely that anis can perceive variation in all of the acoustic variables that we measured, but a DFA model including all of the variables would be difficult to interpret. We therefore simplified the model by using stepwise model selection (p = 0.1 to enter, p = 0.2 to remove).

Stimulus preparation

The 18 filtered recordings of *chlurps* and *ahnee alarms* were used as stimuli in our two playback experiments. Maximum amplitude was standardized with the “maximize” function in Syrinx. Anis commonly give two or more alarm calls in short succession (J. Quinn, L. Grieves, & D. Logue pers. obs.), so playbacks comprised two copies of one stimulus type played 0.3 s apart. Control stimuli were created by synthesizing white noise in Syrinx. The maximum amplitude of controls was matched to the maximum amplitude of the other call types. For each group, the duration of the white noise was matched to the duration of the *chlurp* stimulus in 2011 and to the duration of the *ahnee alarm* stimulus in 2012.

Playback experiments

Our first experiment, conducted in 2011, tested the response of Smooth-billed Anis to *chlurps*, *ahnee alarms*, and white noise (a *control* stimulus) when birds were perched in an exposed position on a tree where they were at risk of attack from aerial predators. We predicted that birds would respond to *chlurp*, but not *ahnee alarm* or *control* stimuli, by diving into vegetation. The second experiment, conducted in 2012, tested birds’ responses to the same classes of stimuli when they were perched on or near the ground where they were vulnerable to a terrestrial attacker. Here, we predicted that *ahnee alarm*, but not *chlurp* or *control* playbacks, would stimulate subjects to fly up off of the ground to higher perches where they would be safer from a terrestrial threat. We again predicted *chlurps* would stimulate subjects to dive into vegetation and seek cover from potential aerial predators.

We measured the average amplitude of a natural *ahnee alarm* call in the field using a sound pressure meter held 30 m (2011) or 21 m (2012) from one vocalizing ani and calibrated the playback speaker to match this output. We chose stimuli randomly for each group after satisfying the constraints that stimuli could not originate from the focal group or from a group on an adjacent territory. We used a balanced design such that all possible playback orders were used with equal frequency. We named groups according to their territory location and tabulated the number of adults and juveniles in each group. We selected 18 groups in each year and each group received three playbacks consisting of a unique *chlurp*, *ahnee alarm*, and *control* stimulus (*n* = 54 trials each year).

We used the same stimuli in both years to control for response variation attributable to within-class variation in stimulus structure. We avoided playing a given stimulus on the same territory in both years. Playbacks were conducted 5 to 10 days apart for each group and playback was postponed for at least 1 h if we detected a predator within 500 m of the focal group or if the focal group interacted with another group. Field methodologies were approved by the McMaster University Animal Research Ethics Board (Animal Utilization Protocol number 09-27-25).

2011 experiment

Experiments were conducted between 0700 and 1130 hours. We approached the groups carefully, attempting to reach a playback distance of 20 m with minimal disturbance (N = 54 trials, $\bar{X}$ = 26.4

m from subjects, range = 10 – 40 m). A 1 min pre-trial period began when at least one adult bird from the focal group was perched on top of vegetation at least 2 m off the ground. All birds that met these criteria were considered “focal birds”. Only data from focal birds contributed to our analyses. If, during the pre-trial period, all of the focal birds moved so that they no longer met the criteria, we stopped the trial and waited for at least one bird to move back into position before beginning a new 1 min pre-trial. When the pre-trial period was over we played the predetermined stimulus from a solid state recorder (Marantz PMD661) driving a loudspeaker (Mineroff Electronics SME-AFS) held at breast height, oriented toward the focal birds. We then observed the group for another 2 min recording the following behaviours: *dive* (birds dive into vegetation and out of sight), *fly up* (birds fly up to a perch, where they would be clearly visible if viewed from above), and *fly to cover* (birds fly low along the ground and take cover in vegetation). We only analysed behaviours that occurred within 5 s of the stimulus. Because birds within a group are not statistically independent of each other, we used a binary approach to scoring responses: if one or more focal birds performed the behaviour we scored a “1”, otherwise we scored “0”.

Our results showed variability in response to *chlurp* stimuli, so we conducted a follow-up set of playbacks to test for effects attributable to particular *chlurp* stimuli or to group ID. Approximately half of the groups that dove in the first set of playbacks (n = 5) were played *chlurp* stimuli that previously elicited dives and the other half (n = 4) were played *chlurps* that did not elicit dives. Similarly, about half of the groups that did not dive in the 2011 experiment received playback of *chlurps* that elicited dives (n = 4) and the others were played *chlurps* that did not elicit a dive (n = 5). Thus, each of the 18 groups received an additional playback of a second *chlurp* stimulus after the initial experiment was completed. No group was played a stimulus that it had heard before.

2012 experiment

Initially, we used a regular *ahnee* call to lure groups to a suitable playback location on their territory. Both lures and stimuli were played from a remote-controlled loudspeaker (Scorpion X1B, FoxPro Inc.) positioned 0.75 m from the ground. The lure consisted of a short (3.1 s) bout of four repeated *ahnee* calls, a common ani vocalization used for group cohesion (Appendix Figure S1; Grieves et al. in review; Chapter I) recorded from a group that was not used in this study. The lure was high-pass filtered at 350 Hz and the amplitude was maximized in Syrinx. We set up the playback equipment at a known foraging location on the focal group's territory, played the lure stimulus and waited for at least one focal bird to get into position (see below). Lures were played at 2 min intervals until the birds arrived and remained at the playback location (n = 21, $\bar{\mathbf{X}}$ = 5.6 repeats of lure, range = 1-16). The lure did not work consistently, so we reverted to the method used in 2011, in which groups were approached discretely without a lure (n = 33 trials).

The 1 min pre-trial period began when at least one bird was on the ground or perched on low vegetation (≤ 1.25 m off the ground), and approximately 20 m from the playback speaker. All birds in position at the beginning of the trial were considered focal birds. After the 1 min pre-trial was completed, we played the stimulus. Responses were scored as in 2011. After each trial we measured the distance from the playback speaker to the focal birds as well as the birds' initial height from the ground. Playbacks during lure trials were, on average, closer to the focal birds than were non-lure trials (lure trials n = 21, $\bar{\mathbf{X}}$ = 28.71 m, range = 11-53 m; non-lure trials n = 33, $\bar{\mathbf{X}}$ = 39.35 m, range = 21.5-80 m).

Playback analysis

Field-scored responses to playback were analysed with randomized G-tests (described above). Randomized G-tests were also used to test whether the lure affected the response to playback in

2012. ANOVA was used to test for differences in the mean number of focal birds observed for each stimulus type (*chlurp*, *ahnee alarm*, and *control*) and in each year. Generalized linear mixed models (GLMM) using group ID as a random factor were used to test whether the distance between focal birds and the playback speaker (independent variable) affected the birds' responses (dependent variable).

Video Scoring

Groups were video recorded throughout the pre-trial, trial, and post-trial periods, although not all of the 54 videos were of sufficient quality for scoring (2011 n = 46; 2012 n = 27). Using the same criteria as in the field, observers (4 in 2011; 2 in 2012) who were blind to the stimulus type and group ID scored ani responses from videos with audio removed (for sample videos with audio, see the online supplementary materials, available from http://www.sciencedirect.com.libaccess.lib.mcmaster.ca/science/article/pii/S0003347214000426).

We scored inter-observer agreement as "1" if all observers agreed and as "0" if there was incomplete agreement, and calculated the percentage agreement for all videos. To calculate field-video agreement, we scored "1" for complete agreement between video and field scores and "0" for no agreement. These were conservative calculations because we scored partial agreements as "0". We report both the percentage agreement and Cohen's kappa (κ), to account for chance agreement.

We used Microsoft Excel 2007 (Microsoft Corp., Redmond,WA, U.S.A) running the PopTools 3.2 plugin (www.poptools.org) for randomization tests. We used SPSS (version 17) for DFA. GLMM and ANOVA were conducted in R (version 3.0.1). The α level for all tests was 0.05.

Results

Context specificity

We observed 31 breeding groups of Smooth-billed Anis in 2011 for a total of 113.25 h (range = 5 – 770 min/group, $\bar{X}$ = 219.19 min/group) and 40 groups in 2012 for a total of 178.33 h (range = 25 – 860 min/group, $\bar{X}$ = 267.5 min/group).

Analysis of the observational data revealed statistically significant associations between call type and context in both years (randomized G tests: 2011 $G_{obs} = 113.29$, average $G_{rand} = 17.79$, $P < 0.0001$; 2012 $G_{obs} = 162.81$, average $G_{rand} = 13.2$, $P < 0.0001$). In both years, *chlurps* were significantly associated with the presence of aerial threats (2011 partial $G_{obs} = 30.54$, average partial $G_{rand} = 8.73$, $P < 0.0001$; 2012 partial $G_{obs} = 41.03$, average partial $G_{rand} = 5.66$, $P < 0.0001$) and *ahnee alarms* were significantly associated with ground threats (2011 partial $G_{obs} = 15.72$, average partial $G_{rand} = 3.11$, $P = 0.0006$; 2012 partial $G_{obs} = 27.38$, average partial $G_{rand} = 3.8$, $P < 0.0001$; Table 1).

Call Analysis

The stepwise procedure selected the following variables for the DFA: mean frequency modulation, minimum peak frequency, mean entropy, and duration. The discriminant functions were statistically significant (function 1: Wilks' $\lambda = 0.051$, $X^2_{10} = 145.8$, $P < 0.001$; function 2: Wilks' $\lambda = 0.288$, $X^2_4 = 61.0$, $P < 0.001$). The DFA correctly classified 94.4% of calls using leave-one-out cross-validation (Fig. 2). One *ahnee* call was misclassified as an *ahnee alarm* and two *ahnee alarms* were misclassified as *chlurps*.

2011 Playbacks

The mean group size was eight individuals (range = 4 – 14) and the mean number of focal birds during the experimental trials was two (range = 1 – 5). One or more focal birds showed the *dive* response in 50% (9/18) of *chlurp* playback trials, but the *dive* response was never observed during *ahnee alarm* or *control* playback trials (G_{obs} = 23.71, average G_{rand} = *2.63, P* < 0.0001, Fig. 3). In all trials in which a dive was observed, the birds dove rapidly (within 5 s) after the *chlurp* stimulus was played. We did not observe the *fly up* or the *fly to cover* response.

There was 96% (44/46 videos; Cohen's kappa, κ = 0.88) agreement in scoring responses between the blind observers (inter-observer agreement) and 96% agreement (44/46 video to field observation matches; κ = 0.86) between blind and field scorers (field-video agreement). We detected no effect of speaker distance from anis (GLMM: Z = -0.093, N = 54, P = 0.93) on responses. The results of the follow-up playbacks showed that neither the previous response to a given *chlurp* stimulus nor the previous response of a group predicted subsequent responses to *chlurp* playback (prior effect of stimulus: G_{obs} = 1.02, average G_{rand} = 0.53, P = 0.34 prior response by group: G_{obs} = 0.11, average G_{rand} = 0.54, P = 1.00).

2012 Playbacks

The mean group size was seven individuals (range = 2 – 14 and the mean number of focal birds during the experimental trials was two (range = 1 – 7). One or more focal birds exhibited *dive* behaviour in 50% (9/18) of *chlurp* trials, but they never dove in response to either of the other treatments (G_{obs} = 20.57, average G_{rand} = 2.63, P = 0.0007; Fig. 3).*Chlurps* also caused birds to *fly to cover* in 28% (5/18) of trials, a response that was never observed during *ahnee alarm* or *control* playbacks (G_{obs} = 12.05, average G_{rand} = 2.7, P = 0.012). In total, 56% (10/18) of groups responded to *chlurp* trials, either by *diving*, *flying to cover*, or both. Anis demonstrated *fly up* responses in 67% (12/18) of *ahnee alarm* playbacks, in 11% (2/18) of control playbacks, and never for *chlurp* trials (G_{obs} = 26.3, average G_{rand} = 2.43, $P < 0.0001$).

We also analysed the 2012 data with lure trials removed and obtained similar results. One or more focal birds performed *dive* behaviour in 46% (6/13) of *chlurp* trials, but they never dove in response to either of the other treatments (G_{obs} = 13.35, average G_{rand} = 2.81, $P = 0.006$). *Chlurps* also caused birds to *fly to cover* in 31% (4/13) of trials, a response that was never observed during *ahnee alarm* or *control* playbacks (G_{obs} = 8.33, average G_{rand} = 2.86, $P < 0.0001$). Anis demonstrated *fly up* responses in 67% (8/12) of *ahnee alarm* playbacks and never for *chlurp* trials or control playbacks (G_{obs} = 21.28, average G_{rand} = 4.93$P < 0.0001$).

The mean number of focal birds tested did not vary systematically by stimulus type (*chlurp*, *ahnee alarm*, and *control*; 2011: $F_{2, 51}$ = 0.246, $P = 0.78$; 2012: $F_{2, 51}$ = 0.138, $P = 0.87$). The mean number of focal birds tested in 2012 was slightly higher than in 2011 (2011: $\bar{\mathbf{X}}$ = 1.8; 2012: $\bar{\mathbf{X}}$ =2.4; $F_{1, 106}$ = 3.90, $P = 0.05$).

Inter-observer agreement in scoring videos was 93% (25/27 videos; κ = 0.85) and field-video agreement was 89% (24/27; κ = 0.75). We detected no effect of speaker distance from anis on responses (GLMM: $Z = 1.301$, $N = 54$, $P = 0.19$), nor was there an effect of using a lure on ani responses to playback of *chlurp* (*dive*: G_{obs} = 0.056, average G_{rand} = 1.08, $P = 1.00$; *fly to cover*: G_{obs} = 0.136, average G_{rand} = 1.15, $P = 0.611$), *ahnee alarm* (*fly up*: G_{obs} = 0.00, average G_{rand} = 1.10, $P = 1.00$), or *control* (*fly up*: G_{obs} = 2.55, average G_{rand} = 1.42, $P = 0.475$) stimuli (we only report tests for responses that were heterogeneous because there cannot be a statistical effect of lure if all groups responded the same way).

Discussion

We have shown that Smooth-billed Anis' *chlurp* and *ahnee alarms* are structurally distinct calls that meet both the production specificity and context-independence requirements of functionally referential signals. C*hlurp* calls are produced in response to aerial threats while *ahnee alarms* are produced in response to terrestrial threats, and our playback experiments confirm that these alarm calls elicit predictable, appropriate responses in signal receivers.

Our observational datasets from both 2011 and 2012 showed strong associations between calling behaviour and context in Smooth-billed Anis. Specifically, *chlurp* calls were associated with the presence of aerial threats and *ahnee alarm* calls were associated with terrestrial threats. We have documented *chlurps* only in conjunction with flying raptors or in rare cases, other birds in flight (e.g. the sudden appearance of a fast-flying dove or vulture under low light conditions), but never for ground-based predators or other terrestrial disturbances. We have documented *ahnee alarms* in response to terrestrial predators such as dogs, cats, mongooses, and humans but rarely for flying or perched raptors or other species. Our observations are consistent with the hypothesis that *chlurps* and *ahnee alarms* meet the stimulus specificity criterion for functionally referential calls.

The DFA revealed that *ahnees*, *ahnee alarms*, and *chlurps* are acoustically distinct calls. The failure of the DFA to correctly classify 100% of calls is probably due to the static nature of our acoustic measurements (the averages, maxima and minima that we measured do not capture specific patterns of modulation). A more detailed acoustic analysis of Smooth-billed Ani calls is forthcoming (Grieves et al. in review chapter I).

In both our 2011 and 2012 experiments, responsive Smooth-billed Anis reacted to playback of *chlurp* alarm calls by immediately diving into vegetation or by flying low along the ground and into vegetation. These behaviours, which were not elicited by *ahnee alarms* or *control* stimuli, appear to be appropriate means of avoiding aerial predators. We conclude that *chlurps* meet the context-independence criterion of functionally referential signals, because the call alone was sufficient to elicit the response. We do not know why *chlurp* playback failed to provoke responses in some trials. The results of our follow-up experiment in 2011, however, allow us to rule out the possibilities that (1) some groups were fundamentally unresponsive, and (2) some stimuli were inadequate to provoke a response.

When subjects were near the ground (2012 experiment), playback of *ahnee alarms* stimulated them to *fly up* to a higher perch. Flying up from the ground is an appropriate response to the threat of attack by a terrestrial predator. Anis on a high perch (2011 experiment) did not move in response to *ahnee alarms*. This lack of movement is not surprising if *ahnee alarms* mark the presence of terrestrial predators, which do not pose an immediate threat to birds perched high off the ground. We conclude that birds respond to *ahnee alarms* as if a terrestrial predator were present, fulfilling the context-independence criterion.

The number of focal birds in our test groups was significantly larger in 2012 than in 2011. If the presence of more birds increases responsiveness to terrestrial alarm playback, the difference in the number of focal birds might explain the heightened responsiveness of the 2012 birds to *ahnee alarm* playback. We doubt this is the case for two reasons. First, the effect of year on number of focal birds was small (2011: $\bar{X}$ = 1.8; 2012: $\bar{X}$ = 2.4) relative to the effect of year on response to the *ahnee alarm* playback (2011: 0%; 2012: 67%). Second, responsiveness to *chlurps* did not increase by a similar margin (2011: 50% dive, 0% fly to cover; 2012: 50% dive, 28% fly to cover), suggesting that the observed increment in number of focal birds is unlikely to explain the large increase in responsiveness to *ahnee alarms*. We conclude that the contextual difference between the two years (birds were high up in 2011, but near the ground in 2012) better explains the different responses to *ahnee alarm* playback than does group size. Context can also explain the *fly to cover* response to *chlurp* playback observed in 2012, as birds perched high in trees would have no need to fly to an alternate source of cover upon hearing *chlurps*.

To our knowledge, this is the first joint-nesting species that has been tested for a functionally referential alarm call system (see Naguib et al. 1999; Platzen & Magrath 2005 for two examples of cooperatively breeding helper-at-the-nest species). Unlike many other cooperatively breeding birds, Smooth-billed Anis in breeding groups experience high levels of egg competition via tossing eggs from the nest and burying eggs under a new nest floor (Quinn & Startek-Foote 2000; Schmaltz et al. 2008). As group size increases, the *per capita* seasonal reproductive success decreases, indicating a high short-term cost to group living (Schmaltz et al. 2008). In addition, anis are frequently exposed to many aerial and terrestrial threats (J. Quinn, L. Grieves & D. Logue pers. obs.) and they are poor fliers (Quinn & Startek-Foote 2000), making them vulnerable to predators. It is possible that more vigilant group members; those who are more likely to detect predators and produce appropriate alarm calls in response, are more tolerated than those who do not alarm. Future research is needed to identify the individuals within a group that produce alarm calls as well as their dominance status and breeding success within the group.

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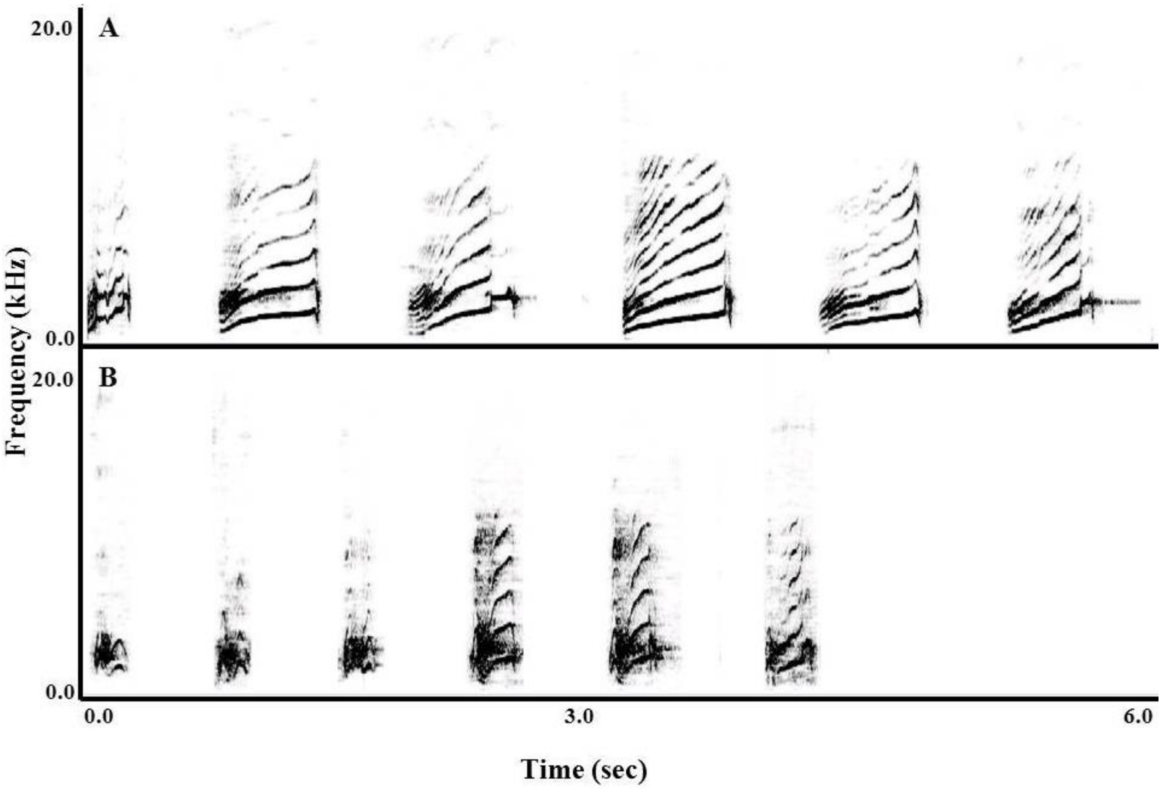

Figure 1. Spectrogram showing six representative examples of the *ahnee alarm* (A) and *chlurp* (B) stimuli used in the playback experiments. *Ahnee alarm*: Duration = 597.5 ms, Frequency = 2335.3 Hz, Pitch = 1593.8 Hz, FM = 14.9 (degrees), AM = -0.00011 (1/*t*), Wiener entropy = -5.3. *Chlurp*: Duration = 295.0 ms, Frequency = 2324.3 Hz, Pitch = 1777.3 Hz, FM = 34.1 (degrees), AM = 0.00019 (1/*t*), Wiener entropy = -4.0. Values are expressed as means (*N* = 6 for each stimulus type). Exemplar *ahnee alarm* stimuli were collected from five different groups in response to human disturbances and *chlurp* stimuli were recorded from four different groups in response to flying Red-tailed Hawks, *Buteo jamaicensis*. Exemplars were chosen to represent both the variation within stimuli of the same type and variation between stimulus types.

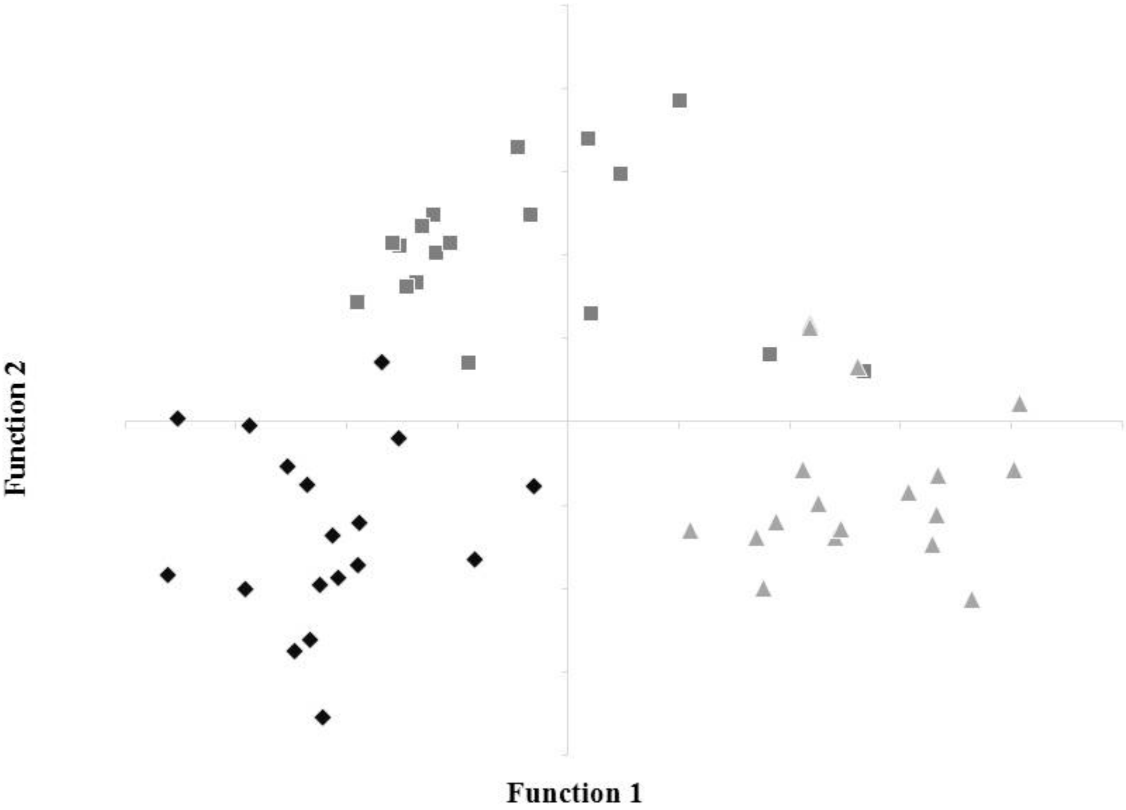

Figure 2. Scatter plot of discriminant function scores for three types of Smooth-billed Ani vocalizations. Discriminant scores are based on acoustic measurements. Prior to analysis, the authors categorized calls as *ahnees* (black diamonds), *ahnee alarms* (dark grey squares), or *chlurps* (light grey triangles). See text for details of acoustic and statistical analyses.

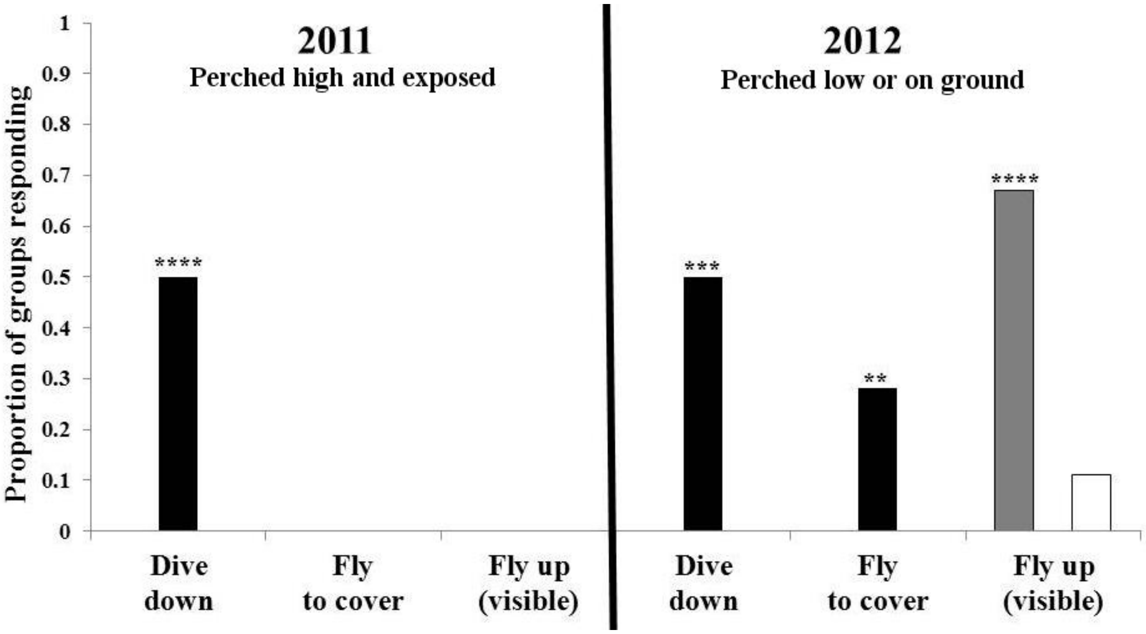

Figure 3. Responses of Smooth-billed Anis to two playback experiments. The Y-axis indicates the proportion of Smooth-billed Ani groups in which one or more group members responded. Treatments are distinguished by histogram bar colours: *ahnee alarms* (grey bars), *chlurp* calls (black bars), and white noise *control* stimuli (white bars). In 2011, birds were perched high off the ground and were exposed when viewed from above. In 2012, birds were perched near or on the ground. $**P < 0.01$, $*** P < 0.001$, $**** P < 0.0001$. See text for a complete description of the responses and statistical analyses.

Table 1. Observations of alarm calls by behavioural context.

	Chlurp	*Ahnee Alarm*	*Neither*	**Total**
Aerial Threat	48	1	10	**59**
Terrestrial Threat	0	15	1	**16**
No Threat	6	0	222	**228**
Total	**54**	**16**	**233**	**303**

Values indicate the number of observation periods (437.6 observation hours; $\bar{X}$ = 7.6 hrs/group) in which groups of Smooth-billed Anis uttered *chlurps* or *ahnee alarms*, or did not utter either of these two call types, in each of the following contexts: *aerial threat* present, *terrestrial threat* present, or *no threat* observed.

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Appendix

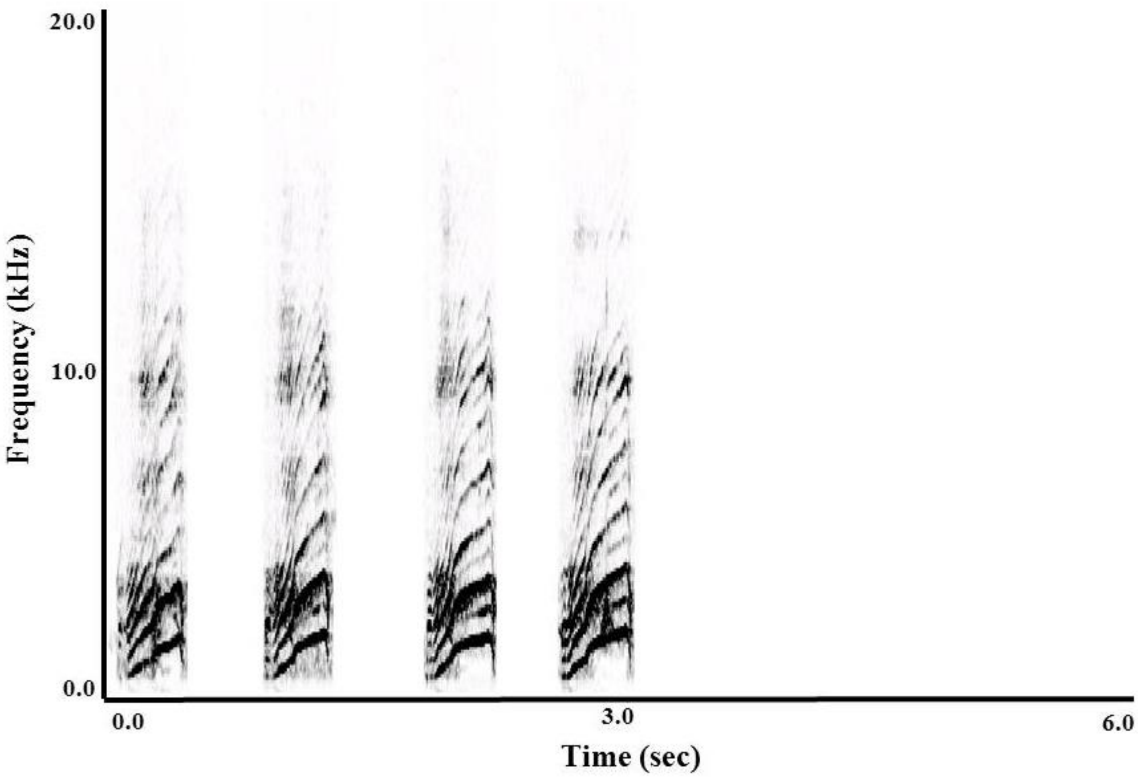

Figure S1. Spectrogram of lure call used in 39% (21/54) of playback trials in the 2012 experiment.

Chapter III

Ready to fight: reliable signals of aggression in a joint-nesting non-passerine, the Smooth-billed Ani

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Abstract

Signals of aggressive intent occur at elevated rates during aggressive contexts, predict subsequent aggression by the signaler, and elicit appropriate responses from receivers. Several recent studies of passerine birds have used a decoy presentation protocol to test the second, 'predictive' criterion. Few studies, however, have used this powerful experimental procedure to investigate aggressive signaling in non-passerines. We tested the predictive criterion in free-living groups of Smooth-billed Anis, a non-passerine with a complex social system. Based on prior observations, we hypothesized that this species uses its *'hoot'* vocalization and 'throat inflation' display to signal aggressive intent. We simulated territorial intrusions and then gave birds the opportunity to attack a conspecific taxidermic mount. In support of our hypothesis, we found that birds that attacked were more likely to give both *hoots* and throat inflation displays than were birds that did not attack. Furthermore, both of these signals increased in the time leading up to the attack. All of the attacking birds were male, suggesting that males play a disproportionate role in territory defense. We conclude that *hoot* calls and the throat inflation display reliably predict attack in this species, and are likely to serve as signals of aggressive intent.

Introduction

Animals use a variety of signals to mediate aggressive encounters. Signals of aggressive intent communicate that the animal giving the signal is likely to escalate its aggressive behaviour toward the animal at whom the signal is directed. It can be difficult to distinguish between signals of aggressive intent and the many other signal types produced during conflict situations (e.g., submissive signals, victory displays; Baker et al. 2012). Searcy and Beecher (2009) offer three criteria that can be used to identify signals of aggressive intent: 1) the signal is given at an elevated rate in aggressive contexts, 2) the signal predicts aggressive escalation by the signaler, and 3) the signal elicits an appropriate response (e.g., attack, retreat) from signal receivers. They also assert that the second, ‘predictive’, criterion should be the focus of more empirical research. This criterion is particularly important because a significant correlation between a signal and subsequent attack indicates that the signal is a reliable (or 'honest') signal of future aggressive escalation.

A recently developed experimental protocol provides a strong test of the predictive criterion (Searcy et al. 2006). First, playback is used to lure a subject to the experimental area. A conspecific taxidermic mount is then revealed, and the test subject is given the opportunity to attack the mount – an unambiguous indicator of aggressive escalation (Searcy et al. 2006; Ballentine et al. 2008; Searcy and Beecher 2009; Hof and Hazlett 2010; Akçay et al. 2011; Baker et al. 2012; Templeton et al. 2012; Anderson et al. 2013; Hof and Podos 2013; Linhart et al. 2013). This protocol can be used to test two predictions of the predictive criterion. First, attackers are predicted to signal more often than non-attackers. Second, signal frequency is predicted to increase in the time leading up to attack. Studies that use this protocol have found evidence that low-amplitude songs, calls, and visual displays predict attack in various bird species (Waas 1991a,b; Searcy et al. 2006; Ballentine et al. 2008; Akçay et al. 2011; Baker et al. 2012).

The ways in which birds use song as a signal of aggressive intent remains controversial (Searcy and Beecher 2009, Naguib and Mennill 2010, Searcy and Beecher 2011). Putative aggressive signals involving song include song type matching, frequency matching, song overlapping, song type switching, and low amplitude (soft) song. Soft song seems to reliably signal aggressive intent in several species (Ballantine 2008; Hof and Hazlett 2010; Akçay et al. 2011), but tests of the other signals have provided mixed or negative results (Searcy and Beecher 2009).

Non-passerine birds do not produce songs, but they may nonetheless have complex acoustic communication systems based on 'calls'. Compared to bird songs, calls tend to be shorter with simpler modulation patterns. Calls are typically given by both sexes, and are involved in a variety of functions including reproduction, alarm, food localization, and group cohesion. As a broad rule then, bird calls are less structurally complex but more functionally diverse than songs (Marler 2004; Benedict and Krakauer 2013).

Tests of the predictive criterion need not be restricted to acoustic signaling. The most common signal modalities used by birds are auditory and visual (Anderson et al. 2013). Visual signals that occur during aggressive encounters in birds include badges of status and postural displays (Searcy and Nowicki 2005). Waas (1991a) identified a distance-reducing display, in which test subjects orient and move toward an opponent, that reliably predicted attack in Little Blue Penguins, *Eudyptula minor*. The wing wave display, also a posture and movement display, is a reliable predictor of attack in male Swamp Sparrows, *Melospiza georgiana*, (Ballentine et al. 2008), Song Sparrows, *M. melodia* (Searcy et al. 2006; Templeton et al. 2012), and Adelaide's Warblers, *Setophaga adelaidae* (Pereira et al. In prep).

Our study species, the Smooth-billed Ani, *Crotophaga ani*, is a joint-nesting, cooperatively breeding cuckoo. Social groups consist primarily of unrelated, socially monogamous breeding pairs (2-17 adults) and their offspring of the year (Blanchard 2000, Quinn and Startek-Foote 2000). Multiple females lay eggs in a single shared nest, and all group members contribute parental care and territory defense. Thirteen different calls, emitted in diverse contexts, have been documented for this species (Davis 1940; Grieves et al. in review).

Field observations suggest that anis use two signals of aggressive intent, one acoustic and one visual. The ‘*hoot’* call is given during territorial chases and in roost (Grieves et al. in review). *Hoots* are short duration, low frequency, broadband calls with an abrupt onset and rapid frequency increase (Grieves et al. in review), consistent with general design rules for acoustic threat signals (Bradbury and Vehrencamp 2008). Throat inflation is a visual display in which the individual cranes the head forward, usually with the bill slightly open, while orienting laterally toward another individual and expanding the throat so that the feather tracts separate, revealing bare skin (L. Grieves, pers. obs.).

This behaviour has features that reduce proximity to a rival (forward leaning), exaggerate size (piloerection), and highlight a body part involved in fighting (the prominent crested bill; Fig. 1), consistent with general design rules for visual threat signals (Bradbury and Vehrencamp 2008). Further, the throat inflation display shares many features with the broadside threat display of the closely related Groove-billed Ani, *C. sulcirostris* (L. Grieves, pers. obs.). This display is made during agonistic territorial interactions between rival males, and can occur either on its own or in conjunction with ‘*hoots’* (*'conks'* in Davis 1940 and Vehrencamp et al. 1986).

Several studies have tested whether passerine songs meet the predictive criterion (Searcy et al. 2006; Ballentine et al. 2008; Hof and Hazlett 2010; Akçay et al. 2011; Templeton et al. 2012; Linhart et al. 2013), but fewer studies have tested this criterion for calls in passerines (Baker et al. 2012) or non-passerines (Waas 1991a,b; Ręk and Osiejuk 2011; Ręk 2013). Tests of the predictive criterion that focus on visual signals in passerines (Ballentine et al. 2008; Baker et al. 2012) and non-passerines (Waas 1991a) are also rare. This pattern reflects the broader paucity of data on signals of aggressive motivation in non-passerine birds (but see Andersson 1976; Mager et al. 2012).

We used playback and presentation of a conspecific mount to test the predictive criterion in cooperatively breeding groups of Smooth-billed Anis. We predicted that both *hoot* calls and the throat inflation display would be more common in attackers than in non-attackers, and that these displays would increase in the time leading up to an attack. Our results also allowed us to describe which group members ultimately attacked the mount, providing evidence that in this species, the sexes have distinct roles in territory defense.

Methods

Study Area

This study was conducted at the Cabo Rojo National Wildlife Refuge in southwestern Puerto Rico (17º59'N, 67º10'W) during the rainy season from October 13 to December 4 2013, a period that spans the peak breeding season for Smooth-billed Anis (Quinn and Startek-Foote 2000). This is a long-term study site, where ani research and population monitoring has been ongoing since 1998 (see Schmaltz et al. 2008 for a site description). All field methodologies were approved by the McMaster University Animal Research Ethics Board (Animal Utilization Protocol number 09-27-25).

Playback Stimuli

Prior to the experiment, we did not know whether birds would need to hear a *hoot* call in order to direct their aggression toward the mount, so we used playback stimuli with and without *hoots*. The *ahnee* call is the most commonly produced Smooth-billed Ani vocalization, and is believed to be a contact call. The *ahnee* stimulus was created by recording an ani giving *ahnee* calls at a natural rate for 25 s. The *ahnee* + *hoot* stimulus was created by adding four copies of the same *hoot* call spaced at 0.5 s intervals to the end of the *ahnee* recording, resulting in a 28 s playback. A repeated phrase of four *hoots* at 0.5 s intervals was chosen because this reflects the natural pattern of *hooting* often heard in the wild (L. Grieves, pers. obs.). *Ahnee* and *ahnee* + *hoot* recordings were high-pass filtered at 350 Hz and the peak amplitude was standardized with the "maximize" function in Syrinx (version 2.6f, John Burt, www.syrinxpc.com). Sample-specific noise was removed using the custom filtering function in Goldwave (version 5.58, Goldwave, Inc.) following Baker and Logue (2007). Both the *ahnee* and *hoot* recordings were taken from groups that were not included in this experiment and were broadcast on a continuous loop during playback trials.

Although we used the same two stimuli on all groups, this experimental design does not suffer from the stimulus pseudoreplication issue first identified by Kroodsma (1989, Kroodsma et al. 2001). The goal of most avian playback studies is to demonstrate that one or more classes of playback stimulus reliably provoke some behavioural response(s). Kroodsma (1989) argued (correctly in our opinion) that for that kind of study the stimulus set should represent the breadth of stimulus types in each class. The goal of the present study was to demonstrate that the subject's own signaling behaviour (*hoot* calling, throat inflation) predicts its future aggressive behaviour (attack on the mount). The playback stimuli and the taxidermic mount attract the subjects to the testing area and prime them to express their signaling and aggressive behaviours by providing the context of a territorial intrusion. Thus, the stimuli do not represent experimental variables, so they should be made as uniform as possible and pseudoreplication is a non-issue.

Experimental Design

Trials were performed between 0700 and 1100 hours at a central location on the test group's territory. With the test group absent, a taxidermic mount of a male Smooth-billed Ani in a neutral position (perched with head facing forward and wings tucked in) was attached to the branch of a tree, 1.6 m above the ground, and covered with a camouflage print cloth. A string attached to the cloth was run to a blind 8 – 30 m away. A Marantz PMD661 recorder with Sennheiser ME62 omnidirectional microphone was placed 15 cm from the mount and a remote controlled loudspeaker (Scorpion X1B, FoxPro Inc.) was placed directly below the mount. A Sony HDR-CX160 Handycam was placed 5 m from the mount and zoomed in to record 1 m on either side of the mount. To aid distance estimates, flagged stakes were placed at 2, 5, and 10 m from the mount. From within the blind, one experimenter (L.A.G.) used a digital voice recorder (Olympus VN-7200) to dictate all activity occurring within 10 m of the mount. Binoculars were used to identify banded birds and make detailed observations. When birds were spread out, observations were focused on birds ≤ 2 m from the mount. We measured the average amplitude of an *ahnee* call using a sound pressure meter held 20 m from one vocalizing ani and calibrated the playback speaker to match this output. Playback began 5 to 15 min after set up was completed and playback loops were broadcast continuously for the duration of the trial. The cloth covering the mount was removed as soon as one or more anis approached to ≤ 10 m from the mount.

We attempted to subject eighteen groups to one trial with the *ahnee* playback stimulus and one trial with the *ahnee* + *hoot* stimulus. Playback order was randomized and balanced across groups. Once the cover was removed, trials lasted until a bird attacked the mount (defined as striking the mount with any part of the body), or for 30 min after the mount was revealed if no birds attacked. If birds did not approach within 10 m of the mount, the cover was not removed and the playback was run for 45 min total before cancelling the trial (n = 4). In cases where birds did not approach the mount within 10 m, the trial was attempted two more times. For each test group, we recorded the times at which playback began, the cover was removed, the first attack was made, and the trial ended.

Four of the 18 groups were nonresponsive to playback (did not approach within 10 m) after three attempts and were excluded from the experiment. Of the remaining 14 groups, two failed to respond during their second trial. Thus, 12 groups were subjected to two trials, and two others underwent only one trial (N_{groups} = 14, N_{trials} = 26). Eight groups received the *ahnee* trial followed by the *ahnee* + *hoot* trial, four groups received the *hoot* trial followed by the *ahnee* trial, and two groups heard playback of the *ahnee* + *hoot* trial only.

Data Collection

One experimenter (L.A.G.) counted the number of *hoots* (Fig. 2) made by focal groups during trials using both video and audio from the microphone placed near the mount. Author L.A.G. scored the presence of the throat inflation display as a state variable from both the videos and detailed behavioural observations made during the trials. The throat inflation display was defined by the skin around the throat being visibly extended or puffed out, and the neck elongated so that the head was oriented and craning toward the mount. Typically, the throat of Smooth-billed Anis has a sleek, relaxed appearance, making this display readily discernible (Fig. 3).

For each group that attacked, we recorded the attacker ID and, where possible, the nocturnal incubator ID. The nocturnal incubator was identified either visually as it left the nest in the morning or by placing a GoPro Hero 3 camera (GoPro, San Mateo, CA) at the nest 1 – 2 hours before sunset and retrieving the camera the following day. Because a small blood sample is taken from all birds when first caught, we were able to determine the sex of all attacking birds using molecular techniques adapted from Griffiths et al. (1998).

We counted the number of *hoots* produced by attacking birds and recorded the time at which each *hoot* was produced for all birds that *hooted* prior to attack (n = 6). We then calculated the mean number of *hoots* made prior to attack and graphed *hoot* production by binning the 120 s leading up to attack (time = 0) into 10 s blocks of time (Fig. 4A). We calculated the mean proportion of time spent in the throat inflation display prior to attack for 10 of the 11 groups that made this display before attacking (one video was of insufficient quality to score and could not be included). We binned the proportion of time spent in the throat inflation display into 10 s blocks and graphed the results for the 120 s prior to attack (Fig. 4B).

Statistical Analyses

We first tested for an effect of playback type (*ahnee* vs *ahnee* + *hoot*) on attack, *hoot* production, and throat inflation with a chi-square test. To estimate whether *hoot* calls and the throat inflation display were reliable predictors of attack, we fitted a binomial generalized linear mixed model (GLMM) with a logit link function (Bolker et al. 2009). In the full model, *hoots* produced prior to attack and throat inflation made prior to attack were the binary predictor variables, attack was the response variable, and group ID was included as a random factor to account for the repeated measures design. In cases where multiple group members *hooted* and/or made the throat inflation display, only *hoots* and throat inflation displays made by the individual that approached the mount most closely (within 2 m) or that eventually attacked were included in our analyses. We analyzed *hoots* and throat inflation as binary (present/absent) variables because our main goal was to determine whether *hoots* and throat inflation predict attack, regardless of trial duration, the number of *hoots* made overall, or the amount of time spent making the throat inflation display prior to attack.

The effect of group ID was negligible in the full model (among-group variance in probability of attack was estimated at $6.15\text{x}10^{-15}$). Further, throat inflation was an almost perfect predictor of attack, so this variable masked any relationship that might exist between *hoots* and attacks when we used the full model. We therefore chose to analyze our predictor variables in two separate models.

For the *hoot* model, we retained group ID as a random factor and fit the GLMM with *hoots* as the sole predictor. To deal with the issue of separation for the throat inflation model, we dropped the negligible random effect (group ID) and used a bias-reduction method to estimate the effect of throat inflation on probability of attack. This bias corrected method was more appropriate than GLMM for both the extreme values in the throat inflation category and our small sample size (Heinze and Schemper 2002; Pasch et al. 2013), but does not account for the repeated measures design. Therefore, we only included *ahnee* playback trials in this analysis (n = 12).

The chi-squared test was run in Microsoft Excel 2010 (Microsoft Corp., Redmond WA) running the PopTools 3.2 plugin (www.poptools.org). The GLMM and bias-reduced GLM were conducted in R (version 3.0.2, R Core Team 2013) using the lme4 (Bates et al. 2013) and brglm (Kosmidis 2013) packages respectively. An alpha level of 0.05 was set for all statistical tests, and all tests were two-tailed.

Results

The average size of test groups was 7 birds (range 4 – 11). Of the 14 groups included in the experiment, one or more birds approached to within 10 m of the playback speaker in 93% (26/28) of trials. Most (57%, 8/14) groups attacked in at least one trial and 46% (12/26) of trials ended in attack. The average latency to attack was 8.3 min (SE = ± 1.87 min, range 2.1 – 26.2 min). Attacks consisted of strikes with the bill and in some cases grasping and clawing with the feet. In one of the eight groups that attacked, the attacker was unbanded and could not be identified or sexed. All of the remaining attackers were male (n = 7).

Half (4/8) of groups that attacked did so in both trials. One of these groups included unbanded birds that could not be identified, so we do not know whether the attacking bird was the same individual in both trials. For the three remaining groups that attacked twice, the attacker was the same individual in both trials. We identified the nocturnal incubator for 38% (3/8) of groups that attacked. In 67% (2/3) of those groups, the attacker was the nocturnal incubator for that group. In the third group, the nocturnal incubator approached within 1 m of the mount, but was not the first to attack.

Playback Type

We found no statistically significant effect of playback type (*ahnee* vs *ahnee* + *hoot*) on attack likelihood (X^2 = 0.001, df = 1, p = 0.976), *hoot* production (X^2 = 0.057, df = 1, p = 0.81), or throat inflation (X^2 = 0.113, df = 1, p = 0.74).

Predictors of Attack

Hoots were statistically significant predictors of attack (GLMM: Wald z = 2.23, p = 0.03). *Hoots* were produced prior to the first attack in 50% (6/12) of trials that ended in attack and in only 7% (1/14) of trials that did not end in attack. The mean number of *hoots* produced prior to attack was 8.5 (range 0 – 66). In the one trial where *hoots* were made that did not end in attack, the number of *hoots* produced was 3. The throat inflation display was also a significant predictor of attack (BRGLM: Wald z = 2.29, p = 0.02). Anis inflated their throats prior to attack in 100% (6/6) of trials that ended in attack, and never in trials that did not result in an attack (0/6). Thus, throat inflation was a perfect predictor of attack. The average amount of time spent in the throat inflation display prior to attack was 35.3 s (range 0 – 97 s). Both the *hoot* rate and the proportion of time spent giving the throat inflation display increased prior to attack (Fig. 4).

Discussion

We show that *hoots* and the throat inflation display reliably predict aggression in Smooth-billed Anis, fulfilling the predictive criterion for classification as signals of aggressive intent (Searcy and Beecher 2009). It remains to be seen whether the response criterion, that signal receivers should respond to *hoots* and throat inflation, is upheld. Throat inflation was a much stronger predictor of attack than *hoots*. Our results indicate that two signal modalities, acoustic and visual, are used by Smooth-billed Anis to communicate aggressive motivation, but more research is needed to investigate whether and how these two signals might alter or reinforce each others' function (Laidre and Vehrencamp 2008). We found no evidence of other signals that predict attack; in fact, anis that did not *hoot* before attacking remained silent.

All of the attackers were male, a finding common to similar studies in both passerines (Searcy et al. 2006; Ballentine et al. 2008; Hoff and Hazlett 2010; Templeton et al. 2012) and non-passerines (Ręk and Osiejuk 2011; Mager et al. 2012), although both males and females attack mounts in at least one non-passerine species (Little Blue Penguins, Waas 1991a). Joint-nesting is believed to be maintained at least in part by the importance of paternal care, especially male incubation, for successful reproduction (Vehrencamp and Quinn 2004). In Groove-billed Anis, cooperatively breeding groups contain a dominant male responsible for all nocturnal incubation and much of the diurnal incubation (Vehrencamp 1977, 1978). In Smooth-billed Anis, the primary nocturnal incubator is male, but little is known about dominance relationships in this species (Quinn and Startek-Foote 2000; Vehrencamp and Quinn 2004).

We were unable to identify the nocturnal incubator in all of our test groups but we did find that, in two of the three groups for which we had this information, the attacking male was the nocturnal incubator. We also found that in the three groups that attacked in both trials and for which the attacker identity was known, the attacker was the same in both trials. While these data are insufficient to draw strong conclusions, our experimental design could easily be modified to test for behavioural (attack) consistency across multiple trials within groups. Pairing our design with identification of the nocturnal incubator for all test groups would allow future researchers to test the hypothesis that the nocturnal incubator is more aggressive than other group members.

In summary, we have shown that *hoot* calls and the throat inflation display are reliable predictors of attack in male Smooth-billed Anis. The throat inflation display is a highly reliable predictor of attack. Our study adds to the growing body of research on calls and visual displays as reliable predictors of attack in non-passerines and, to our knowledge, is the first to test the predictive criterion in a cooperatively breeding, joint-nesting species.

Acknowledgments

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Figure 1. Smooth-billed Ani, *Crotophaga ani*, showing prominent crested bill. Anis often attack using their beak to bite, peck and hold opponents. Photo: © Alfredo Irizarry 2014.

Figure 2. Smooth-billed Ani in A) relaxed position and B) throat-inflation display. Asterisks (*) denote the focal (attacking) individual while the other bird in A is the taxidermic mount. Arrows indicate A) the relaxed throat and B) the inflated throat with skin exposed. Note that in B, the focal individual is crouched with wings spread slightly and tail extended, typical of this display and similar to the broadside display in Groove-billed Anis (Vehrencamp et al. 1986; Bradbury and Vehrencamp 2008, p. 509). Also note that in B, the focal bird’s bill is slightly open, common during throat inflation.

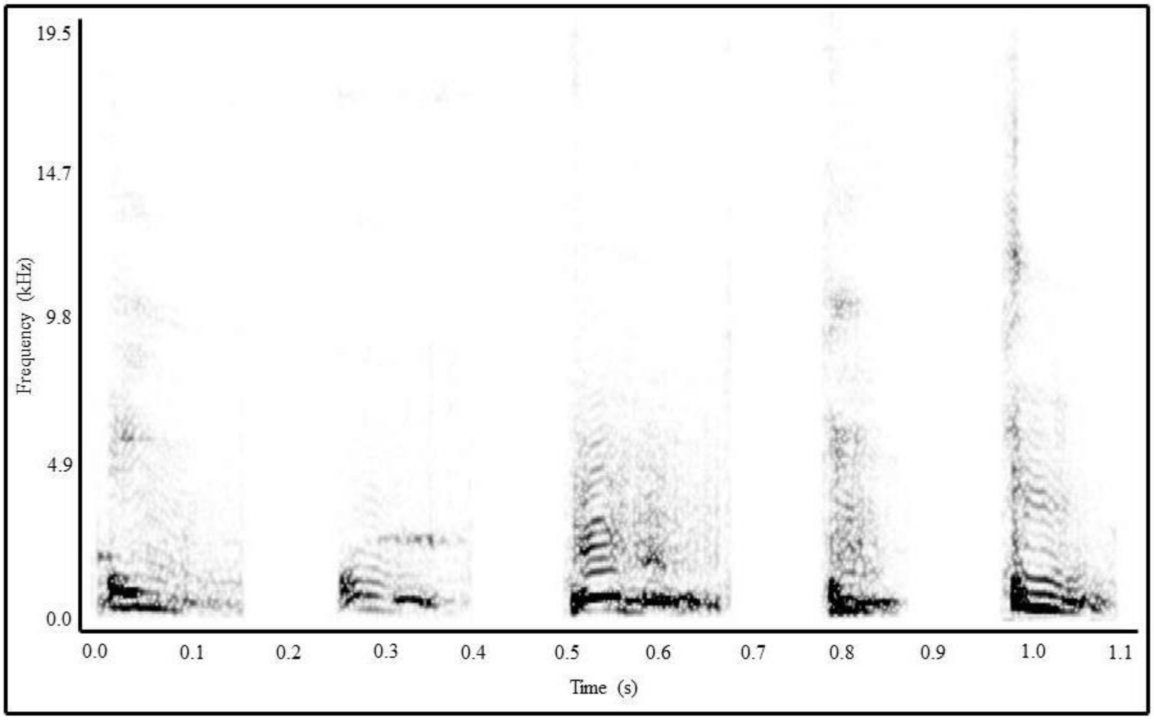

Figure 3. Spectrogram showing five *hoot* calls produced by different groups prior to attacking a conspecific mount during experimental trials.

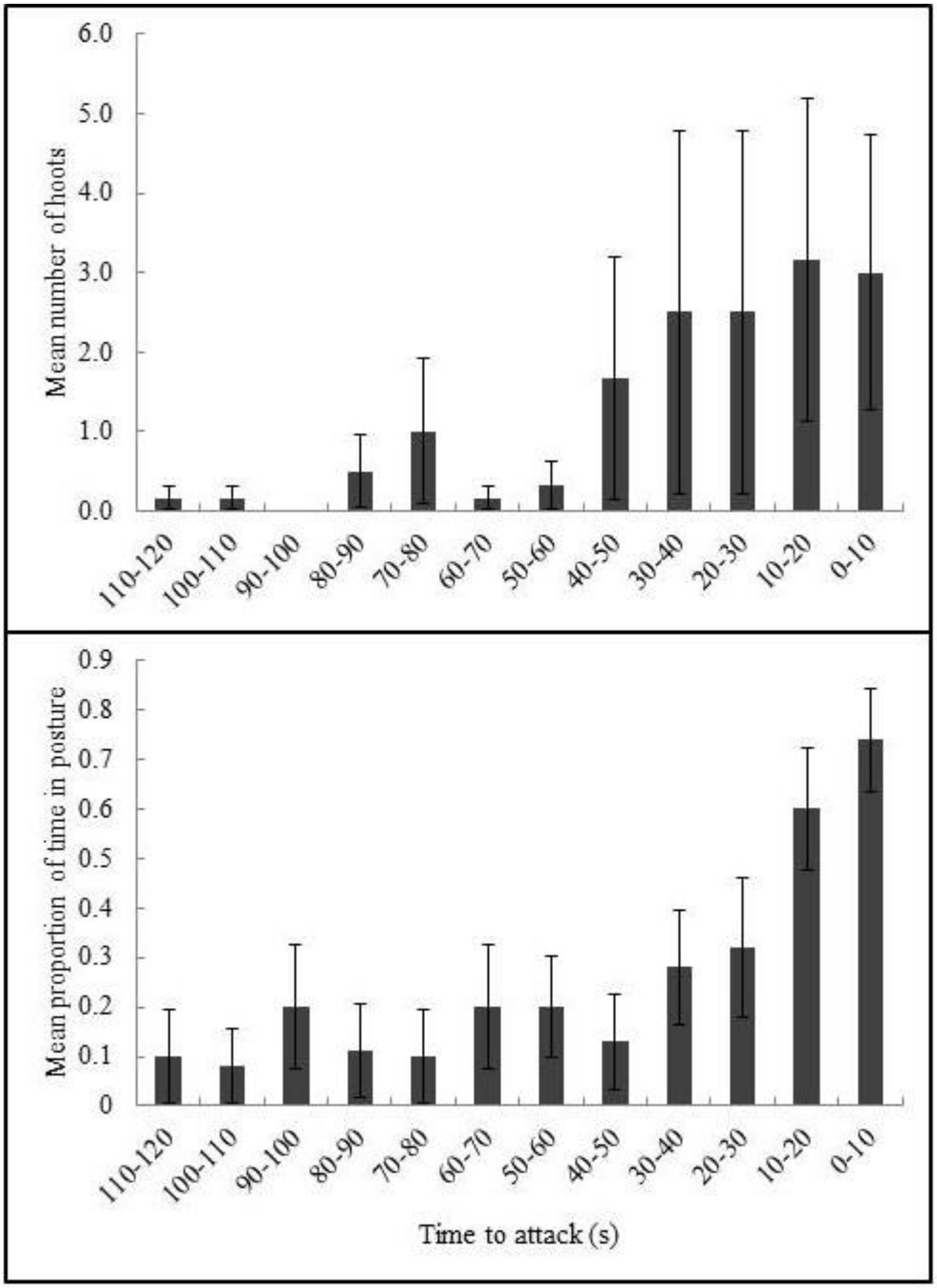

Figure 4. Mean number of *hoots* produced (A) and mean proportion of time spent in the throat inflation display (B) by Smooth-billed Anis in the 120 s prior to attack (time 0). Time was binned into 10 second intervals leading up to the attack time (time 0) using the shortest latency to attack (150 s) as a starting point. A: $n_{\text{hoot + attack}} = 6$, B: $n_{\text{throat inflation + attack}} = 10$. Error bars indicate the standard error of the mean.

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Synthesis & Future Directions

Joint-nesting is a rare breeding system and in Smooth-billed Anis, unlike many cooperatively breeding species, group members are unrelated. Previous researchers identified short-term costs to joint-nesting in the form of increased egg loss, via egg tossing and egg burial, with increasing group size (Schmaltz et al. 2008). Schmaltz et al.'s (2008) research indicated the need for more data on the benefits of group living in this species. My thesis represents the first exploration of acoustic communication and its associated potential benefits in joint-nesting Smooth-billed Anis.

I found that anis engage in complex communication in at least two ways: 1) through the use of a referential alarm call system, and 2) via acoustic and visual signals that predict aggressive escalation in the form of physical attacks. The use of a sophisticated alarm call system provides information about predator type and elicits appropriate evasive responses in signal receivers, and may provide a benefit to group-living individuals through early detection of predators and increased likelihood of survival.

The ability to communicate accurately and effectively about willingness to attack provides a second potential benefit to group living. Signalers can indicate their aggressive motivation by *hooting*, throat inflation, or both. Receivers can then choose how to respond, either by escalating or capitulating. While the receiver response to these aggressive signals has not yet been tested in anis, research in other species indicates that aggression plays an important role in both dominance and group stability (Blanchard et al. 1988; Verbeek et al. 1996; Issa & Edwards 2006). More aggressive individuals are often the most dominant in their group, and overall aggression decreases once dominance relationships stabilize, leading to reduced fighting and risk of injury for all group members (Issa & Edwards 2006). While I tested whether *hoot* calls and throat inflation were reliable predictors of attack in Smooth-billed Anis using a conspecific mount representing an intruder from outside the group, the above logic holds.

Animals need to communicate effectively about aggressive motivation with individuals from outside their group as well as with group members. Accurate communication with outsiders in aggressive interactions should allow individuals to assess whether a contest is likely to escalate to a fight and whether the risk is worth taking (Maynard-Smith & Harper 2003; Searcy & Nowicki 2005).

My experiments on aggressive and alarm signaling indicate that both acoustic and visual communication are important for anis. My analysis of the vocal repertoire of Smooth-billed Anis should aid future communication research by standardizing the call nomenclature and making accurate identification and classification of call types easier.

In chapter I, I described the vocal repertoire of the Smooth-billed Ani. I provided spectrograms and acoustic measurements to help future researchers differentiate and classify ani calls and demonstrated that multivariate analysis can be used to corroborate visual classification of call types. I also described the contexts in which the different calls were heard. Knowing the context in which calls are produced can help researchers classify calls, but it also provides a starting point for future acoustics research in this species.

For example, I noted that the *whine* call was produced between individuals, during territorial chases, and in response to *hoots* and *grunts* from other group members. The *whine* may function as a submissive or appeasement call (Purton 1978; Engh et al. 2006; Reber et al. 2013). This hypothesis could be tested by presenting groups with a conspecific taxidermic mount in conjunction with playback of *whines* and other calls, and looking for differences in call production and behavioural response depending on the call type broadcast. Detailed observations on naturally produced *whines* and subsequent behaviours, using individually recognizable, colour banded individuals could also aid in determining possible functions for this call.

I also identified three alarm calls: *ahnee alarms*, *chlurps*, and *shouts*. *Ahnee alarms* were usually produced in response to terrestrial threats and *chlurps* in response to flying raptors, while *shouts* were often heard after a raptor had flown past or when a perched raptor was detected. These alarm calls may represent a form of graded signal, a signal that transmits information by varying frequency, intensity or both (Wilson, 1975; Manser et al. 2002; Wilson & Evans 2012). If so, I would predict that *ahnee alarms* represent the lowest threat level, *chlurps* the highest, and *shouts* an intermediate level of threat. Predator presentation experiments could be designed to test call type production in response to different threat levels, such as flying (higher threat) versus perched (lower threat) raptors, and near (higher threat) versus far (lower threat) terrestrial predators.

In chapter II, I showed that Smooth-billed Anis use a functionally referential alarm call system in which two distinct call types are produced in response to two different predator classes. I used observational data to demonstrate the production specificity of Smooth-billed Ani alarm calls, and showed that *ahnee alarms* and *chlurps* were significantly associated with terrestrial and aerial threats respectively. I also attempted to test the production specificity of these alarm calls experimentally; details of this pilot research can be found in the appendix (A3).

I showed that adult Smooth-billed Anis can distinguish between *chlurp* and *ahnee alarm* calls and respond differently to each call type. *Ahnee alarms* cause anis to fly up from the ground or low perches, while *chlurps* cause anis to dive down or fly to cover and out of sight. I hypothesized that nestlings could also distinguish between these two alarm calls and may have different, adaptive responses to playback of these alarms, as has been shown in other species (Platzen & Magrath 2005; Suzuki 2011).

I designed a playback experiment to test nestling responses to *chlurp* and *ahnee alarm* call playback. I encountered several difficulties with the experimental set up and found no evidence of differential nestling responses to adult alarm call playback. Further, nestlings failed to respond to *ahnee alarms* produced by adults during a natural encounter with a nest predator and the nestlings were killed. Full details of this work, and anecdotal evidence of innate *chlurp* production and response in juvenile anis, can be found in appendix A4.

In chapter III, I designed an experiment to test whether a vocal signal, the *hoot* call, and a threat display, throat inflation, were reliable predictors of attack in Smooth-billed Anis presented with a conspecific mount. Both the number of *hoot* calls produced and the proportion of time spent in the throat inflation display increased prior to attack. Both *hoots* and throat inflation were reliable predictors of attack, with throat inflation being a stronger predictor than *hooting*.

My experimental set up was efficient, requiring only about 20 minutes to set up, and the results were clear and easy to interpret. During this experiment, I found some preliminary evidence of behavioural consistency in Smooth-billed Anis. In 43% (3/7) of groups that attacked in both trials, where the attacker ID was known, it was the same individual that attacked each time. My experimental design could be modified to conduct multiple, identical trials on breeding groups to test the hypothesis that groups contain individuals who are more likely to attack than other group members.

I also found that in 67% (2/3) of cases where the nocturnal incubator of the test group was known, it was the nocturnal incubator who attacked. In joint-nesting Groove-billed Anis, *Crotophaga sulcirostris*, the nocturnal incubator is considered to be the dominant male in each breeding group (Vehrencamp 1977, 1978), and the same has been assumed for Smooth-billed Anis (Vehrencamp & Quinn 2004), but there is currently no method of assaying or quantifying dominance in Smooth-billed Ani breeding groups.

Future researchers could use my experimental design to test the prediction that the nocturnal incubator is more likely to attack than other group members. Such a result would suggest that the nocturnal incubator is more aggressive, a trait often associated with dominance (Blanchard et al. 1988; Verbeek et al. 1996), than other group members, lending support to the tenet that the nocturnal incubator is in fact the dominant male of the group.

In summary, I have quantitatively described the vocal repertoire of the Smooth-billed Ani for the first time, shown that anis use a sophisticated alarm call system to communicate information about predator class (aerial and terrestrial), and respond appropriately to these signals in the absence of other cues, and provide evidence that anis use both acoustic (*hoot* calls) and visual (the throat inflation display) modalities to signal aggressive motivation and willingness to attack. Vocal communication has not been studied in our lab before and, to my knowledge, has never been studied in Smooth-billed Anis. My thesis demonstrates that this is a rich area for productive future research.

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Appendix

A1. Lab protocols

A1.1. Molecular Sexing

Smooth-billed Anis can be sexed using molecular techniques based on two conserved chromo-helicase-DNA-binding (CHD) genes located in avian sex chromosomes. The PCR products amplify across an intron in the CHD-Z gene found on the Z chromosome of both males and females, and in the female-specific CHD-W gene. These introns usually differ in length between males and females. Thus, when viewed on a gel, one will see a single CHD-Z band for males and a second CHD-W band of different size in females (Griffiths et al. 1998).

Since January 2012, I have been using the following reaction mixture (Table A1.1) and cycling conditions (following Fridolfsson & Ellegren 1999) to sex Smooth-billed Anis. Cycling conditions: initial denaturing at 95 ºC for 2 min, 10 cycles of denaturation at 94 ºC for 30 s followed by 60 ºC ‘touchdown’ annealing for 30 s (-1 ºC/cycle until a temperature of 50 ºC is reached) and extension at 72 ºC for 30 s, 30 cycles of denaturation at 94 ºC 30 s, followed by annealing at 50 ºC for 30 s, and extension at 72 ºC for 30 s, and final extension at 72 ºC for 5 min. A Peltier thermal cycler (PTC-200) was used for all PCR.

PCR products were run by electrophoresis on 2.5% agarose gels for 3 h and visualized under UV light. Should PCR products ‘suddenly’ fail to amplify, replacing the aliquots of ddH_2O, buffer, dNTPs, primers, and BSA often solved the problem, as these reactants degrade after multiple freeze/thaw cycles.

Table A1.1. PCR reaction mixture for DNA sexing of Smooth-billed Anis. Values reported are those required for a single sample with a final volume of 10µl.

Reagent	**Initial concentration**	**Volume added**	**Final concentration**
ddH_2O	--	5.9 µl	--
Buffer (with $MgCl_2$)	10X	1.0 µl	1X
dNTPs	10mM	0.2 µl	0.2mM
P8-F	10µM	0.8 µl	0.8 µM
P2-R	10µM	0.8 µl	0.8 µM
BSA	3mg/ml	0.2 µl	0.6mg/ml
Taq DNA polymerase	5U/µl	0.1 µl	0.5U
DNA	~20ng	1.0 µl	~2ng

-F denotes the forward primer, -R denotes the reverse primer.
The nucleotide sequence of the forward primer, P8, is: 5’-CTCCCAAGGATGAGRAAYTG-3’ and the nucleotide sequence of the reverse primer, P2, is: 5’-TCTGCATCGCTAAATCCTTT-3’ (Griffiths et al. 1998).

A1.2. Multiplex PCR

Multiplex PCR using microsatellites is a technique that can reduce lab costs by including multiple primer sets in a single reaction, allowing the amplification of multiple loci in a single reaction (Edwards & Gibbs 1994; Reed et al. 1994; Henegariu et al. 1997; Neff et al. 2000). I used five polymorphic microsatellite loci developed by Blanchard & Quinn (2001) and Gregory & Quinn (2005).

Using Blanchard's PCR reaction mixtures and cycling conditions as a starting point (Blanchard 2000; Blanchard & Quinn 2001), I used the following PCR reaction mixture (Table A1.2.1) and cycling conditions: initial denaturation at 94 ºC for 2 min, 3 cycles of annealing at 58 ºC for 30 s, and extension at 72 ºC for 30 s, followed by 32 cycles of denaturation at 94 ºC for 15 s, annealing at 58 ºC annealing for 30 s, and extension at 72 ºC extension for 30 s and a final extension at 72 ºC for 2 min. A Peltier thermal cycler (PTC-200) was used for all PCR.

I attempted to incorporate the 9546 microsatellite primer (Blanchard 2000) into the ANI1 multiplex, but was unable to successfully do so. This is most likely due to the difference in suggested annealing temperatures (Blanchard 2000; Quinn Lab unpublished data). I recommend the following PCR reaction using the above cycling conditions with an annealing temperature of 56 ºC (Table A1.2.2). I tested one other PCR reaction mixture (Table A1.2.3) using the cycling conditions given above, with an annealing temperature of 58 ºC, but was unable to obtain results.

Table A1.2.1. Multiplex PCR (ANI1) reaction mixture for Smooth-billed Anis using primers ANI500C5 and ANI450B2 for two microsatellite loci. Values reported are those required for a single sample with a final volume of 10µl.

Reagent	**Initial concentration**	**Volume added**	**Final concentration**
ddH_2O	--	4.86µl	--
Buffer (with $MgSO_4$)*	10X	1µl	1X
dNTPs	10mM	0.4µl	0.4mM
ANI500C5-F	10µM	0.25µl	0.25µM
ANI500C5-R	10µM	0.25µl	0.25µM
ANI450B2-F	10µM	0.27µl	0.27µM
ANI450B2-R	10µM	0.27µl	0.27µM
BSA	3mg/ml	1.0µl	0.3mg/ml
Taq DNA polymerase	5U/µl	0.2µl	1U
DNA	~20ng	1.5µl	~3ng

-F denotes the forward primer, -R denotes the reverse primer.

*With *New England Biolabs ThermoPol Reaction Buffer* (#B90045), the final concentration of $MgSO_4$ in a 1X buffer mixture is 2mM.

The sequence of the forward primer ANI500C5-F is: 5’-ATCTTCAGTAGTACATGTGC-3’ and the sequence of the reverse primer ANI500C5-R is: 5’-TGTGTAATAGAGCAGCCAG-3’ (Blanchard & Quinn 2001).

The sequence of the forward primer ANI450B2-F is: 5’-GCTTCTTTTAGGATTAACCGT-3’, and the sequence of the reverse primer ANI450B2-R is: 5’-CCTGGTTTGTAGCACTGAC-3’ (Blanchard & Quinn 2001).

Table A1.2.2. PCR (ANI3) reaction mixture for Smooth-billed Anis using primer ANI9546 for one microsatellite locus. Values reported are those required for a single sample with a final volume of 10µl.

Reagent	**Initial concentration**	**Volume added**	**Final concentration**
ddH_2O	--	5.5µl	--
Buffer (with $MgSO_4$)*	10X	1µl	1X
dNTPs	10mM	0.4µl	0.4mM
ANI9546-F	10µM	0.2µl	0.2µM
ANI9546-R	10µM	0.2µl	0.2µM
BSA	3mg/ml	1.0µl	0.3mg/ml
Taq DNA polymerase	5U/µl	0.2µl	1U
DNA	~20ng	1.5µl	~3ng

-F denotes the forward primer, -R denotes the reverse primer.

*With *New England Biolabs ThermoPol Reaction Buffer* (#B90045), the final concentration of $MgSO_4$ in a 1X buffer mixture is 2mM.

The nucleotide sequence of the forward primer, ANI9546-F is: 5’-TAAAACTATAGAAGGCGGAATG-3’, and the sequence of the reverse primer, ANI9546-R is: 5’-ACACGGAGCCGCAGCCA-3’ (Blanchard & Quinn 2001).

Table A1.2.3. Multiplex PCR (ANI2) reaction mixture for Smooth-billed Anis using primers ANI500C14 and CANSNX17 for two microsatellite loci. Values reported are those required for a single sample with a final volume of 10µl.

Reagent	**Initial concentration**	**Volume added**	**Final concentration**
ddH_2O	--	5.0µl	--
Buffer (with $MgSO_4$)*	10X	1µl	1X
dNTPs	10mM	0.4µl	0.4mM
ANI500C14-F	10µM	0.3µl	0.3µM
ANI500C14-R	10µM	0.3µl	0.3µM
CANSNX17-F	10µM	0.15µl	0.15µM
CANSNX17-R	10µM	0.15µl	0.15µM
BSA	3mg/ml	1.0µl	0.3mg/ml
Taq DNA polymerase	5U/µl	0.2µl	1U
DNA	~20ng	1.5µl	~3ng

-F denotes the forward primer, -R denotes the reverse primer.

*With *New England Biolabs ThermoPol Reaction Buffer* (#B90045), the final concentration of $MgSO_4$ in a 1X buffer mixture is 2mM.

The sequence of the forward primer ANI500C14-F is: 5’-GGGGATGGTTTATTTTGAGG-3’, and the sequence of the reverse primer ANI500C14-R is: 5’-GGATACGGGTGGGGCCT-3’ (Blanchard & Quinn 2001).

The sequence of the forward primer CANSNX17-F is: 5’-GTCTCTGGCCGTCTTCACTG-3’, and the sequence of the reverse primer, CANSNX17-R is: 5’-GGTAAGTTTCCCACAAGATCA-3’ (Gregory & Quinn 2005).

A2. Nest monitoring

I developed new methods for video monitoring Smooth-billed Ani nests and marking nestlings for individual identification. As ani breeding groups consist primarily of unrelated individuals (Blanchard 2000; Vehrencamp & Quinn 2004), and many nestlings are therefore unrelated to their nest mates, collecting provisioning data in which both the adults feeding and nestlings receiving food can be individually identified is of interest.

I used two Sony Handycams (HDR-PJ260, HDR-CS160) equipped with either an NP-FV50 or NP-FV100 battery and mounted using either a collapsed tripod or a Joby Gorillapod. The NP-FV50 battery allowed continuous recording for ~ 2.5 hours, while the NP-FV100 battery allowed recording for up to 4 hours. With the tripod, I had to anchor the camera using zip-ties and gorilla tape and the length of the tripod legs made it difficult to position the camera. The Joby Gorillapod has shorter (25.7 cm), bendable legs that made mounting cameras faster and easier.

With the Handycams, I collected high-quality provisioning data and was able to identify adult birds by reading their band combinations and, where video quality was highest, even band numbers. This is particularly helpful in situations where the bird lacks a full band complement and can only be identified by band number. The ability to individually identify nestlings has been limited because their dark skin pigment makes colour-marking difficult (Samuelsen 2008). I used silver metallic Sharpie markers to apply unique patterns (randomly selected dots and lines) to ani chicks' heads. These markings were clear and easy to distinguish on videos and, with re-application every 1 – 2 days, remained visible throughout the video monitoring period. Sony Handycams are an excellent means of collecting provisioning data, identifying colour-banded birds, colour-marked nestlings, and in recording other interesting nest behaviours such as egg tossing and egg burial by adults (Quinn & Startek-Foote 2000; Schmaltz et al. 2008). I recommend the following protocol for video monitoring, specifically to collect provisioning data:

1. Attach Sony Handycam units (HDR-PJ260 or newer) equipped with NP-FV100 and 64GB SD card to 25.7 cm long Joby Gorillapods.
2. Protect Handycam with sealed Ziploc bags, cutting open a hole for the lens and taping the edges securely to the camera frame using gorilla tape, to give some protection from the elements (Handycams are not waterproof and cannot be left out in inclement weather).
3. Mount camera 0.5 to 1.5 m from the nest and check display to ensure a good view into the nest. Ideally, the recording frame should include the entire nest and at least 6 inches around the nest, to capture adult and/or predator activities in the nest vicinity.
4. Use manual spot focus and automatic light level adjustment settings to ensure the video is focused clearly on the nest and that exposure levels will adjust automatically with changes in light levels. Check the camera settings and viewing field to ensure the best recording possible, but do not begin recording at this time.
5. Collect all eggs and chicks from nest and complete regular morphometric data collection.
6. Randomly select and apply a unique dot and line pattern to the top of each chick's head using a silver metallic Sharpie. Record chick ID and colour patterns in a field book.
7. Set up camera between 0630 and 0730 h to collect data for the entire morning.
8. Collect camera between 1130 and 1230 h when 4 h of data have been recorded.
9. Data can be downloaded and battery packs charged during the afternoon break so cameras are ready for use the following morning.
10. On subsequent visits, chicks can be collected for reapplication of colour marks and morphometric measurement prior to beginning the day's nest recording.

A3. Pilot research – predator presentation

I planned to present ani breeding groups with taxidermic mounts of a) a cat or mongoose, b) a hawk with wings spread that would be run down a wire to simulate flight, c) a perched hawk with wings folded, and d) a Smooth-billed Ani. I predicted that the cat or mongoose would elicit *ahnee alarm* calls and the flying hawk would elicit *chlurps* but that the perched hawk and ani mounts would not elicit either alarm type from Smooth-billed Ani groups. Unfortunately, I was unable to obtain mounts for this experiment.

In lieu of predator mounts, I attempted to elicit *chlurps* by flying a Frisbee over groups. Problems with this method included our lack of skill in accurately throwing the Frisbee over groups, especially in windy conditions, and the limited distance from which we could throw the Frisbee. As a result, the birds became aware of our presence before we could complete the trials, and tended to either not respond to the Frisbee or to duck down silently when we threw it.

We also attempted to elicit *chlurps* by flying a remote-controlled helicopter over ani groups, but we had critical problems with this technology we could not resolve. We could not reliably get the helicopter off the ground and flying. Based on pilot trials in which we did raise the helicopter, I would be concerned about the mechanical noise produced during flight, altitude limitations, the shape, and slow flight speed of this machine. If the helicopter flew too slowly, in combination with its blocky shape that does not resemble a raptor, the anis may not perceive the helicopter as a real threat and the machine may not be effective in eliciting *chlurps*.

I also attempted to elicit *chlurps* and *ahnee alarms* using live predators (Templeton et al. 2005). I found a falconer willing to train a young Red-tailed Hawk to make short flights on a lead, from one experimenter's glove to another, such that the hawk could be flown from person A to person B over ani groups, mimicking a real aerial threat. Unfortunately, my wildlife permit was denied by the Department of Natural Resources in Puerto Rico.

I conducted pilot trials for the terrestrial predator simulation by running a dog at or past ani groups, attempting to elicit *ahnee alarms*, but encountered several difficulties. First, I was unable to find a pre-trained dog or a volunteer who could train the dog to make a directed run at ani groups on command. Second, it was challenging to get the experimenters and the dog into place without disturbing the anis and causing them to move out of position. Third, I found that if the anis were not perched low or in the grass before we arrived, they were usually aware of the experimenters and the dog, and the trials were ineffective. The anis would often fly up out of reach and silently watch the dog running around below them.

I also attempted to elicit *ahnee alarms* by having a human run directly at anis perched low to the ground. As in the dog trials, anis usually became aware of the runner from a distance and flew up silently. Humans may have been too slow and obvious to be perceived as a real threat, though this method has been used effectively in other species (e.g., Murphy 2006). To be effective, I think this design would require setting up equipment while keeping both researchers and the dog out of sight, potentially using a vehicle as a blind, waiting for anis to fly down low, and then releasing a dog trained to run directly at the birds. Alternately, a lure call might bring the groups to the experimental area after set up, and the dog could be released from a blind.

From these pilot trials, and given the difficulty in obtaining a permit to use live animals, I recommend using taxidermic mounts, as has been done successfully in other studies (Koivula et al. 1995; Digweed & Rendall 2009; Clarke et al. 2012; Haff & Magrath 2013). I advise searching for mounts several months in advance of the experimental start date and, failing that, to make one's own or contact a taxidermist. I contacted the Royal Ontario Museum, Puerto Rican ornithological societies and the U.S. Fish and Wildlife Service branch in Puerto Rico, but was unable to obtain any mounts. Perhaps contacting the corresponding authors of previously published work, taxidermists and hunters would be more productive avenues for securing mounts.

A4. Pilot research – nestling alarm experiments

Smooth-billed Ani nestlings are mobile by five days of age and often flee the nest in response to researchers performing nest checks (J. Quinn & L. Grieves pers. obs.). I predicted that *ahnee alarms*, indicative of a terrestrial threat, would cause nestlings to flee the nest and scramble to the outer edges of branches, as most terrestrial predators (e.g. cats, rats and mongooses) are capable of climbing to and raiding the nest. I predicted that *chlurps*, indicating an aerial threat, would cause nestlings to hunch down and lie still at the bottom of the nest because raptors hunting from the air may not detect inconspicuous nestlings. I also predicted that both *chlurp* and *ahnee alarm* call playback would cause nestlings to cease begging and go silent, reducing their likelihood of detection by nest predators.

To test these predictions, I placed a remote-controlled playback speaker 1 m from the nest and a video camera 1 – 2 m from the nest to record nestling responses. From a blind, I waited until three criteria were met: 1) adult group members were greater than 2 m from the nest, 2) adults were silent, and 3) nestlings resumed normal begging behaviour. Then, I played either an *ahnee alarm* or a *chlurp* stimulus (described in chapter II), and documented nestling responses. Nests were selected based on the presence of at least one five day old nestling.

There were several difficulties with this set up. First, camera placement is often difficult, as many nests are constructed such that anchoring a camera with a quality view to the nest is challenging if not impossible. Second, I could not always hear when the nestlings were begging. Third, given the mobility of nestlings by day five, there is a danger they will flee the nest during the set up and either not return within a reasonable time needed to conduct the experiment or fall to the ground, risking injury or death. Fourth, anis usually became agitated during the set up and it sometimes took over one hour for the experimental criteria outlined above to be met. Together, these problems made it almost impossible to set up and perform the experiment efficiently.

Further, in three pilot trials, I found no evidence that nestlings responded to either *ahnee alarm* or *chlurp* playback. I also collected video data of a nest predation event by a mongoose during which adults gave many *ahnee alarm* calls. The nestlings, at least three of which were five days old, did not appear to respond to either the alarms or the predator and were killed.

Given the difficulty of a clean experimental set up and the lack of evidence to support my predictions, I do not think this experiment would be worth attempting again. It may be that the responses to *ahnee alarms* and *chlurps* do not develop until the nestlings are closer to fledging than was tested in the experiment I attempted.

Despite the lack of response observed in five day old and younger nestlings, Smooth-billed Anis are able to produce and respond to *chlurps* as early as 28 days of age (L. Grieves pers. obs.). We hand-raised three ani chicks taken from three different nests for use as lure birds to trap adult anis. These birds were kept indoors and were not exposed to *ahnee alarm* or *chlurp* stimuli. I documented six instances of *chlurp* production in response to flying Turkey Vultures (N = 1), Red-tailed Hawks (N = 1), and doves (N = 2) visible through the window. In some cases, a reason for the *chlurp* could not be determined (N = 2). *Chlurps* caused at least one bird to dive down out of sight in 50% (3/6) of cases, once in response to a Red-tailed Hawk, and twice where the reason for the *chlurp* could not be determined. My observations suggest that both *chlurp* production and the dive down response are innate.

Designing an experiment to test these hypotheses would require removing newly-hatched chicks from their nest and raising them in isolation to guarantee they were not exposed to any Smooth-billed Ani vocalizations or behaviours. These birds could then be both played *chlurp* stimuli and presented with aerial predators. Unfortunately, it would be impossible to release these birds back into the wild after the experiment, and they would have to be either held in captivity or killed. Thus, I did not attempt this experiment.

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