Reproductive Competition and Success in a Joint-Nesting Cuckoo

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TITLE: Reproductive Competition and Success in a Joint-Nesting Cuckoo

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Abstract

Joint-nesting species experience complex and diverse social environments which can influence individual reproductive success. When social groups are formed by non-relatives, competition for representation in incubated clutches can be intense and result in substantial reproductive losses. Because conflicts of this nature have direct implications on fitness, resolutions and outcomes of such are of particular interest to evolutionary research. For this reason, I explored patterns of reproductive competition and skew in joint-nesting Smooth-billed Anis (*Crotophaga ani*). In chapter I, I show marked differences in multivariate bill and body size between sexes of Smooth-billed Anis (*Crotophaga ani*) despite reportedly reduced sexual dimorphism in co-operative breeders. I also show that multivariate bill size in males correlates with annual reproductive success and individual contribution to clutches from multiple parents. A similar trend is not observed in females, suggesting potential for sexual selection on male bill size. Together, these data provide evidence for morphological influences on reproductive fitness in joint-nesting species. In chapter II, I show that relative parental effort is positively correlated with reproductive fitness in social groups of Smooth-billed Anis. Nocturnal incubation is risky and is biased toward a single male - akin to Groove-billed (*Crotophaga sulcirostris*) and Greater Ani (*Crotophaga major*). I report heightened reproductive contribution by nocturnally incubating males when compared to other males within social groups. In chapter III, I tested whether social group size is correlated with endocrine markers of stress in adult male and female Anis. Using a novel enzyme linked immunosorbent assay, I show that corticosterone (the primary glucocorticoid in birds) deposited in feathers is highest in birds from atypically large social groups. While the direct consequences of elevated corticosterone on reproductive fitness in Anis is yet unknown, these results suggest that breeding in large social settings is likely to be physiologically expensive.

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Preface

This thesis contains three chapters. Each chapter has been written as a manuscript to be submitted for publication in independent, peer-reviewed journals. The first and second chapters will be submitted to The Condor and The Journal of Avian Biology respectively and the third chapter will be submitted to General and Comparative Endocrinology. Detailed information pertaining to working titles, authorships and individual contribution is presented below:

Chapter 1: Bill Size Correlated with Reproductive Success in Male Smooth-billed Anis (Crotophaga ani): Sexual Dimorphism and Evidence for Sexual Selection in a Joint-Nesting Cuckoo

- Authors: J.K. Robertson, M. Barclay, G. Schmaltz, J.R. Caldwell, X. Mu and J.S. Quinn. Contribution: Field data were collected by L. Grieves, L. Blanchard, A. Samuelsen, G. Schmaltz, B. Bravery, M. Cruz, H. Darrow, H. Reider, K. Peiman, M. Beaucreux, S. Schopman, L. Barabas, A. Demko, J. Eyster, A. Boon, R. Land, T. Pope, J. Haselmayer, F. Tarazona, I. Rodriguez, B.-M. Wadien, E. Rutherford, and the candidate under the supervision of Dr. James Quinn. Microsatellite genotyping was completed by Dr. Gregory Schmaltz, John Ryan Caldwell (BSc) and the candidate. Parentage analyses, morphometric analyses and statistical analyses were conducted by the candidate with guidance from Drs. Jonathon Dushoff and Ben Bolker.

Chapter 2: Does Parental Effort Correlate with Reproductive Contribution in Communal Nests? Patterns of Reproductive Skew in a Social Cuckoo.

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and J.S. Quinn. Contribution: Field data were collected by L. Grieves, L. Blanchard, A. Samuelsen, G. Schmaltz, B. Bravery, M. Cruz, H. Darrow, H. Reider, K. Peiman, M. Beaucreux, S. Schopman, L. Barabas, A. Demko, J. Eyster, A. Boon, R. Land, T. Pope, J. Haselmayer, F. Tarazona, I. Rodriguez, B.-M. Wadien, E. Rutherford, and the candidate under the supervision of Dr. James Quinn. Microsatellite genotyping was completed by Dr. Gregory Schmaltz, John Ryan Caldwell (BSc) and the candidate. Paternity assignments, and statistical analyses were conducted by the candidate with guidance from Dr. Ben Bolker.

Chapter 3: The Effect of Social Group Size on Stress in a Co-operatively Breeding Bird: Consequences of Extreme Social Living

- Authors: J.K. Robertson, C.S. Hurd, Hing, J.S., C. Muir and J.S. Quinn. Contribution: Field samples were collected by Leanne Grieves (MSc) and the candidate under the supervision of Dr. James Quinn. Endocrine analyses were conducted by the candidate under supervision of Drs. Cameron Muir and Gabriela Mastromonaco, with assistance from Conner Hurd (BSc) and Jing Sheng Hing (MSc Candidate). Statistical analyses were conducted by the candidate.

General Introduction

Social animals must overcome obstacles of both the physical and more abstract social environment to survive and reproduce. Though surpassing challenges of the physical environment is essential for locating base requirements (ie. food, water), the social environment can often determine whether such resources can be accessed and is therefore a powerful selective force. For species who breed in close proximity to others (ie co-operative breeders), social conflict through competition for reproductive opportunities can be costly and understanding the nature of such competition is necessary to develop a holistic view of how mating systems influences selection.

Co-operatively breeding species care for offspring that are not their own, often to the detriment of their own reproduction. In most co-operative breeders, reproductive rights are exclusive to a dominant pair (high reproductive skew), and non-breeding subordinates help rear their offspring. This breeding framework is more commonly known as "helper-at-the-nest" and typically involves kinship between dominant breeders and subordinates. By helping to ensure the survival of closely related juveniles, subordinate helpers acquire indirect fitness benefits (Hamilton, 1964), while securing resources from shared territories and avoiding the often high costs of dispersal. In these societies, subordinate breeding attempts are relatively infrequent and typically limited by costs of eviction (the subordinate restraint model; Johnstone & Cant, 1999; Buston et al, 2007), inbreeding avoidance (Koenig et al, 1997; Cockburn, 1998), and rarity of extra-group

copulations (but see Dunn & Cockburn, 1999; Richardson et al, 2001).

Kinship between social group members, however, is not a required precondition of co-operative breeding. In fact, non-kin breeding groups have been documented in a number of bird species (ie. Pied Kingfishers (*Ceryle rudis*; Reyer, 1984), Galapagos Hawks (*Buteo galapagoensis*; DeLay et al, 1996), Taiwanese Yuhinas (*Yuhina brunneiceps*; Yuan et al, 2004), Superb Fairy Wrens (*Malurus cyaneus*; Dunn et al, 1995)). In these species, the indirect fitness benefits are absent and breeding co-operatively is putatively better explained by investment in future breeding opportunities (ie the "pay-to-stay" model; Kokko et al, 2002), development of parental skills (Lancaster, 1971; Snowdon & Cronin, 2007) or heightened per capita reproduction in group settings (Shen, 2009). In non-kin social groups, breeding attempts by subordinates are inconsistent though classical concession models of skew posit higher subordinate reproductive contribution (low skew) than in kin groups (Vehrencamp, 1979; Emlen, 1982b; Emlen and Vehrencamp, 1983; Reeve, 1991; Reeve et al, 1998.)

Regardless of kinship between social groups members, reproductive 'power struggles' can be intense and individually taxing (ie. infanticide in Guira Cuckoos, *Guira guira*; (Macedo et al, 2001) and abortion in Banded Mongooses, *Mungos mungo* (Cant et al, 2010)). Because conflicts of this nature have direct implications on individual fitness, resolutions and outcomes in a species are of particular interest to modern evolutionary biologists. For this reason, I chose to explore consequences of reproductive competition and patterns of reproductive success in a joint-nesting framework, in which multiple females contribute to a communal clutch and compete for representation.

Study Species

The Smooth-billed Ani (*Crotophaga ani*) is a neotropical cuckoo that lives across most of South America, the Caribbean Islands, parts of Central America and southern Florida. Smooth-billed Anis form breeding groups composed of socially monogamous pairs during breeding seasons (typically during local rainy seasons), which share in nest building, territory defense and predator defense (Quinn & Startek-Foote 2000). Multiple females contribute genetically to clutches, however competition for representation in final broods is intense. Members of both sexes partake in brood reduction by egg tossing or egg burial, resulting in per capita reproductive losses which increase with group size (Schmaltz et al, 2008; Quinn, unpublished observations).

Adult Smooth-billed Ani (*Crotophaga ani*) captured on a nest in December, 2014. Female.

I studied male reproductive skew and endocrine correlates of reproductive competition in Smooth-billed Anis over two consecutive field season (2014-2015) at the Cabo Rojo, National Wildlife Refuge (south-western Puerto Rico). In Chapter 1, I explore how male

phenotype influences reproductive success and skew in Smooth-billed Anis. In cross-species reviews, sexual selection and dimorphism appear more modest in co-operatively breeding compared to solitary breeding and lekking species (Dunn et al, 2001; Dale et al, 2015). Due to the high proportion of helper-at-the-nest societies amongst co-operative breeders, joint-nesting species dynamics are probably under-represented in such reviews. I argue that joint-nesting species fall under different selection paradigms, and non-kin group membership may promote sexual selection and the evolution of sexual dimorphism by permitting within-group extra-pair fertilizations. To explore this hypothesis, I assess sexual dimorphism in beak and body size of adult Anis and how each measure correlates with annual reproductive success and brood contribution.

In chapter II, I further examine male patterns of reproductive skew and how they correlate with male parental role in Anis. While the majority of parental effort appears evenly distributed amongst adults (Quinn & Startek-Foote, 2000), nocturnal incubation appears to be biased to a single male within each social group (Quinn, J.S, Grieves, L.A. and Robertson, J.K. unpublished observations). In the closely related Groove-billed Anis (*Crotophaga sulcirostris*), nocturnal incubation is risky and comes with a higher risk of mortality (Vehrencamp et al, 1986). I therefore explore whether nocturnally incubating males sire more offspring in communal nests, thereby matching their parental effort to their genetic contribution in incubated broods.

Beyond patterns of reproductive skew, I also examine how reproductive conflict influences endocrine markers of stress in adults during breeding seasons. In chapter III, I validated an enzyme-linked immunosorbent assay (ELISA) to quantify corticosterone (the primary glucocorticoid in birds) deposited in Ani rectrices. I then used this assay to test correlations between corticosterone deposition and social group size (here, a proxy for degree of reproductive competition)(Schmaltz et al, 2008). I also tested whether corticosterone deposition differed between sexes as a result of reproductive competition.

Joint-nesting is a rare breeding framework that has only been documented in 15 species to date (Vehrencamp &, Quinn 2004). Exploring reproductive and endocrine trends in such a complex breeding system may shed light on how individual fitness, and subsequent selection, is influenced by social conflict and mating system as a whole.

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1 | Sexual Dimorphism and Evidence for Sexual Selection in a Joint-Nesting Cuckoo

Master of Science– Joshua Kenneth Robertson; McMaster University– Biological Sciences

1.1 Abstract

Co-operative breeders typically display weaker degrees of sexual dimorphism and sexual selection than solitary breeding and lekking species. Given regular breeding by subordinate group members, however, joint-nesting species likely fall under different selection paradigms than species with helper-at-the-nest breeding systems but are seldom represented in cross-species reviews (ie Dunn et al, 2001). In this study, we examined sexual dimorphism in bill and body size of the Smooth-billed Ani (*Crotophaga ani*) – a non-kin, joint-nesting cuckoo species. In this species, competition at the nest by ovicide is intense and due to compensation for egg losses, physiological investment by female breeders is high. Shared reproduction between non-kin group members permits reproductive competition and within-group extra-pair copulations without inbreeding. We therefore anticipated sexual selection on males and resultant sexual dimorphism. Indeed, we show significant differences in multivariate bill size (Bill Score) and body size (Body Score) between sexes (n_{males}=180, $n_{females}$=173). Furthermore, sexual dimorphism of bill size could not be explained by allometry alone. We also draw a positive correlation between male multivariate bill size and number of nestlings sired in year of capture, based on microsatellite genotypes at 5-13 polymorphic loci for 101 adults (57 males, 44 females) and 88 nestlings. Male bill size but not body size also exhibited a positive correlation with the proportion of communal broods sired. Body size was not correlated with either measure of reproductive success. Contrary to results for males, neither bill size nor body size was correlated with either measure of reproductive success of females - consistent with predictions of male-biased trait enhancement. While we lack behavioural observations suggestive of sexual selection, our results allude to sexual selection on male bill size. To our knowledge, this study is the first to address dimorphism and morphological correlates of reproductive success in a non-kin, joint-nesting species.

1.2 Introduction

Darwin's theory of sexual selection (1871) has received extraordinary research attention since its conception. Despite remarkable theoretical and empirical advances (Møller & Birkhead, 1994; Owens & Hartley, 1998; Dunn et al, 2001; Rubenstein & Lovette, 2009; Young & Bennet, 2013; Dale et al, 2015), our modern understanding of how breeding system may influence sexual selection and sexual dimorphism in a species remains incomplete. Across most taxa, relative parental investment and reproductive variance are correlated with intensity of sex-specific sexual selection (Bateman, 1948; Trivers, 1972; Andersson, 1994), and represent parameters that are influenced by a species' breeding strategy. For example, where female investment is high and exceeds that of males, theory predicts that females should be choosy, and selection for male-biased trait enhancement through male-male competition and female choice is likely (Darwin, 1871). Alternatively, where parental investment is more closely matched between sexes, sexual selection is thought to be relaxed and the degree of sexual dimorphism, more modest (Trivers, 1972; Andersson, 1994). Similarly, sex specific reproductive variance (which is typically highest in the sex with lowest reproductive investment) may influence sex specific trait enhancement. In a sex with high reproductive variance, better competitors are greater rewarded with heightened reproductive success or monopolization, while poorer competitors are more limited in reproductive opportunities. In such a case, traits influencing reproductive success are subject to strong selection. Indeed, Møller and Birkhead (1994) and Dunn et al. (2001) highlight enhanced dimorphism in bird species with polygynous mating systems where variance in male, but not female, reproductive success is high (Dunn et al, 2001).

Relative parental investment and reproductive variance are likely to differ amongst different social breeding systems (ie. co-operative and solitary breeding). For this reason,

social breeding system is thought to influence intensity of sexual selection and sexual dimorphism (Dunn et al, 2001; Rubenstein & Lovette, 2009; Young & Bennet, 2013; Dale et al, 2015). In a phylogenetically controlled species survey, Dunn et al. (2001) showed relatively low levels of sexual size dimorphism in co-operatively breeding birds. Similarly, Dale et al. (2015) report reduced sexual dichromatism in co-operative breeders across all known passerine species. Such a reduction in sexual dimorphism across co-operative breeders is thought to be the result of reproductive monopolization (high skew and therefore high reproductive variance) in both sexes, contrary to higher dimorphism in solitary breeders where only males tend to experience high reproductive skew (Rubenstein & Lovette, 2009; Tobias et al, 2012; Lyon & Montgomerie, 2012; Young & Bennet, 2013). In analyses by Dunn et al. (2001) and Dale et al. (2015), however, the majority of co-operative breeding species assessed display helper-at-the-nest breeding systems. In such social frameworks, extra-group copulations are uncommon (Cockburn, 1998; but see Dunn & Cockburn, 1999; Richardson et al, 2001) and breeding opportunities by subordinates are often limited by inbreeding avoidance (Cockburn, 1998), therefore facilitating high reproductive skew in both males and females. In joint-nesting species, female reproductive skew is low and forming socially breeding groups with non-relatives may permit within-group competition for mates and extra-pair mating. We therefore hypothesized that non-kin joint-nesting species fall under different selection paradigms than helper-at-the-nest species. Indeed, joint-nesting Taiwanese Yuhinas (*Yuhina brunneiceps*), exhibit a high degree of sexual dimorphism in wing-chord and body size, with males being the larger sex. Relatedness between social group members is low (Liu et al, 2015) and within-group extra-pair paternity is strikingly high in this species (21.4% of female offspring) (Yuan et al, 2005), alluding to sexual selection for larger males. Further examination of selection in non-kin, joint-laying species may shed light on reproductive benefits of communal nesting.

Here, we examine sexual dimorphism in a joint-nesting cuckoo species with reportedly low male and female reproductive skew within groups (Vehrencamp, 2000; Vehrencamp & Quinn, 2004). Smooth-billed Anis (*Crotophaga ani*) form groups of socially monogamous pairs and pairs vigorously compete for maternal representation in incubated clutches (Schmaltz et al, 2008). Ovicide by egg tossing and burial are common in communal nest, causing females to frequently lose eggs (Schmaltz et al, 2008). Female parental effort is therefore high due to costs of oviposition and egg replacement. Unlike social groups of joint-nesting Acorn Woodpeckers (*Melanerpes formicivorus*), or Pukeko (*Porphyrio melanotus melanotus* from the north island of New Zealand), preliminary research suggests that Smooth-billed Ani groups are primarily composed of non-kin breeders (Koenig & Pitelka, 1979; Craig & Jamieson, 1990; Blanchard, 2000; Vehrencamp & Quinn, 2004; Riehl, 2013). While extra-pair copulations in social groups of Anis are thought to be uncommon, parentage analyses have supported their occurrence (Blanchard, 2000; J.S. Quinn personal observation), suggesting an opportunity for selection of higher quality mates (Kempenaers et al, 1992; Hasselquist et al, 1996; Møller & Tegelström, 1997; Foerster et al, 2003; Estep et al, 2005). We therefore anticipated sexual selection and dimorphism in certain traits. Smooth-billed Anis exhibit a pronounced bill crest (Fig. 1.1) that may signal quality or be used during aggressive encounters. Body size is also a very common sexually dimorphic trait across animal species (reviewed in Fairbairn, 1997; Fairbairn, 2007) and may promote mating success in Anis by enhancing intra-male competitiveness or indicating condition.

In this study, we examined sexual dimorphism in bill and body size of joint-nesting Smooth-billed Anis using multivariate measures derived from principal component analyses (PCA). To examine sexual selection on male traits, we then assessed whether bill and body size correlated with measures of annual reproductive fitness, and whether correlates were specific to adult males. We predicted that multivariate bill and body size would be sexually dimorphic, and that both bill and body size would positively correlate

with annual reproductive fitness in males, but not females. Few studies have investigated sexual dimorphism in joint-nesting species (but see Craig & Jamieson, 1990; Koenig et al, 1995; Yuan et al, 2006; Dey et al, 2012) and to our understanding, the intensity and direction of sexual selection are yet to be examined.

1.3 Methods

Field Site

Smooth-billed Anis were studied at the Cabo Rojo National Wildlife Refuge (NWR) in the southwest of Puerto Rico (1759°'N, 6710°'W) between 1998 and 2014. The Cabo Rojo NWR is comprised of semi-open, dry scrubland in secondary succession and supports a year-round population of Smooth-billed Anis. There, Anis breed predominantly during the rainy season (typically September to December).

Adult Capture, Banding and Blood Sampling

Adult Smooth-billed Anis were captured using paired and horizontally stacked mist-nets (6.6 cm mesh, 18 m long, 7.2 m high) (Meyers & Perdieck, 1993; Schmaltz et al, 2008), hardware cloth walk-in nest traps (Mock & Schwagmeyer, 1999), or funnel 'lure' traps placed within a central region of a social group's territory with a hand-raised ani lure bird protected in a small cage (Vehrencamp, 1977). Immediately after capture, we drew approximately 50 µl to 200 µl of blood by brachial venipuncture and capillary tube collection, or by jugular puncture and syringe collection (Sheldon et al, 2008). Blood samples were stored in Queen's Lysis Buffer (Seutin et al, 1999) and kept at 4°C until further use. Adults were given unique combinations of three colored bands and one USGS numbered aluminum leg-band. Each were measured and released on home territories. Morphometric measurements taken included; mass (to the nearest 0.1 g using a spring scale), maximum depth of bill and culmen length (measured to the nearest 0.1 mm using mechanical calipers) and right wing chord (measured with a wing ruler). Recaptured individuals were not measured unless the most recent capture occurred in a previous breeding season. In all 353 adults were captured, blood sampled, sexed using molecular

markers (as described below) and measured as part of a longitudinal research program.

Nestling Capture, Banding and Blood Sampling

Social groups were routinely monitored for evidence of nest building and egg laying. Nests were located by searching known territories or by following adults and nest locations were marked with a handheld GPS. Following initial discovery, we visited nests every one to three days until the youngest nestling had reached roughly three days post-hatch (dph). Upon hatching, nestlings were given a unique nail-clipping per nest for downstream identification. At three dph, chicks were banded with one USGS aluminum band and one darvic colour band in a combination that was unique to the brood. We drew 50 µl to 100 µl of blood from nestlings between one and three dph by femoral venipuncture and collection with non-heparinized capillary tubes (Sheldon et al, 2008). A total of 859 nestlings were captured and blood sampled, 479 of which were banded as part of a longitudinal research program.

Social Group Size and Composition

Group sizes were determined by counting the number of adults leaving and entering communal roosts, and were repeated throughout the breeding season (Schmaltz et al, 2008). If not all group members were captured, we estimated the number of breeding males and females using group size counts and assuming equal sex ratio (non-breeding helpers are uncommon; Loflin, 1983; Quinn & Startek-Foote 2000). Juveniles are readily identifiable and were excluded from adult group size measurements. If group counts were odd, we recorded half group sizes as decimal values. In this study, data were used from a total of 45 nests.

Sex Determination and Microsatellite Genotyping

We isolated whole genome DNA from blood-lysis samples using phenol-chloroform or ammonium acetate salt extraction and stored samples at -20°C until further use. The sex of adult Anis was determined by PCR amplification of Chromo-Helicase DNA-binding gene (CHD1) intron 16 (Griffiths et al, 1996; Fridolfsson & Ellegren, 1999) and amplicon size separation on 3% agarose gels (353 adults). DNA quantity and quality were then assessed by NanoDrop spectrophotometry (NanoDrop Technologies). Where DNA quality was sufficient, 5-13 microsatellite loci (ANI450B2, ANI9546, ANIC5, CrMa3-142, CrMa327, CrMa4-6, JJQ0536, SNX7, SNX14, SNX17, JSQ1, JSQ5, JJQ0536) (Blanchard & Quinn, 2001; Gregory & Quinn, 2005; Riehl & Bogdanowicz, 2009) were PCR amplified with fluorescently labeled primers. PCR amplicons were then ethanol purified and submitted to the Natural Resource DNA Profiling and Forensic Centre (NRDPFC; Trent University, Peterborough, ON) (101 adults, 360 nestlings) where they were run on an ABI 3730 DNA analyzer with GS-500 size standards. Microsatellite fragment lengths were scored by eye in GeneMarker 2.0-2.2 (SoftGenetics, LLC).

Sexual Dimorphism Analyses

Complete measurements were available for 353 adult Smooth-billed Anis (180 males, 173 females). To derive multivariate metrics of bill and body size for each adult, we conducted two independent principal component analyses (PCA). In the first PCA, we loaded measurements of wing-chord (mm) and mass (g) and selected the component explaining the highest measurement variance to generate a unique Body Score (Body PC1) for each individual. Wing chord and mass were selected as loading variables to account for both skeletal size and condition (Supplementary Fig. A1). Mass scaled linearly with wing chord (Linear Model: $p < 0.001$, $t = 5.400$, $df = 351$, $SE = 0.1136$, $n = 353$) rendering log transformations unnecessary. In the second PCA, we selected length

of exposed culmen (mm) and maximum depth of bill (mm) as loading variables. For the body size PCA, the component explaining the greatest variance in measurements was used to produce a unique 'Bill Score' (Bill PC1) (Supplementary Fig. A2). To simplify visual interpretation, both Body Score and Bill Score were scaled between zero and one. Both PC analyses were conducted using the prcomp package in R (R Core Team 2015). All components were scaled and centred.

To test for sexual dimorphism in bill and body size, we compared Body Score and Bill Score between sexes using Welch Two Sample T-Tests in R. To assess whether sex had an effect on the relationship between bill and body size, we then constructed a generalized linear model (R package lme4) (Bates et al, 2015) with Gaussian distribution, where Bill Score was selected as the response variable, and Body Score and sex were chosen as predictor variables. An interaction between Body Score and sex was also chosen as a fixed effect predictor in our model to test for proportional differences between sexes.

Trait variance should be comparable between sexes under equal selective pressure (Rubenstein & Lovette, 2009). We therefore compared Body Score and Bill Score variance between sexes. Comparisons were conducted with a Levene test (Levene, 1960) via the lawstat (Hui et al, 2008) package in R.

Parentage Assignments

All genotyped adults with complete measurement profiles were considered as potential parents of nestlings from communal nests at territory of capture (57 males, 44 females). Because morphometry may change across breeding seasons, adults who were not captured within the year nestlings were sampled at resident territories were excluded from the remainder of analyses. Complete measurements and genotyping of at least one adult from a social group with sampled nestlings was required for parentage assessment (45

nests).

We used CERVUS software (Marshall et al, 1998) to assign paternity and maternity independently at 95% confidence. Genotypes at a minimum of 5 microsatellite loci were required for consideration in parentage assessments. CERVUS assumes Mendelian inheritance of loci and uses maximum-likelihood methods to assess parentage (Marshall et al, 1998).

Reproductive Success Analyses

To assess whether bill size and body size were correlated with reproductive success, we constructed a sex-specific generalized linear mixed model using the R package glmmADMB (Bolker et al, 2012). First, the number of nestlings sired or dammed in year of capture was selected as the response variable. Body Score, Bill Score and social group size were chosen as fixed effect predictors, with breeding year, territory and individual ID as random effects. Given stringency of parentage assignments and the large number of unsampled adults, a high proportion of adults were assigned zero nestlings at 95% confidence. We, however, have no reason to anticipate biased representation in parentage assignments. We therefore assumed a zero-inflated Poisson distribution to account for an observed zero bias.

As social group size may influence per capita reproductive success (Schmaltz et al, 2008) we also tested whether bill and body size correlated with within-nest contribution in either sex. To measure within-nest contribution, we calculated the observed over expected number of nestlings sired [nO/nE] for each adult in the previous analysis. Here, the expected number of nestlings sired [nE] was equal to the total offspring within nests divided by the estimated number of males in group sizes [$n_{nestlings}/n_{males}$]. We then constructed a glmm with nO/nE as the response. Again, Body Score and Bill Score were

selected as fixed effect predictors, while breeding year, territory and individual ID were selected as random effects. Social group size was not a fixed effect predictor in this model because group size was implicit in our measure of within nest contribution (the response variable). As our response variable was continuous with Poisson-like distribution and a spike at zero, a compound Gamma-Poisson, or 'Tweedie' distribution was assumed (Tweedie, 1984). We therefore constructed our model using the cpglmm function in the R package cplm (Zhang, 2013).

Because the strength of sexual selection is proportional to reproductive variance (Fisher, 1958), we also compared the variance in number of nestlings sired between sexes by a Levene test using the R package lawstat (Hui et al, 2008) with zero-correction. In all analyses, alpha-levels were set to 0.05.

Mate acquisition and reproductive success are influenced by age in numerous avian species (Weatherhead, 1984; Weatherhead & Boag, 1995; Lozano et al, 1996; Alonso et al, 2010). To tease apart the effects of age and morphology on reproductive success, we examined morphological changes during adulthood in both male and female Anis. Using historic recapture data, we compared Body Score and Bill Score between years of capture by paired student's t-tests. Where multiple recapture events were present, we compared measurements separated by the greatest number years to best detect subtle morphometric changes. For visual interpretation, change in Body and Bill Score were plotted according to time between samplings (Supplementary Fig. A3).

1.4 Results

PCA and Size Dimorphism in Adult Smooth-billed Anis

Our analyses detect sexual size dimorphism in adult Smooth-billed Anis. The primary vector from a body size PCA (Body Score) explained 63.8% of variance in measures of wing chord (mm) and mass (g), while the primary vector from a bill size PCA (Bill Score) explained 86.0% of variance in measures of exposed culmen (mm) and bill depth (mm). Multivariate dimensions of Body Score and Bill Score differed significantly between sexes (Tab. 1.1), with males having both larger body and bill scores (Fig. 1.2). Proportional bill size (Bill Score/Body Score) was also significantly larger in males than females (t= -2.8365, df = 314.98, p = 0.005; n_{males} = 180; $n_{females}$=172, where one female with a Body Score of zero was removed) suggesting that male bill size is larger than female bill size beyond allometric expectations (further illustrated in (Fig. 1.2)). In a general linear model, Body Score, sex and an interaction between Body Score and sex were significant predictors of Bill Score (Tab. 1.2), supporting both sexual size dimorphism and a sex specific exaggeration of bill size beyond allometric expectations. Student's paired t-tests show no significant changes in male morphology between capture events (Male Body Score : t = -0.792, df = 19, p = 0.438; Male Bill Score: t = 0.204, df = 19, p = 0.841) suggesting limited changes in morphology during adulthood. Female Body Score but not Bill Score, however, differed significantly between capture events (Female Body Score : t = -2.197, df = 30, p = 0.036 ; Female Bill Score: t = -1.268, df = 30, p = 0.214).

Bill Size and Reproductive Success

In total, paternity (n = 57) or maternity (n = 44) were assigned for 88 nestlings at 95% confidence and 180 nestlings at 80% confidence. Of 57 adult males and 44 adult

females, 30 males (52.6%) and 19 females (43.2%) were assigned at least one nestling at 95% confidence. In males, Bill Score but not Body Score was a significant predictor of nestlings sired in year of capture (Tab. 1.2) (Fig. 1.2) and within-nest reproductive success (Tab. 1.3) (Fig. 1.3). Group size was not a significant correlate of nestlings sired in year of capture (Tab. 1.2). Neither Body Score nor Bill Score was significantly correlated either measure of reproductive success in females (Tab. 1.2)(Tab. 1.3).

Variance in Reproductive Success and Phenotype

Variance in the number of nestlings sired or dammed was not significantly different between sexes ($\bar{x}_{\text{females}} = 1.638$, $\bar{x}_{\text{males}} = 1.179$, W = 0.112, p = 0.739). Variance in Bill Score differed significantly between sexes (W = 7.452, p = 0.007, n_{males}= 180, n_{females}= 173, df = 1), with male variance (0.015 units) exceeding that of females (0.008 units). Similar to Bill Score, variance in male Body Score (0.034 units) exceeded that of females (0.030 units), though not significantly (W = 0.145, p = 0.704, n_{males}= 180, n_{females}= 173, df = 1).

1.5 Discussion

Sexual size dimorphism and dichromatism appear reduced among co-operative breeders (Dunn et al, 2001; Dale et al, 2015). Our results, however, show distinct morphometric differences between male and female Smooth-billed Anis. Both bill and body size appear larger in adult males than adult females, supporting our predictions. Notably, sex differences in bill size could not be explained by allometry alone (Fig. 1.2), nor age (Supplementary Fig. A3).

The evolution of sexual dimorphism in body size has been a topic of ongoing research since Darwin (1871) and enhanced body size in males in most taxa is typically thought to be the result of sexual selection (Searcy, 1979; Price, 1984; Björklund, 1990; Andersson, 1994 for example). Larger body size in male Anis may have promoted mating opportunities either by signaling condition or providing advantages in intra-male competition. Interestingly, our analyses, show no significant correlation between male body size (Body Score) and annual reproductive success, suggesting body size is not currently under strong sexual selection. Indeed, variance in male Body Score did not differ significant from female Body Score as predicted if trait selection is equal between sexes (Rubenstein & Lovette, 2009). Arguably, male-biased size dimorphism in Anis may also be explained by sex-specific territory defence. Male Pukeko conduct the majority of territory defense in joint-nesting social groups (Dey & Jamieson 2013), and a similar pattern may be at play in Anis. Male exclusive territory defence, however, appears unlikely in Smooth-billed Anis as members of both sexes have been captured by lure trapping techniques and therefore respond aggressively to intruders.

While ancestral sexual selection may explain evolutionary origins of sexual size dimorphism, maintenance of such may be explained by natural selection in response to

male-biased nocturnal incubation. Nocturnal incubation in Smooth-billed Anis is typically carried out by a single male (Robertson et al, unpublished), as in Groove-billed Anis (*Crotophaga sulcirostris*) (Vehrencamp et al, 1986). Diurnal incubation patterns have not been documented in Smooth-billed Anis, however, daytime incubation in the closely related Groove-billed Anis appears male biased (Vehrencamp et al, 1986). Increased body size in male Anis may promote more efficient incubation of large communal clutches (Vehrencamp, 2000) and is a likely target of positive directional selection. Joint-nesting Pukeko and Acorn Woodpeckers show similar asymmetry in nocturnal incubation and body size, with males being the larger sex (Craig & Jamieson, 1990; Koenig et al, 1995) and the sex to conduct all nocturnal incubation (Craig, 1980a). While promoting incubation efficiency, a large body size may also be useful for nest defense during risky nocturnal incubation.

Contrary to trends of body size, our results show a correlation between male bill size and both measures of reproductive success (Fig. 1.3) and (Fig. 1.4). A parallel trend was not shown in females (Fig. 1.3) as would be expected if bill size in males alone is under sexual selection (Bateman, 1948). Supporting selection on male bill size, variance in male Bill Score was significantly larger than female Bill Score (Kotiaho et al, 2001). We found no significant changes in bill and body size across capture events (Supplemental Fig. A3), suggesting that morphological correlates of reproductive success are not an artifact of age, although further study is advised given a small sample size. Unexpectedly, reproductive variance did not differ between sexes as would be predicted when sexual selection is acting upon one sex (Bateman, 1948). The presence of non-breeding adults in social groups (as shown by zero-inflation in parentage assignments), however, may be interfering with an accurate interpretation of selective gradients. Hauber and Lacey (2005) have raised similar concerns and report higher reproductive variance in co-operatively breeding females when non-breeding alloparents are present. Beyond the presence of non-breeding adults in our analyses, the presence of small sample size and an inability

to assess lifetime reproductive variance is likely to limit a meaningful understanding of differences in reproductive variance.

While our data provide evidence for selection on male bill size in Smooth-billed Anis, the direct benefit of enhanced bill size remains unclear. We recognize that enlarged bill morphology may play a role in survival through food acquisition, as in Song Sparrows (*Melospiza melodia*) (Schluter & Smith, 1986), House Finches (*Carpodacus mexicanus*) (Badyaev et al, 2008) and Darwin's Finches (*Geospiza fortis*) (Herrel et al, 2005), and may correlate with provisioning efficiency. Given a lack of evidence for sex-specific diets and provisioning roles, however, we argue that the evolution of a prominent bill crest in male Smooth-Billed Anis (Fig. 1.1) is better explained by mechanisms of sexual selection through inter-sexual mate choice or male-male competition for mating opportunities. In Anis, a large bill crest may signal quality and therefore facilitate female mate choice. Although studies have substantiated bill coloration as a signal of quality in species such as Zebra Finches (*Taeniopygia guttata*) (Bolund et al, 2006), Mallards (Anas platyrhynchos) (Peters et al, 2004), and House Sparrows (*Passer domesticus*) (Laucht et al, 2010), remarkably few have substantiated bill size as a positive signal of quality (but see Møller, 1989). Condition dependent bill size, however, is supported in the literature. Male bill size but not body size was associated with telomere length in late juvenile and adult American Redstarts (*Setophaga ruticilla*) (Angelier et al, 2015). Telemore length is often linked to individual fitness (Haussmann et al, 2005; Bauch et al, 2012; Angelier et al, 2013; Angelier et al, 2015; Lewin et al, 2015), suggesting a measurable correlation between bill size and condition.

Beyond signaling condition, exaggerated bill size may also enhance intra-male competitive ability for mating opportunities by revealing dominance status or enhancing fighting ability. Male Atlantic Puffins (*Fratercula arctica*) and Crested Auklets (*Aethia cristatella*) have significantly larger bills than females (Harris, 1979; Jones, 1993) that

are thought to promote success in intra-male aggression (Jones & Hunter, 1999). Male Hermit Hummingbirds (*Phaethornis longirostris*), a lekking species, also have longer bills and pointier bill tips than female conspecifics; both of which are advantageous as weaponry during intra-sexual competition (Rico-Guevara & Araya-Sala, 2015). Smooth-billed Anis are highly aggressive towards conspecifics from neighboring territories (Quinn & Startek-Foote, 2000) and it is possible that males engage in similar antagonistic behavior to establish dominance hierarchies or acquire mating opportunities.

Together, our results show sexual dimorphism in both bill and body size of adult Smooth-billed Anis and provide evidence for sexual selection on bill size in a co-operatively breeding species. We argue that non-kin composition of social groups may increase opportunities for competition for access to social mates and for extra-pair fertilizations, permitting higher reproductive variance and sexual selection in males. Bill size in males appears to be under sexual selection, though it remains unclear whether female mate choice, intra-male competition, or both are responsible. Mount presentations have been used to assess signals of aggression in Smooth-billed Anis (Grieves et al, 2015) and analysis of bill size and sex in individuals willing to aggress against male conspecific mounts may help clarify method of selection. Behavioural observations supporting mating assortment are also required to clarify selection for larger bills in males. Mating system is an essential factor in unraveling complexities of selection (Andersson, 1994), and the extent to which extra-pair and subordinate-sired offspring occur in Anis will affect the strength of sexual selection and resultant degrees of sexual dimorphism. Future studies should address extra-pair paternity in communal clutches to elucidate whether extra-pair sires or acquisition of higher quality mates explain observed selection gradients.

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1.7 Figures and Tables

TABLE 1.1: Results from a general linear model with Bill Score (Bill PC1) as the response variable. Bill Score correlates with Body Score and sex. Body Score (Body PC1) and Bill Score were derived from a principle component analysis using measurements from 180 adult male and 173 adult female Smooth-billed Anis (*Crotophaga ani*). Asterix (*) indicates significance at an alpha of 0.05.

Coefficient	Estimate	Std. Error	t-value	p-value
Intercept	0.443	0.019	23.600	<0.001*
Body Score	0.117	0.044	2.646	0.0091*
Sex	0.068	0.031	2.167	0.031*
Body Score*Sex	0.157	0.060	2.602	0.010*

TABLE 1.2: The effects of Bill Score (Bill PC1), Body Score (Body PC1) and social group size on annual reproductive success in male and female Smooth-billed Anis (*Crotophaga ani*). Asterix (*) indicates significance at an alpha of 0.05.

Sex	Sample Size (n)	Coefficient	Estimate	Std. Error	Z-value	p-value
Male	57	Intercept	0.438	0.418	1.05	0.295
		Bill Score	0.464	0.171	2.71	0.007*
		Body Score	-0.198	0.197	-1.00	0.316
		Group Size	-0.060	0.047	-1.27	0.203
Female	44	Intercept	0.444	0.899	0.49	0.620
		Bill Score	1.448	2.331	0.62	0.530
		Body Score	-2.290	1.615	-1.42	0.160
		Group Size	0.036	0.058	0.62	0.530

TABLE 1.3: The effects of Bill Score (Bill PC1) and Body Score (Body PC1) on proportion of communal nests sired or dammed. Results from a generalized linear mixed model. Asterix (*) indicates significance at an alpha of 0.05.

Sex	Sample Size (n)	Coefficient	Estimate	Std. Error	t-value	p-value
Males	57	Intercept	-2.767	1.047	-2.643	0.027*
		Bill Score	4.593	1.497	3.068	0.013*
		Body Score	-1.933	0.952	-2.030	0.073
Females	44	Intercept	-0.458	1.255	-0.365	0.724
		Bill Score	-1.382	2.840	-0.478	0.639
		Body Score	0.610	1.487	0.410	0.691

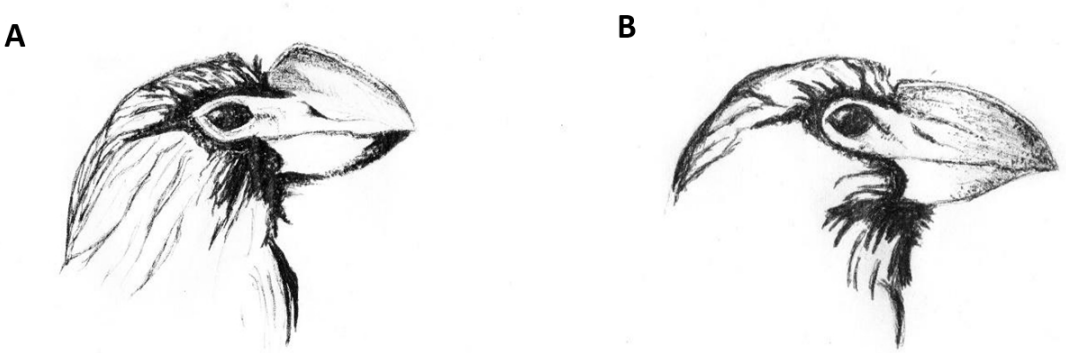

FIGURE 1.1: Lateral depiction of a male Smooth-billed Ani (*Crotophaga ani*) with bill crest often exceeding top of head. (B) A similar lateral depiction of a female Smooth-billed Ani (*Crotophaga ani*) with markedly reduced bill crest and proportional bill length. Drawing by Kimberley A. Tasker.

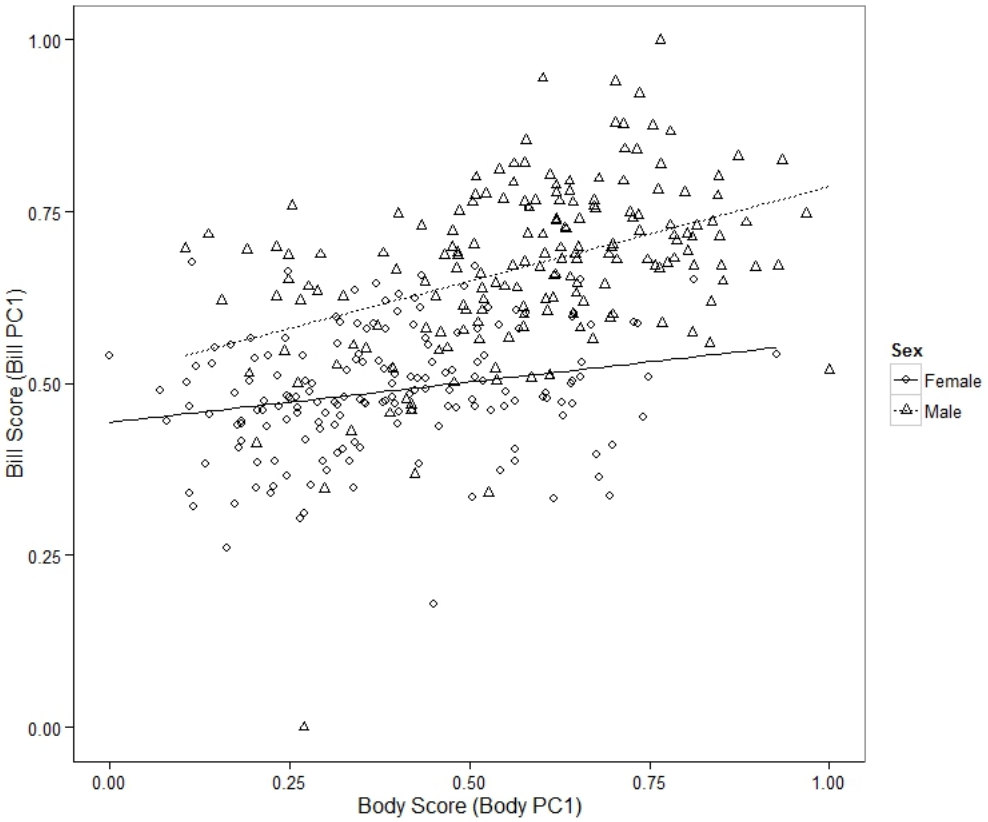

FIGURE 1.2: Bill Score (Bill PC1) according to Body Score in adult male and female Smooth-billed Anis (*Crotophaga ani*). Proportional bill size is distinct between sexes. Body Score (Body PC1) and Bill Score were derived from a principle component analysis using measurements from 180 adult male and 173 adult female Smooth-billed Anis. Individual males are represented by open triangles and individual females by open circle. Dashed and solid lines represent trends in male and female adults respectively.

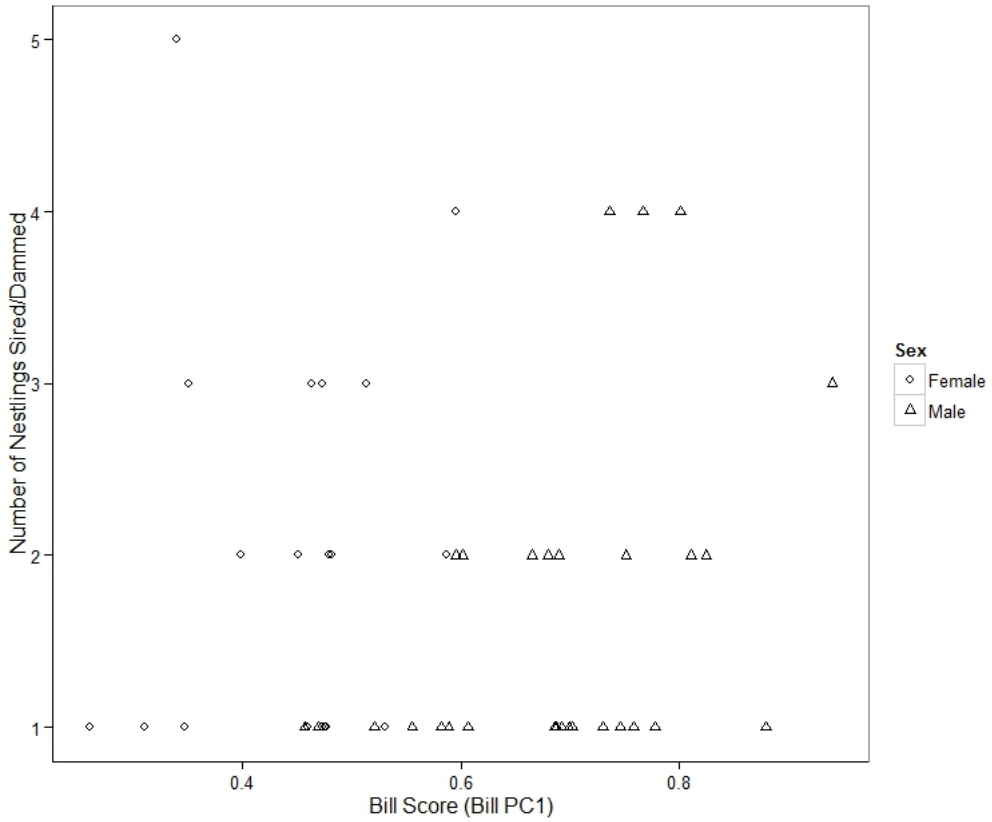

FIGURE 1.3: Annual reproductive success according to Bill Score in male and female Smooth-billed Anis (*Crotophaga ani*) ($n_{males} = 57$, $n_{females} = 44$). Individual males are represented by open triangles and individual females by open circles. Reproductive success is represented as the number of offspring sired or dammed in year of adult capture.

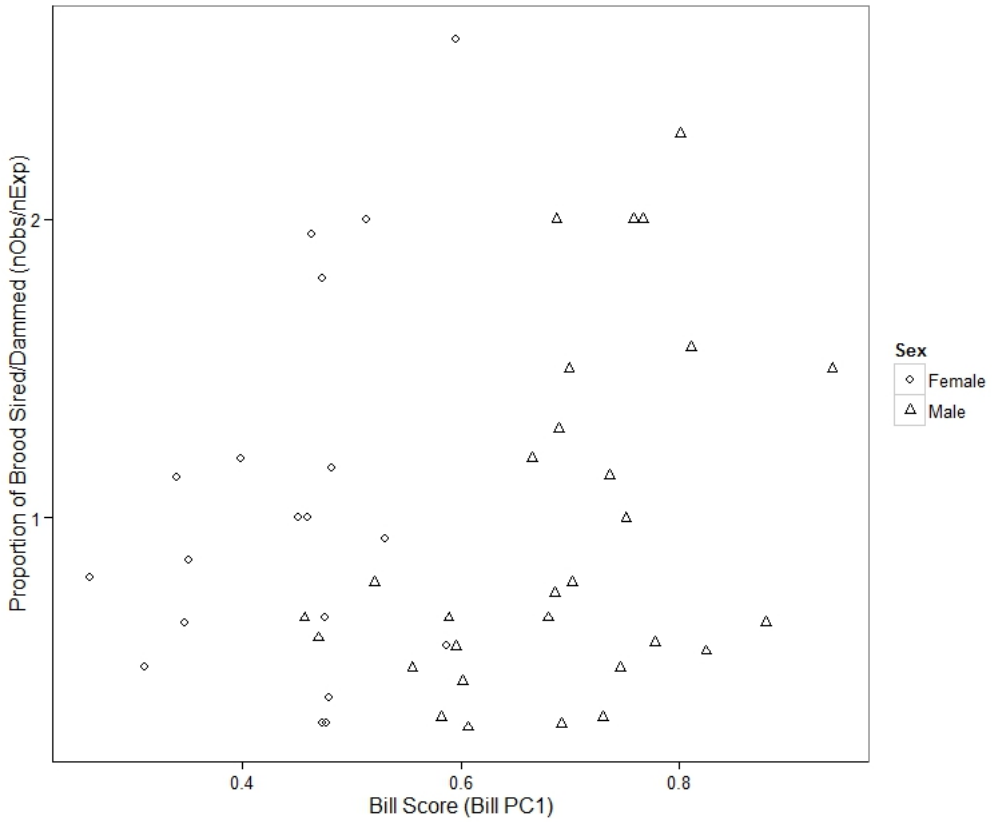

FIGURE 1.4: Proportion of communal nests sired according to multivariate bill size in male and female Smooth-billed Anis (*Crotophaga ani* (n_{males} = 57, $n_{females}$ = 44). Individual males are represented by open triangles and individual females by open circles. Reproductive success is represented as the proportion of offspring in brood sired or dammed in year of capture, divided by expected proportions sired or dammed in broods (or, 1/(0.5 x group size)).

2 | Does Parental Effort Correlate with Reproductive Contribution in Communal Nests? Patterns of Reproductive Skew in a Social Cuckoo

Master of Science– Joshua Kenneth Robertson; McMaster University– Biological Sciences

2.1 Abstract

Co-operative breeders provide parental care to non-filial offspring – a behaviour known as alloparental care. Hypotheses for the evolution and stability of alloparental care often follow inclusive fitness theory; however, genetic analyses have revealed that co-operatively breeding groups are sometimes comprised of non-kin. In species in which relatedness among group members is low, theory predicts that the degree of parental and alloparental effort should therefore be proportional to one's genetic contribution to mixed broods, depending upon reproductive options. We used genotyping data across 5-13 microsatellite loci for Anis from 17 social groups (50 adults and 117 nestlings) to assess the hypothesis of proportional parental effort in a population of Smooth-billed Anis (*Crotophaga ani*), a joint-nesting cuckoo species. Nocturnal incubation in this species appears to be performed almost exclusively by a single male. We first confirm low relatedness among social group members ($\bar{r}$= 0.159, n = 44 dyads), and low juvenile retention (2.577-17.526%) suggesting that inclusive fitness benefits are unlikely to explain uneven allocation of incubation effort. Second, we show that nocturnal incubation status was a significant predictor of reproductive success in males, where nocturnal incubators sire a greater proportion of mixed broods. Our results differ from those reported for another species of Crotophaginae and detect moderate reproductive skew in a reportedly egalitarian species.

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2.2 Introduction

Co-operative breeders extend parental care to offspring that are not their own (Emlen & Vehrencamp, 1983). This extension of care to non-filial offspring, or alloparental care, can impose costs on annual reproductive success and has therefore garnered significant research attention (e.g., Riedman, 1982; Birkhead & Nettleship, 1984, Redondo et al, 1995; Kokko et al, 2000; Da Silva Mota et al, 2006; Zöttl et al, 2012). While paradoxical to conventional fitness models, alloparental care has evolved in several taxa (Grimes, 1976; Woolfenden, 1976; Zahavi, 1976; Solomon & French, 1997; Cockburn, 2006) and has been documented in 9% of avian species (Cockburn, 2006). Putative hypotheses for the stability of alloparental care often follow inclusive fitness theory (Hamilton, 1964), but research has shown that group relatedness is not obligatory (ie Pied Kingfishers (*Ceryle rudis*; Reyer, 1984), Galapagos Hawks (*Buteo galapagoensis*; DeLay et al, 1996), Taiwanese Yuhinas (*Yuhina brunneiceps*; Yuan et al, 2004), and Greater Anis (*Crotophaga major*; Riehl, 2011). In fact, a recent species survey estimated that 15% of co-operative breeding avian species form socially breeding groups primarily with non-kin (Riehl, 2013). In such cases, indirect fitness benefits are not available and the costs of alloparental effort are likely to impose pressures on relative parental effort (Trivers, 1972; Emlen, 1978). Indeed, theory predicts that individuals should reduce parental care directed toward non-filial offspring (Clutton-Brock, 1991; Heinsohn & Legge, 1999) in favour of their own fitness. In co-operatively breeding species where multiple males and females contribute to communal broods (joint-nesting), patterns of parental care may therefore be predicted to match patterns of reproductive skew.

Alloparental behaviours are diverse and rarely limited to provisioning of nestlings. Nest defence, nest construction, predator mobbing and territory defence are commonly carried out by most sexually mature, co-operatively breeding group members (reviewed

in Brown, 1987; Zack, 1986; Komdeur, 1994; Heinsohn & Legge, 1999; Arnold, 2005), and such efforts cannot be directed to favour individual offspring in communal nests. Therefore, current hypotheses state that adult co-operative breeders should adjust their degree of parental and alloparental effort (or together defined as 'inclusive parental care') according to their proportional genetic contribution to a brood (Trivers, 1972; Emlen 1978; Werren et al, 1980; Burke et al, 1983; Westneat & Sherman, 1993). Indeed, inclusive parental effort appears highly variable across adult group members in a number of avian spcies (Mumme et al, 1990; Komdeur, 1994; Heinsohn & Cockburn, 1994; Clutton-Brock et al, 1998; Vehrencamp et al, 1986; Riehl & Jara, 2009), which may suggest differences in investment. Direct support for whether parental effort is strategically matched to genetic contribution, however, is limited and inconsistent. In the Carrion Crow (*Corvus corone corone*) and Greater Ani, distribution of inclusive parental effort appears uneven, though reproductive skew is remarkably low (Canestrari et al, 2005; Riehl, 2012). Similarly, relative alloparental effort in co-operatively breeding Meerkats (*Suricata suricata*) was not correlated to relatedness to pups when sex, age and foraging success were controlled (Clutton-Brock et al, 2001). In contrast, helping Seychelles Warblers (*Acrocephalus sechellensis*) reduce provisioning effort significantly when relatedness to the brood is low (Komdeur, 1994) and male polyandrous Dunnocks (*Prunella modularis*) increase foraging effort when perceived and realized paternity are high (Davies et al, 1992). Relatedness to broods also plays a key role in determining relative alloparental effort in Pied Kingfishers that form social groups with kin and non-kin (Reyer, 1984).

In this study, we explore the hypothesis of proportional parental effort in a population of joint-nesting neotropical cuckoos. The Crotophagids (Crotophaginae) are a sub-family of highly social, neotropical cuckoos that have gained considerable research attention for their egalitarian, joint-nesting behaviours. The Greater Ani (*Crotophaga major*), Groove-billed Ani (*Crotophaga sulcirostris*) and Guira Cuckoo (*Guira guira*) form social groups composed of unrelated, monogamous pairs during breeding seasons

(Vehrencamp, 1978; Macedo, 1992; Riehl & Jara, 2009) and all social group members are thought to take part in nest building and provisioning (Vehrencamp, 1978;Macedo, 1992; Riehl & Jara, 2009). In Greater and Groove-billed Anis, however, incubation effort is not so evenly distributed. Nocturnal incubation is predominantly performed by a single male in breedings groups of this species and represents a high risk and costly behaviour (Vehrencamp et al, 1986; Tinbergen & Williams, 2002; Riehl, 2012). In the Groove-billed Ani, nocturnal incubators suffer higher risk of mortality (Vehrencamp et al, 1986; Vehrencamp et al, 1988). In both Groove-billed and Smooth-billed Anis, adult group members roost communally and therefore gain selfish herd (Hamilton, 1971) and energetic advantages that are not shared by nocturnal incubators. Interestingly, reproductive skew is also reportedly low for both species (Vehrencamp, 2000; Riehl, 2012) which is consistent with theoretical predictions for joint-nesting species with non-kin social groups (Vehrencamp, 1983).

Unpublished field observations point to similarities in nocturnal incubation patterns in Smooth-billed Anis, where a single male appears to conduct most nocturnal incubation (Grieves, L.A., Quinn, J.S., and Robertson, J.K. personal observations) Further, preliminary research suggests patterns of within group relatedness and reproductive skew in the Smooth-billed Ani (Blanchard, 2000) are similar to other Crotophagids, though broad-scale genetic analyses have yet to be published. We therefore sought to observe nocturnal incubation patterns in Smooth-billed Anis and explore two hypotheses to explain differences in male inclusive parental effort, by combining field observations and microsatellite genotyping. Firstly, we explored whether adult group members are close kin and whether indirect fitness benefits gained through kin selection might explain differences in male effort. Secondly, we tested whether nocturnal incubators contribute more offspring to mixed broods. While reproductive competition by ovicide and infanticide in Smooth-billed Anis has received some research attention (Schmaltz et al, 2008; Quinn et al, 2010), relative parental effort and skew in final incubated clutches remains

largely unexplored and may shed light on evolutionary differences between members of Crotophaginae.

2.3 Methods

Field Site

Smooth-billed Anis were studied at the Cabo Rojo National Wildlife Refuge (NWR) in south-western Puerto Rico (17°59'N, 67°10'W) from 2013 to 2014. The Cabo Rojo NWR is comprised of mixed open field and dry forest in secondary succession, and experiences discrete rainy and dry seasons. Smooth-billed Anis inhabit the Cabo Rojo NWR year-round and typically breed after the onset of the rainy season (September-December). Observations were therefore limited to the rainy season.

Adult Capture and Identification

We captured adult Smooth-billed Anis using two horizontally stacked mist-nets (6.6 cm mesh, 18 m long, elevated to a maximum height of 7.2 m) (Meyers & Perdieck, 1993; Schmaltz et al, 2008) and hardware cloth, funnel traps with a hand-raised lure Ani in a protective cage. Funnel traps were placed within a central region of known territories to entice territorial aggression (Vehrencamp, 1977).

Immediately after capture, we sampled 50 µl to 200 µl of blood by basilic venipuncture and capillary tube collection (Sheldon et al, 2008). Blood samples were stored in approximately 1.0 mL of Queen's Lysis Buffer (Seutin et al. 1999) at 4°C until later use. Adults were randomly assigned a unique combination of three Darvic coloured leg bands and one USGS numbered aluminum band for field identification. Maximum depth of bill (mm) and length of exposed culmen (mm) were measured to a tenth of a millimeter using analogue calipers, while right wing chord was measured to the nearest millimeter using a wing ruler. Mass (g) was measured to the nearest tenth of a gram using a spring scale. Following measurement, captured birds were released on home territories. Recaptured

adults, however, were not measured unless they had been captured in a previous breeding season. All capture, banding and measurements were taken as part of a long-term population monitoring program.

Nocturnal Incubator Capture and Identification

Where nest sites permitted, nocturnal incubators were captured on the nest at dawn using walk-in nest traps (Mock & Schwagmeyer, 1999). Nest trapping was largely restricted to late egg stage as to not jeopardise offspring survival if nocturnal incubators exhibited 'trap-shy' behaviour (ie. avoidance of the nest due to trap presence). Following capture, nocturnal incubators were processed as previously described and immediately released on home territories. Where capture was not possible, video observations (infrared at night) were used to identify incubators. Cameras were placed at nests at least one hour before dusk and collected the following day to minimize nest disturbance. Together, we identified nocturnal incubators at 17 territories (9 individuals by capture in 2014, 3 previously sampled individuals by video observation in 2013, 3 previously sampled individuals by observation in 2012 and 2 by observation in 2003). Repeat observations were made at 5 territories in 2013 and 4 territories in 2014 and no observations reported inconsistences in nocturnal incubator identification (similar to Riehl, 2012). A total of 33 non-nocturnally incubating adults were captured from social groups with known nocturnal incubators (16 males and 17 females), though only males were considered in downstream analyses.

Social Group Size and Composition

To determine the number of adults in social groups, we counted the number of adults leaving and entering roosts through the breeding season (Schmaltz et al, 2008). The

maximum number of adults observed on at least three different occasions was used as a measure for social group size. Juveniles are easily recognized and excluded from group size censuses. Unique coloured leg band combinations were used to identify group members that had been previously captured.

Nest Searching, Nestling Capture, and Nestling Identification

Adults were routinely monitored for signs of nest building and egg laying. Gravid female Anis are distinguishable by the bulge of a developing egg, and such sightings promoted increased nest searching efforts. Following nest discovery, nest locations were marked with a hand-held GPS and visited every one to three days until the onset of hatching. Nests were then checked daily until the youngest nestling reached three to four days post-hatch (dph). Once hatched, each nestling was marked with a unique toe-nail clipping for future identification. We then sampled 50 µl- 100 µl of blood from each nestling by femoral venipuncture and capillary tube collection. Blood was stored in Queen's Lysis Buffer at 4°C until processing. At three dph, chicks were banded with one USGS aluminum band and one darvic colour band in a combination that was unique to the brood. In total, 116 nestlings were sampled across 17 territories with known nocturnal incubators.

Morphometric Principle Component Analysis

Bill size is correlated with reproductive success in male Smooth-billed Anis (Robertson et al, unpublished). We therefore generated a multivariate bill size measurement using a Principle Component Analysis (PCA) for downstream reproductive success analyses. To do so, we loaded length of exposed culmen (mm) and maximum bill depth (mm) into a scaled and centered principle component analysis (PCA) using the R package

prcomp (R Core Team 2015). From the PCA, we selected the component explaining the highest measurement variance to assign a multivariate 'Bill PC1' to each adult. To simplify interpretation, each Bill PC1 was scaled from zero to one. To control for body size, we then loaded measurements of wing chord (mm) and mass (g) into a second scaled and centered PCA. Measurements of wing chord did not scale linearly with body mass (Linear model: $t = 0.529$, $p = 0.601$, $n = 59$). We therefore took the natural logarithm of each variable prior to loading. Again, the component explaining the greatest measurement in both loading variables was selected and used to calculate a multivariate 'Body PC1' for each individual.

Molecular Sex Determination and Microsatellite Genotyping

Genomic DNA was isolated from blood samples by phenol-chloroform extraction and stored at -20°C until needed. Quantity and quality of DNA was assessed by NanoDrop spectrophotometry (NanoDrop Technologies). We determined the sex of all captured adults (n=50) by PCR amplification of Chromo-Helicase DNA-binding gene (CHD1) intron 16 and amplicon separation on 1-3% agarose gels (Griffiths et al, 1996; Fridolfsson & Ellegren, 1999). Following molecular sexing, candidate parents and sampled nestlings were genotyped at 5-13 microsatellite loci (ANI450B2, ANI9546, ANIC5, CrMa3-142, CrMa327, CrMa4-6, JJQ0536, SNX7, SNX14, SNX17, JSQ1, JSQ5, JJQ0536) (Blanchard & Quinn 2001; Gregory & Quinn, 2005; Riehl & Bogdanowicz, 2009) by PCR amplification with fluorescently labelled primers. Fluorescent PCR amplicons were then ethanol purified and submitted to the Natural Resource DNA Profiling and Forensic Centre (NRDPFC; Trent University, Peterborough, ON) for size assessment on an ABI 3730 DNA analyzer with GS-500 size standards. Microsatellite genotypes were manually called from electropherograms in GeneMarker 2.0-2.2 (SoftGenetics, LLC) to reduce automatic binning errors (Dewoody et al, 2006). For this study, microsatellite genotypes

were determined for 166 individuals ($n_{adults} = 50$, $n_{nestlings} = 116$).

Parentage Analyses and Relatedness

We used Cervus Software 3.0 (Kalinowski et al, 2007) to assign paternity in social groups where nocturnal incubators were identified. All males observed in social groups with known nocturnal incubators were assessed as candidate fathers. Cervus uses maximum likelihood to assign parentage from allele frequencies by comparing likelihood scores of candidate parents to randomly simulated adults. For our analysis, we simulated 10,000 adults and estimated that 60% of breeding males were represented in analyses, according to estimated group sizes and assuming an even sex ratio. The 'proportion of loci genotyped' across our sample population was 0.80 and the proportion mistyped (expected error) was set to 0.05 for conservative analyses. Only individuals genotyped at > 5 microsatellite loci were considered for analysis. Parentage was assigned at both 80% and 95% confidence.

Using the previous genotyping data, we then used ML-Relate (Kalinowski et al, 2006) to calculate the coefficient of relatedness (r) between adults in known social groups. ML-Relate also uses maximum likelihood methods to calculate r from allele frequencies and estimates relationships according to likelihood of having zero, one or two alleles at each locus identical by descent.

Group Retention

Capture of all adults within social groups was not feasible, consequently limiting interpretation of relatedness measures among individuals within groups. For this reason, we estimated stringent and relaxed juvenile retention to provide indirect evidence for patterns of intra-group relatedness and inclusive fitness benefits of nocturnal incubation.

To estimate stringent juvenile retention, we quantified the number of individuals captured at natal territories one year following fledging ('n_c'), divided this value by the total number of juveniles banded (or 'n_T') and represented the proportion as a percentage. Recaptured individuals were identified by aluminum leg band numbers (USGS aluminum bands). By identifying individuals through recapture alone, false positives were eliminated though retention is likely to be underestimated due to limited recapture. Relaxed juvenile retention was estimated in a similar manner, however, individuals sighted with a colour band combination matching that of a nestling banded in the territory of sighting one year prior were included in retention counts (n_c). Furthermore, those captured in natal territories two years after fledging were also included in n_c. Due to leg size limitations, nestlings were banded with one numbered aluminum leg band (USGS) and one colour leg band. Colour combinations are therefore repeated across territories during an individual breeding season and may limit accurate interpretation of retention by permitting false positives. In the relaxed retention measure only capture and sighting records from 2011-2014 were used for analysis. Given high dispersal (Quinn, J.S. unpublished observations) and an inability to track individuals during non-breeding seasons, estimating and accounting for juvenile survivorship was not possible. By not accounting for survivorship in estimates of n_T, both stringent and relaxed measures are therefore likely to be underestimates.

Statistical Analyses

To test whether intra-group adults are more closely related than inter-group adults, we compared average r ($\bar{r}$) between intra-group and inter-group dyads using an unpaired Students T-Test in R (R Core Team 2015). To examine whether nocturnal incubation behaviour correlated with genetic contribution to mixed broods, we first constructed a generalized linear mixed model (glmm) with number of nestlings sired at 80% confidence

as the response variable and nocturnal incubation status, Bill PC1 and Body PC1 as fixed effect predictors. Social group size was not a significant predictor of reproductive success in previous analyses (Robertson et al, unpublished) and within out data ($z = 0.168$, $p = 0.867$). We therefore excluded group size as a fixed effect from analyses. Territory ID and year of sampling were included as random effects and the R package lme4 (Bates et al, 2015) was used for model construction. A Poisson distribution of the response variable was assumed. We repeated this analysis with number of nestlings sired at 95% as the response variable and minor adjustments to account for sample size. Again, social group size was not significantly correlated with reproductive success in preliminary analyses ($z = 0.800$, $p = 0.426$) and was therefore excluded from analysis. Due to high stringency in paternity assignments and the number of unsampled adults, non-assignment rate was high. We, however, have no reason to anticipate a biased distribution of males in paternity assignments. As in prior analysis, a Poisson distribution was assumed, however, with zero-inflation to account for high non-assignment. The R package glmmADMB was used to construct the secondary model (Bolker et al, 2012). Finally, average body size (Body PC1) and bill size (Bill PC1) were compared between nocturnal incubators and non-nocturnally incubating males using an unpaired Student's T-Test. Alpha levels were set to 0.05 for all analyses.

2.4 Results

Within Group Relatedness and Juvenile Retention

We calculated $\bar{r}$ of dyads within groups (n = 44) and between groups (n = 821). Within social groups, $\bar{r}$ of dyads was low ($\bar{r}$= 0.150) and comparable to that of dyads between groups ($\bar{r}$= 0.159; t = 0.274, df = 47, p = 0.786) (Fig 2.1). Of all within group dyads, ML-Relate estimated three parent-offspring relationships, three full sibling relationships and 8 half-sibling relationships. Stringent juvenile retention was calculated as 2.577% (n_c = 5, n_T = 189) and relaxed retention as 17.526% (n_c = 34, n_T = 189).

Morphology, Parental Effort and Reproductive Success

The primary vector of a principle component analyses (PCA) for body size (Body PC1) explained 54.9% of variance in body size measurements (n = 50), while the primary vector of a bill size PCA (Bill PC1) explained 78.2% of variance in body size measurements (n = 50).

Of 116 genotyped nestlings, paternity was assigned for n = 90 (77.6%) at 80% confidence and n = 35 (30.2%) at 95% confidence. Both role in nocturnal incubation and Bill PC1 were significant predictors of the number of nestlings sired (annual reproductive success) at 80% confidence (Tab 2.1) (Fig 2.2) however, role in nocturnal incubation alone was significantly correlated with the number of nestlings sired at 95% confidence (Tab 2.2) (Fig 2.3). Body PC1 was not significantly correlated with reproductive success at either 80% and 95% confident parentage assignments (Tab 2.1) (Fig 2.2). While average bill size and body size of nocturnally incubating males (Bill PC1= 0.535, sd = 0.206 n = 17; Body PC1= 0.742, sd = 0.231 n = 17) exceeded that of non-nocturnally incubating males (Bill PC1= 0.439, sd = 0.263, n = 16; Body PC1= 0.549, sd = 0.152,

n = 16), body size alone differed significantly between nocturnally incubating and non-nocturnally incubating males (Bill PC1: t = 1.169, df = 28.447, p = 0.252; Body PC1: t = 2.846, df = 27.829, p = 0.008).

2.5 Discussion

Caring for offspring that are not one's own is a costly behaviour most often explained by close kinship between co-operatively breeding group members (Hamilton, 1964; Reyer, 1984; Komdeur, 1994; Clutton-Brock, 2002; Blackmore & Heinsohn, 2008). Our results, however, show low degrees of relatedness, and low juvenile retention (2.577 – 17.526%) among communally breeding groups of Smooth-billed Anis. Interestingly, our juvenile retention estimates are similar to those reported by Koford et al (1990) for closely related Groove-billed Anis (12% retention). Contrasting Koford et al (1990) however, an inability to estimate and account for juvenile survivorship may suggest underestimation in our results.

Co-operative breeding with non-kin is not uncommon, though the implications of nesting communally with strangers have gained little research attention (but see Vehrencamp et al, 1986; Schmaltz et al, 2008; Riehl, 2012). Theory predicts that individuals should match inclusive parental effort to genetic contribution in broods (Trivers, 1972; Emlen, 1978; Werren et al, 1980; Burke et al, 1983; Westneat & Sherman, 1993). In joint-nesting species with low skew, inclusive parental effort should be equally distributed among adult group members. Inclusive parental care in males, however, does not appear evenly distributed in our study population. Like Greater and Groove-billed Anis (Vehrencamp et al, 1986; Riehl, 2012), nocturnal incubation behaviour was exclusive shown in males and the identity of specific incubators was not subject to change within a breeding season. Given our reported patterns of group relatedness, inclusive fitness benefits are not likely to explain divisions in male parental care.

Our results provide support for the hypothesis of proportional parental effort (Trivers, 1972; Emlen, 1978; Werren et al, 1980; Burke et al, 1983; Westneat & Sherman, 1993)

in male Smooth-billed Anis. Males contributing to nocturnal incubation sired more offspring than non-nocturnally incubating males according to parentage assigned at both relaxed (80% confidence) and stringent (95% confidence) criteria. As costs of thermoregulation (Tinbergen & Williams, 2002) and risk of predation (Vehrencamp et al, 1988) are likely high, nocturnal incubation represents a considerable level of inclusive parental effort. Notably, behavioural studies have reported heightened territory defense and predator mobbing by nocturnal incubators in both Greater and Groove-billed Anis (Vehrencamp et al, 1986; Koford et al, 1990; Riehl & Jara, 2009), suggesting disproportional allocation of parental effort in these individuals extends beyond incubation alone. Interestingly, nocturnal incubators were significantly larger than their male group mates in our sample population. Large body size in nocturnal incubators may enhance defence against nest predators while also enhancing incubation efficiency during nights. Importantly, our results highlight that morphometry alone is not likely to explain patterns in male reproductive skew (Tab 2.1).

Interestingly, our results regarding the relationship between genetic contribution to the clutch and proportional care contrast those reported for Greater Anis, although patterns of group relatedness are similar. Riehl (2012) reported remarkably low reproductive skew in Greater Anis, with nocturnal incubators contributing no more or less to mixed broods than the remainder of males in social groups. Such inconsistencies between species may signify specialization of male parental care, reliance on fitness contributions of alternate direct benefits or an evolutionarily unstable strategy in Greater Anis (Riehl, 2012), but support for any hypothesis remains limited.

The proximate mechanisms behind our reported patterns of male reproductive skew are not clear. Smooth-billed Anis form socially monogamous pairs which assemble in breeding groups during breeding seasons. Extra-pair fertilizations in Anis have been detected using molecular analyses (Blanchard, 2000). It is therefore possible that costs

of nocturnal incubation are off-set by enhanced breeding opportunities through extra-pair matings. Nocturnal incubation has been considered a defining feature of behavioural dominance in Groove-billed Anis and appears to play a role in acquisition of high quality mates and better laying positions (Vehrencamp et al, 1986). Given low female sampling within our 17 territories, however, our ability to confidently assign parentage trios and identify extra-pair offspring was extremely low, therefore limiting our ability to compare extra-pairing matings between nocturnal incubating and non-nocturnal incubating males. Broad scale analyses of within-group extra-pair fertilizations may help shed light on sources of male reproductive skew.

Frequent access to communal nests may also facilitate modification of joint clutches to favour genetic contribution by the nocturnal incubator and his mate. Smooth-billed Anis partake in intense at-the-nest competition during breeding seasons, resulting in extreme egg loss by tossing and burial (Schmaltz et al, 2008). Though most acts of brood reduction are thought to be carried out by females in Groove-billed Anis (Vehrencamp et al, 1986), observations of egg tossing by males have been made (Quinn, personal observations). Nocturnal incubators and their mates may be more likely to evict eggs which are not their own, though this would require a mechanism of egg recognition which has not been identified in Anis. More likely, mates of nocturnal incubators may bias broods in their favour by delaying oviposition until after all other females have laid, thereby avoiding at-the-nest competition and ensuring representation in the incubated clutch. In Groove-billed Anis, Vehrencamp et al (1986) report pairing of nocturnal incubators and last laying females in 68% of communal clutches. In this species, optimizing time of oviposition is thought to be a regular component of reproductive strategy (Vehrencamp et al, 1986). Similar behaviours in Smooth-billed Anis may explain our reported patterns of reproductive skew. Future research should identify which group members compete most intensively for contribution to communal clutches, and how social role correlates with laying order.

Taken together, inclusive parental effort is not evenly distributed among male Smooth-billed Anis and participation in nocturnal incubation correlates with genetic contribution to communal clutches. Our results provide evidence for reproductive skew amongst males, though the proximate mechanisms driving this require further exploration. Despite taxonomic affiliation, we report a less egalitarian breeding system in Smooth-billed Anis than in closely related Greater Anis. While there may be a number of explanations for this disparity, tolerance of reproductive skew in Smooth-billed Anis may suggest that there are direct benefits of group membership which are not experienced by Greater Anis and yet to be identified.

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2.7 Figures and Tables

TABLE 2.1: Annual reproductive success of Smooth-billed Ani (*Crotophaga ani*) males according to nocturnal incubation and morphology; results of a generalized linear mixed model. Paternity is assigned at 80% confidence. Asterix (*) indicates significance at an alpha of 0.05.

Coefficient	Estimate	Std. Error	z-value	p-value
Intercept	-0.945	0.520	-1.815	0.070
Body PC1	0.600	0.711	0.843	0.399
Bill PC1	1.356	0.508	2.670	0.008*
Nocturnal Incubation Status	1.123	0.314	3.581	<0.001*

TABLE 2.2: Male reproductive success as it correlates with relative male parental effort and morphology; results of a generalized linear mixed model. Parentage is assigned at 95% confidence. Asterix (*) indicates significance at an alpha of 0.05.

Coefficient	Estimate	Std. Error	z-value	p-value
Intercept	-0.439	0.970	-0.450	0.650
Body PC1	1.282	1.242	1.030	0.302
Bill PC1	0.214	0.878	0.240	0.808
Nocturnal Incubation Status	2.384	0.758	3.140	0.002*

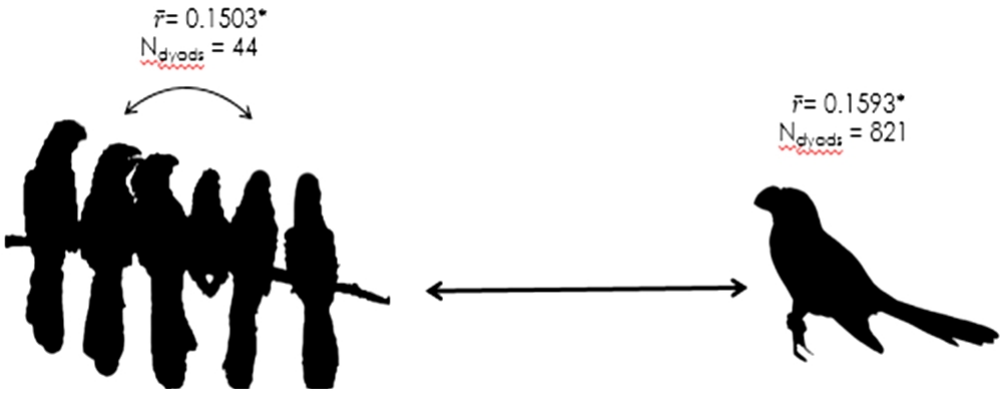

FIGURE 2.1: Relatedness of dyads within and between social groups of Smooth-billed Anis (*Crotophaga ani*). Mean coefficient of relatedness ($\bar{r}$) within groups is represented above silhouette of Ani social group (left side) and $\bar{r}$ between groups is represented about solitary silhouette (right side).

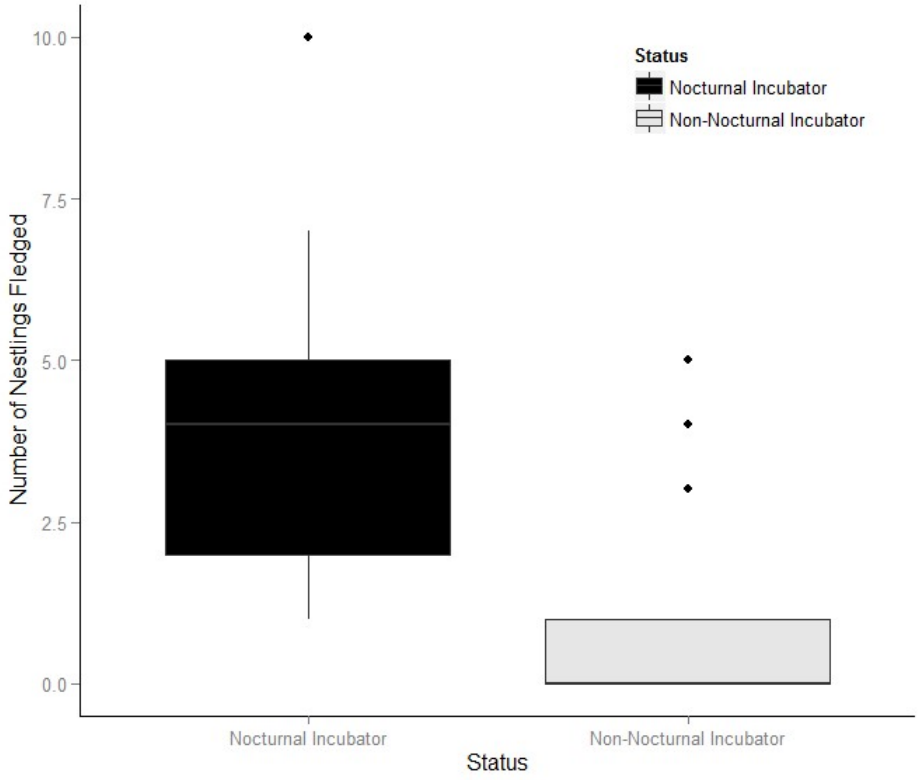

FIGURE 2.2: Annual male reproductive success according to nocturnal incubation status within social groups of joint-nesting Smooth-billed Anis (*Crotophaga ani*) ($n_{\text{nocturnal incubators}} = 17$, $n_{\text{non-nocturnal incubators}} = 16$). Paternity is assigned at 80% confidence and reproductive success is represented as number of offspring sired. Noctunal incubators represented in black and non-nocturnal incubators in grey. Medians are represented by grey and black lines through boxes, respectively. Whiskers represent ± 1.5 multiplied by the interquartile range (the distance between the first and third quartile).

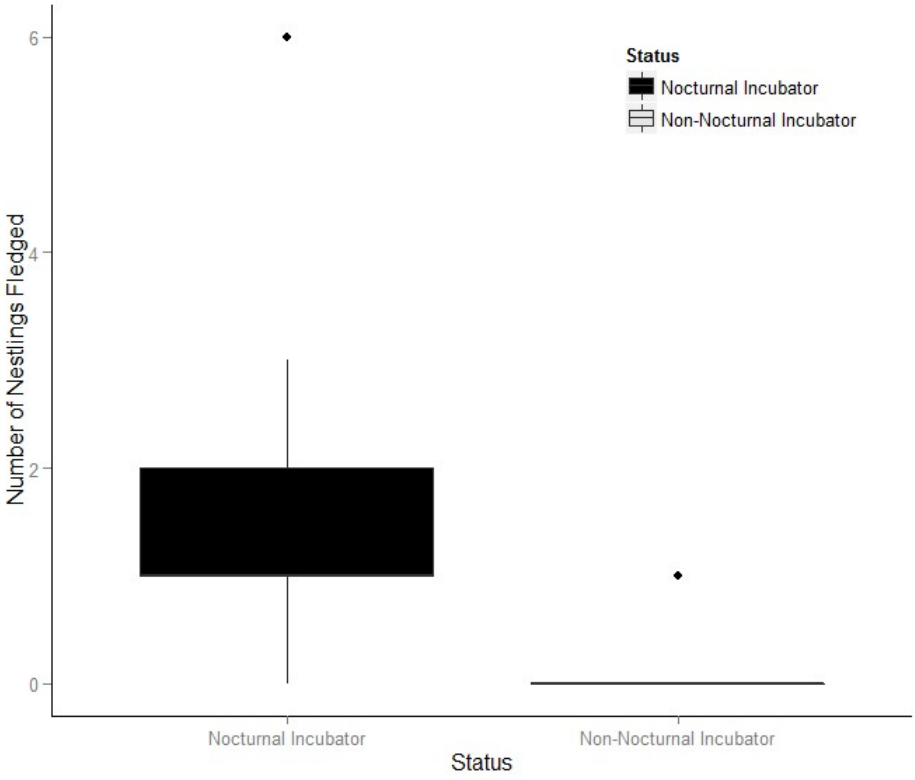

FIGURE 2.3: Annual reproductive success in male Smooth-billed Anis (*Crotophaga ani*) according to nocturnal incubation status of communal clutches ($n_{\text{nocturnal incubators}} = 17$, $n_{\text{non-nocturnal incubators}} = 16$). Paternity is assigned at 95% confidence and reproductive success is represented as the number of offspring sired. Medians are represented by grey and black lines through boxes, respectively. Whiskers represent ± 1.5 multiplied by the interquartile range (the distance between the first and third quartile).

3 | Chapter 3: The Effect of Social Group Size on Stress in a Co-operatively Breeding Bird: Consequences of Extreme Social Living

Master of Science– Joshua Kenneth Robertson; McMaster University– Biological Sciences

3.1 Abstract

Living closely with others can provide myriad fitness benefits, from shared territory defense and predator mobbing to shared parental care and cooperative resource acquisition. Costs of aggregation are not absent, however, and are likely to influence optimal and realized (or observed) group sizes in a social species. Studies of co-operatively and communally breeding species posit that observed group sizes may be explained by net fitness benefits at a given group optimum, which are outweighed by costs of social living when groups are atypically large, and lost when groups are atypically small. Here, we explored optimal groups sizes in a species of joint-nesting, neotropical cuckoo (the Smooth-billed Ani, *Crotophaga ani*) using endocrine markers of stress (corticosterone, or CORT). We captured 47 adult Smooth-billed Anis (29 males, 18 females) during the breeding seasons of 2012-2014 at the Cabo Rojo, National Wildlife Refuge in south-western Puerto Rico, and documented social group sizes as part of a long-term monitoring program. Tail feathers were sampled and used to quantify deposited CORT (pg/mg) in enzyme-linked immunosorbent assays (ELISAs) (n=50). Feather-CORT is thought to reflect plasma titres during growth and provide a stable and long-term measure of stress in birds. We therefore used feather-CORT as an indicator of stress in Smooth-billed Anis. Our analyses revealed a significant, positive correlation between log-transformed feather-CORT and categorical group size, but not continuous group size, with individuals from atypically large social groups ($\geq \bar{x}$ + 1SD) showing highest mean concentrations (66.606 pg/mg;) and individuals from atypically small social groups ($\leq \bar{x}$- 1SD) showing lowest mean concentrations (26.257 pg/mg). Females also showed higher mean feather-CORT than males (Females = 68.438 pg/mg, Males = 15.246 pg/mg). Our results suggest that living in atypically large groups is physiologically expensive and may represent an evolutionarily unstable strategy. To our knowledge, this is the first study to explore a correlation between stress and group size in a joint-nesting species.

Master of Science– Joshua Kenneth Robertson; McMaster University– Biological Sciences

3.2 Introduction

Living in groups can provide numerous fitness benefits, either directly (through shared territory defense, predator defense, resource acquisition, and parental effort) (Skutch, 1961; Brown, 1974; Reyer, 1986; Heinrich, 1988; Farabaugh et al, 1992; Arnold, 2000; Arnold et al, 2005; Kokko et al, 2001 for example) or indirectly (through dilution of predation risk and inclusive fitness benefits) (Hamilton, 1971; reviewed in Clutton-Brock, 2002). Costs of social aggregation, however, are not absent and are thought to influence optimal and realized (or observable) group sizes in gregarious species (Wrangham, 1980; Chapman & Chapman, 2000; Pride, 2005; Raouf et al, 2006). Indeed, theoretical research suggests that realized group sizes can be explained by net fitness benefits that are outweighed by costs when group membership is atypically large or small (Wrangham, 1980; Chapman & Chapman, 2000; Pride, 2005). Where group membership is atypically high, competition for access to resources and mating opportunities, frequent agonistic interactions and heightened spread of disease may limit incentive to join, or remain in social groups (Al-Rawi & Craig, 1975; van Schaik et al, 1983; Brown & Brown, 1996; Raouf et al, 2006). In atypically small groups, advantages of group living such as shared territory and predator defense are diminished and provide incentive to join larger social clusters (Wrangham, 1980). In co-operative breeders (particularly those with multiple breeding males and females), competition for reproductive opportunities, and benefits of shared parental effort, are strong antagonistic forces that likely influence optimal group size. Facultative co-operative breeders, therefore, provide an ideal framework to investigate the costs and benefits of group formation and optimal group size theory.

Across vertebrates, activation of the hypothalamic-pituitary-adrenal (HPA) axis is widely used as an indicator of stress (Wingfield & Romero, 2001). HPA activation in response to perceived stressors results in the secretion of glucocorticoids from the adrenal

cortex, which mobilizes energy stores through lipolysis, gluconeogenesis and proteolysis (Sapolsky et al, 2000; Romero, 2004). Temporary elevation of serum glucocorticoids is therefore thought to act adaptively by routing energy stores to solve immediate problems. Indeed, temporarily heightened glucocorticoids in birds has exhibited positive correlations with cognition (Sandi et al, 1997; Akirav et al, 2003; Pravosudov, 2003), caching behaviour (Saldanha et al, 2000; Pravosudov, 2003; Pravosudov et al, 2003) and survivorship (Breuner et al, 2008; Rivers et al, 2012). Chronic HPA activation, however, presents considerable metabolic demands and can yield detrimental effects on reproduction and survival (Schoech et al, 1991; Boonstra & Singleton, 1993; Smith & French, 1997; Sapolsky et al, 2000; Koren et al, 2012). While numerous studies have examined the effects of social hierarchy on circulating corticosterone titers in birds (Kotrschal et al, 1998; Pravosudov et al, 2003; Poisbleau et al, 2005), few have investigated the influence of social group size on stress (Raouf et al, 2006; Schmaltz et al, 2016) and fewer still in joint-nesting species (Schmaltz et al, 2016). Here, we explored the relationship between social group size and corticosterone deposited in feathers of joint-nesting, Smooth-billed Anis (*Crotophaga ani*). Feather-CORT is thought to reflect plasma titres during growth and provide a stable and long-term measure of stress in birds.

Smooth-billed Anis are socially monogamous, neotropical cuckoos that breed in social groups and share in nest building and care of young. Competition for maternal representation in communal nests is intense and costs associated with compensation for egg losses by egg tossing and burial are thought to be high (Schmaltz et al, 2008). Indeed, Schmaltz et al (2008) reported a negative correlation between per capita reproductive success and group size, with increased per capita egg loss in large groups. Despite these reproductive losses, direct benefits of group membership, such as alarm calling (Grieves et al, 2014), shared provisioning, and shared territory defence (Quinn & Startek-Foote, 2000) have been reported and may provide incentives for group formation

or joining. Solitary nesting is not uncommon and presents an opportunity to ensure complete genetic contribution to a brood, though the aforementioned direct benefits of group living must be forgone. Trends of group size and CORT have been explored in yolks of Smooth-billed Ani eggs (Schmaltz et al, 2016), however similar analyses are lacking for breeding adults and may shed light on the evolutionary mechanisms behind grouping behaviour and group maintenance. Though moult patterns are unclear in Smooth-billed Anis, previous research (Snow & Snow, 1964) and unpublished observations (Robertson, J.K.) suggest an overlap between breeding seasons and moult. Feather samples collected during breeding seasons may then represent a useful medium to quantify and compare baseline CORT from adults.

In this study, we used an enzyme linked immunosorbent assay (ELISA) to quantify CORT deposited in Smooth-billed Ani rectrices and explored trends of feather-CORT and social group size. We hypothesized that intra-group competition mediates stress in adult Smooth-billed Anis. We therefore predicted that; 1) feather-CORT is highest in large social groups where social competition is highest, and 2) feather-CORT differs between sexes in adults. Previous research posits that sexual selection may be acting on male bill size (Robertson et al, unpublished), implying intra-male competition for breeding opportunities. Intra-male competition is likely to be costly (Duyse et al, 2004) and may suggest higher feather-CORT in male Smooth-billed Anis. Alternatively, females may exhibit higher feather-CORT than males due to heightened costs of egg loss by ovicide and reproductive compensation. To our knowledge, this is the first study to investigate the effects of social group size on endocrine markers of stress in a joint-nesting species.

3.3 Methods

Field Location

Wild Smooth-billed Anis were captured and observed at the Cabo Rojo National Wildlife Refuge (NWR) in south-western Puerto Rico from 2012-2014. The habitat of the Cabo Rojo NWR is dry forest and pasture land with discrete, annual wet and dry seasons. Smooth-billed Anis inhabit the wildlife refuge year-round, though breeding is limited to the wet season when invertebrate prey is abundant (September to December). As we were interested in effects of reproductive competition on stress in adults, all capture and observations were limited to breeding seasons.

Adult Capture, Sampling and Group Size Determination

Adults were primarily captured using two horizontally stacked mist-nests (6.6 cm mesh, 18 m long, 7.2 m high) on telescopic poles (Meyers & Perdieck 1993; Schmaltz et al. 2008) as they left night roosts. We also captured adults using funnel traps, "baited" with a hand-raised, conspecific lure bird in a protective cage (Vehrencamp, 1977) and using hardware cloth, walk-in nest traps (Mock & Schwagmeyer, 1999). Once captured, we collected 50 µl to 200 µl of blood by brachial or basilic venipuncture and capillary tube collection and stored samples in a small volume (approximately 1 mL) of Queen's Lysis Buffer (Seutin et al, 1999). Blood samples were then kept at 4°C until further use for whole genomic DNA isolation and molecular sexing. We then collected the left, outermost rectrix of each individual and stored them in sealed and labeled plastic bags. All captured adults were assigned a unique combination of three Darvic coloured leg bands and one USGS numbered aluminum band for future identification and released in their respective home-territories. Social group sizes were estimated by counting the

number of individuals leaving from, and returning to communal roosts (Schmaltz et al, 2008). Juveniles are easily recognized and excluded from group size counts.

Molecular Sex Determination

Genomic DNA was isolated from blood-lysis samples by phenol:chloroform extraction and precipitation with isopropyl alcohol, then stored in TE (Tris-EDTA, pH = 7.4) buffer at -20°C until further use. Smooth-billed Anis exhibit sexual dimorphism in bill and body size, however, overlap is considerable (Robertson et al, unpublished) and is limiting to accurate sex determination by morphology. We therefore determined the sex of all captured adults by PCR amplification of the Chromo-Helicase DNA binding (CHD) gene (Griffiths et al, 1996; Fridolfsson & Ellegren, 1999) and amplicon size separation on 1-3% agarose gels.

Steroid Hormone Extractions

Extraction of CORT from rectrices followed protocols by Bortolotti et al (2008), with slight modification. Briefly, rectrix samples were washed sequentially under pure water and 100% methanol to remove fecal contamination and to strip preen oils. The calamus was then removed to eliminate contamination by dried blood. Once dried, we sampled a 40-125 mm section of each rectrix, measured from the proximal end of the feather. Samples were then diced into 5 x 5 mm sections and weighed in 25 mL sterile scintillation vials to the nearest tenth of a milligram. To each sample, we added 1 mL of 100% methanol for every 5 mg of rectrix fragments prior to incubation at 50°C for 24 hours. Methanol extracts were then sterile filtered using 0.22 µm syringe filters into new scintillation vials. Feather fragments were vortexed in 1 mL of diethyl ether for 30 seconds, and ether extractions were sterile filtered and pooled with methanol extracts

(Koren et al, 2012). All samples were stored at -20°C until further use. Prior to analysis, we evaporated methanol-ether extracts under a fume hood at room temperature until dry and reconstituted steroid samples to the appropriate volume in phosphate assay buffer (pH 7.0) (approximately 20x concentration).

Enzyme-Linked Immunosorbent Assays

We quantified CORT in feather isolates using enzyme-linked immunosorbent assays (ELISA). All assays were conducted using NUNC Maxisorp plates and a corticosterone antibody (Esoterix B3-163, Calabasas Hills, California, USA) shown to have low cortisol (0.4%) and cortisone (0.1%) cross-reactivity (Lattin et al, 2009). CORT standard was obtained from Steraloids Inc. (Newport, Rhode Island, USA) and a horse-radish peroxidase (HRP) conjugate was obtained from Creative Diagnostics Inc. (Shirley, New York, USA). NUNC plates were first coated with 50 µl of anti-CORT diluted to 1:10,000 in coating buffer (50 mM bicarbonate buffer, pH = 9.6) and incubated at 4°C for 12 hours. Wells were then washed five times with 100 µl of a Tween wash solution (0.15 M NaCl and 0.5 mL Tween 20/L) to remove excess, unbound antibody and incubated at room temperature for 2 hours with 50 µl of phosphate assay buffer. Following incubation, assay buffer was removed and CORT standard, samples and HRP conjugate was added to each well. Here, 50 µl of standard or sample were added to each well, followed by 50 µl of conjugate, diluted to 1:10,000 from a 1 mg/mL stock in assay buffer. On each plate, we used a twelve-point standard curve ranging from 5,000 pg/well to 2.44 pg/well of CORT standard. All samples and standards were run in duplicate. Following addition of samples, conjugate and CORT standard, plates were left to incubate for 1.5 hours, then washed again with Tween wash solution to remove unbound HRP-conjugate. Next, 100 µl of a substrate solution containing citrate buffer, H_2O_2 and 2,2'-azine-bis [3-ethylbenzthiazoline-6-sulfonic acid] were then added to each well and plates were shaken

for 1-2 hours. Optical density at 405 nm of wells was then measured using a microplate reader and a regression line was plotted to the standard curve. Mass of CORT per well of sample was quantified by interpolation from the plotted standard curve. Though Bortolotti et al. (2008, 2009, 2010) recommend representing feather-CORT by unit of feather length (pg/mm), Lendvai et al. (2013) and Grunst et al. (2014) suggest measurement error across samples is likely and urge representation by unit of mass (pg/mg). Indeed in previous studies and across our samples (linear model, $t = 43.926$, $p < 0.0001$), CORT standardized by unit of mass and length were highly correlated (Kennedy et al, 2013; Grunst et al, 2014). We therefore chose to represent feather-CORT per unit mass (pg/mg).

Assay Validations – Parallelism

We first tested parallel displacement of CORT-HRP by CORT standard and CORT extracted from feather samples. To do so, we created five pooled samples, each containing methanol-ether extracts from 10 randomly selected feather samples. Each pooled sample was concentrated 48-fold, then serially diluted twelve times in phosphate assay buffer. Serially diluted samples were then assessed in parallel with standard curves.

Assay Recovery

We assessed the accuracy of CORT detection in our assay by quantifying the percent recovery of exogenous CORT added to five randomly selected sample extracts. Each sample was concentrated 20-fold in assay buffer and spiked with a known concentration of CORT standard (1000, 500, 250, 125 and 62.5 pg). Samples were run according to assay protocols and percent recovery was assessed as prior.

Fault Bar Assessment

Punctuated stress in birds is capable of modulating feather growth and can cause physical deformities in feather barbs and barbules known as fault bars (Bortolotti et al, 2008). Indeed, feather segments containing faults bars have been shown to contain heightened concentrations of deposited CORT (Bortolotti et al, 2009). We therefore tested the ability of our assay to detect expected differences in CORT deposition between adjacent feather segments (12 mm) with and without visible fault bars (within-feather) according to protocols described in Bortolotti et al (2009).

Statistical Analysis

All analyses were conducted in the statistical program R (R core, 2016). To first test whether feather sections containing fault bars have differing quantities of deposited CORT than adjacent sections without fault bars, we compared sample means using a one-tailed, paired Student's T-Test. Next, we tested whether social group size was positively correlated with feather-CORT using a linear mixed effect model with Gaussian distribution in the package lme4 (Bates et al, 2015). Social group size and sex were selected as fixed effect predictor variables, while year sampled, territory and individual ID (to account for multiple samplings of the same individual) were selected as random effects. Feather-CORT (pg/mg) (the response variable) was log transformed to meet normality. Because optimal social group size can vary between sexes (ie. Wrangham, 1980), an interaction between sex and group size was initially included as a fixed effect predictor in our model, however this interactions was later removed due to a non-significant effect ($p = 0.266$, $t = -1.140$). To test the robustness of our model to correctly reject a null hypothesis, we conducted a power analysis for $n = 50$ and $r = 0.3$ (moderate correlation) using the package 'pwr' (Champely et al, 2016). In the case that our linear model did not provide sufficient power to reject the null hypothesis, we also compared mean, log

transformed feather-CORT between sexes using an unpaired students T-test.

To test whether relationships between social group size and feather-CORT were non-linear, we compared feather-CORT (pg/mg) of birds from intermediately sized social groups to those from atypically large and atypically small groups using an analysis of covariance (ANCOVA). Here, log transformed feather-CORT was selected as the Gaussian distributed response variable. Again, categorical group size, sex and an interaction between sex and categorical group size were selected as fixed effect predictors but removed due to a non-significant effect ($f = 0.769$, $p = 0.470$). Groups' sizes were categorized according to deviation from the mean. Group sizes exceeding one standard deviation from the mean ($\geq \bar{x}$ + 1SD) were considered atypically large, while those below one standard deviation from the mean were considered small ($\leq \bar{x}$ - 1SD). Alpha levels for all analyses were set to 0.05.

3.4 Results

Assay Validations

Serially diluted, pooled feather extract showed parallel displacement to corticosterone standard ($r^2 = 0.99$) (Fig 3.1a). Recovery of exogenous CORT from spiked samples was 86.5% ± 10.8% (n=5) and correlated with expected concentrations ($r^2 = 0.97$) (Fig 3.1b). Feather segments with fault bars contained significantly greater concentrations of deposited CORT by a paired Student's T-Test (t = 1.826, df = 10, p = 0.049, n = 11) (Fig. 3.2).

Adult Capture, Observation and Rectrix Sampling

We collected 50 rectrices from 47 individuals for analysis during the breeding seasons of 2012 to 2014 ($n_{Females}$ = 18, n_{Males} = 29). Two rectrices were collected from two individuals in 2014 and one individual was captured and sampled within two consecutive breeding seasons. Social group size ranged from 2 to 13 individuals (n=50), with a mean of 6.4 ± 3.169 individuals across all years. Group size categories (small, intermediate and large) contained 8 (n_{Males}= 5, $n_{Females}$= 3), 32 (n_{Males}= 22, $n_{Females}$= 10) and 10 individuals (n_{Males}= 4, $n_{Females}$= 6) respectively. All adults were captured from 31 independent social groups.

Feather Corticosterone and Group Size

Our assay detected CORT in all feather segments (n=50). Feather-CORT concentrations ranged from 3.33 to 314.97 pg/mg with a mean of 28.36 ± 6.3 pg/mg. Both social group size and sex were not significantly correlated with log transformed feather-CORT

(pg/mg), (Tab 3.1)(Fig 3.3) in a linear mixed effect model, however given our sample size, the statistical power of our model was low ($\pi = 0.572$). Log-transformed feather-CORT differed significantly between sexes by a Student's T-test (t=2.274, df = 23.476, p = 0.032), with mean female concentrations exceeding that of males ($\bar{x}_{\text{males}} = 15.25$ pg/mg $\pm$ 11.95; $\bar{x}_{\text{females}} = 71.82$ pg/mg $\pm$ 88.77) (Fig 3.3) (Fig 3.4). An ANCOVA identified significant differences in mean feather-CORT between categorical group sizes (Tab 3.2) and visualization revealed highest values in those within atypically large groups (Fig 3.5). A tukey's post-hoc revealed significant differences between log feather-CORT from adults in intermediately sized and large groups (Tab 3.3), but not between adults from small groups and large groups, or adults from small and intermediately sized groups (Tab 3.3). Sex was again significantly correlated with feather-CORT (Tab 3.2).

3.5 Discussion

Though previous analyses have detected CORT in feathers (Bortolotti et al, 2008; Lattin et al, 2009; Harms et al, 2010; Fairhurst et al, 2012; Koren et al, 2012; Kennedy et al, 2013; Bourgeon et al, 2014 for example), few have used enzyme linked immunosorbent assays (ELISAs) for quantitation (Bourgeon et al, 2014; Carbajal et al, 2014) and to our knowledge, none have done so for feathers of joint-nesting species. Our results show that ELISAs are a robust technique to quantify CORT deposited in adult rectrices with high accuracy (as shown by assay recovery) and are sufficient to detect punctuated differences predicted by feather deformities (ie. fault bars) (Fig 3.2) (Bortolotti et al, 2009).

Despite direct fitness benefits of group membership (Quinn & Startek-Foote, 2000; Grieves et al, 2014), our analyses suggest living in large social groups is stressful for Smooth-billed Anis. Adults in atypically large groups showed significantly higher CORT deposited in rectrices than those in intermediately sized groups (Fig 3.5), and heightened but not significantly different deposition when compared to atypically small groups. Interestingly, we show no significant difference in feather-CORT between intermediate and atypically small groups, suggesting that the effects of social interaction on CORT mobilization are exaggerated in atypically large groups alone, as predicted.

Elevated CORT deposition experienced by individuals in atypically large groups is most likely explained by reproductive competition. During breeding seasons, Smooth-billed Anis engage in intense at-the-nest competition for maternal representation in incubated clutches. Members of both sex engage in ovicide by egg tossing and burial, resulting in remarkable per capita egg losses (Schmaltz et al, 2008; Quinn unpublished observations). In closely related Groove-billed Anis (*Crotophaga sulcirostris*), eggs losses are compensated for by increasing oviposition which are generally compensated for by increased oviposition (Vehrencamp et al, 1986). Competitive interactions have long been

known to elevate baseline glucocorticoids in socially living species (Schoech et al, 1991; Sapolsky, 2000; Sloman et al, 2000; Abbot et al, 2003; Goymann & Wingfield, 2004) and competitive interactions behind egg burial and tossing are likely to follow suit. Aside from at-the-nest dynamics, enhanced competition for access to mates and resources in large social groups may also act to increase glucocorticoids in both sexes. Such patterns are common in social birds (Pravosudov et al, 2003; Duyse et al, 2004; Poisbleau et al, 2005; Landys et al, 2010) and may outweigh benefits of social aggregation.

As predicted, feather-CORT differed between sexes. Interestingly, female feather-CORT deposition exceeded that of males (Fig 3.3). It is likely that this pattern reflects physiological expenses of initial and compensatory oviposition. In previous studies, plasma CORT was dramatically elevated during egg-laying periods in female American Kestrels (*Falco sparverius*) (Rehder et al, 1986) and in domestic laying hens (*Gallus gallus domesticus*) (Johnson & Tienhoven, 1981). Similarly, nesting and egg-laying stages in female House Sparrows (*Passer domesticus*) coincided with elevated plasma CORT (Hegner & Wingfield, 1986). While all laying females are likely to experience elevations in baseline glucocortoids during breeding seasons, females in larger groups partake in compensatory egg production, therefore increasing annual oviposition and likely exacerbating physiological costs. Indeed, female Smooth-billed Anis from multi-female social groups have been shown to deposit higher concentrations of CORT in eggs than those in single female groups (Schmaltz et al, 2016) – an action thought to occur by passive diffusion and therefore reflect circulating maternal CORT (Hayward & Wingfield, 2004; Rettenbacher et al, 2005; Almasi et al, 2012). As measures of feather-CORT have been shown to reflect plasma titers during the time of growth (Bortolotti et al, 2008; Hörak et al, 2013) our analyses are unlikely to capture CORT mobilization in response to compensatory oviposition posterior to egg loss. However, observations of Groove-billed Anis posit that primary laying females prepare for expected ovicide by increasing egg deposiston in early nesting (Vehrencamp et al, 1986). In Smooth-billed Anis, per-capita clutch

size increased with group size (Schmaltz et al, 2008). Similar mechanisms of early compensatory oviposition may therefore be at play in female Smooth-billed Anis to prepare for losses in large social groups, thus stimulating CORT production and elongating periods of heightened circulation. By prolonging CORT elevation, likelihood of deposition in growing feathers and capture in our assay is increased and may explain our reported trends.

In all, we show that ELISAs are valuable tools for quantifying CORT deposited in feathers. Unlike classically used radio-immunoassays (RIAs), ELISAs are equivalently sensitive tools that do not require the use of harmful, gamma-emitting isotopes (Yolken, 1980). We therefore encourage others to use similar tools for detection and measurement of feather-CORT. We also show that the use of feathers as a stable endocrine archive is sufficient to detect, and ask questions about CORT mobilization in response to social stressors. Interestingly, our results illustrate that feather-CORT deposition is highest in joint-nesting Smooth-billed Anis from atypically large social groups and this elevation in baseline CORT is most likely due to intensive reproductive competition. Not surprisingly, atypically large groups are relatively infrequent in our field study (6 of 31 groups observed), but not rare. Given reduction of per capita reproductive success (Schmaltz et al, 2008) and elevations of baseline CORT in large social groups, formation and maintenance of such may represent an evolutionarily unstable strategy if not offset by undescribed direct fitness benefits. We therefore recommend further investigation of the mechanisms driving group formation and maintenance in this species.

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3.7 Figures and Tables

TABLE 3.1: Log transformed feather corticosterone (pg/mg) according to integral group size and sex: results of a linear mixed effect model (n=50). Asterix (*) indicates significance at an alpha of 0.05.

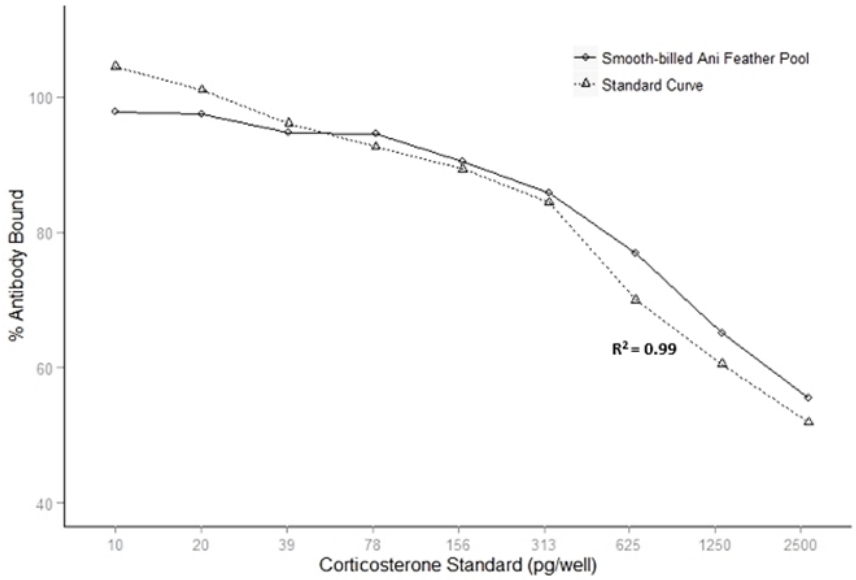
Coefficient	Estimate	t-value	df	p-value
Intercept	2.605	5.623	24	<0.001*
Group Size	0.101	1.826	23	0.080
Sex	-0.610	-1.981	23	0.059

TABLE 3.2: Effect of categorical group size and sex on adult, log transformed feather corticosterone (pg/mg): results of an analysis of covariance (ANCOVA) (n=50). Asterix (*) indicates significance at an alpha of 0.05.

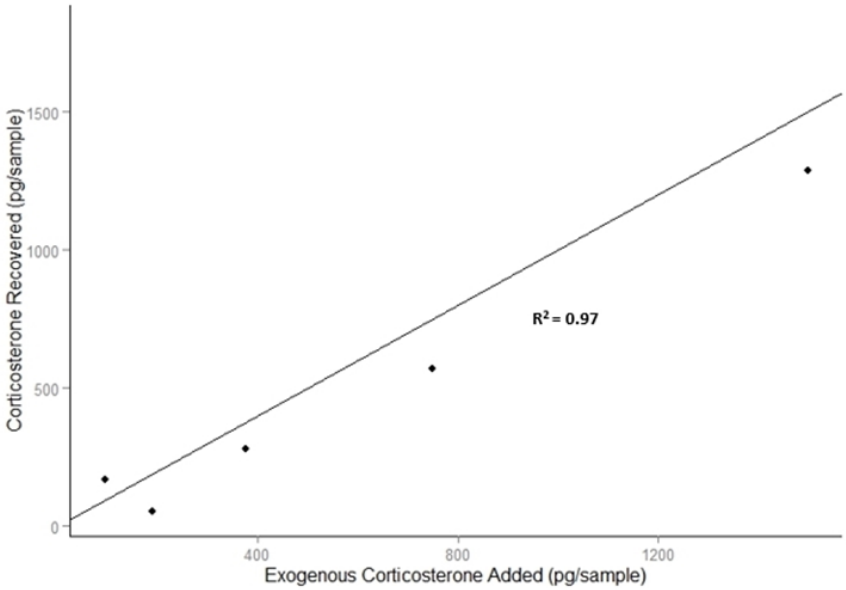
Coefficient	Sum of Squares	F-Value	df	p-value
Group Size Category	8.740	4.370	2	0.026*
Sex	5.220	5.219	1	0.035*
Residuals	50.660			

TABLE 3.3: Post-hoc comparison of mean log transformed feather corticosterone (pg/mg) between categorical group sizes. Tukey's method used for post-hoc analyses (n=50). Asterix (*) indicates significance at an alpha of 0.05.

Group Size Category Pair	Difference in Means	p-value
Small-Intermediate	-0.174	0.908
Small-Large	-1.173	0.058
Intermediate-Large	0.999	0.031*



(A)



(B)

FIGURE 3.1: Feather corticosterone assay validations. (a) Parellel displacement of serially diluted, pooled Smooth-billed Ani (*Crotophaga ani*) feather extracts and corticosterone standard. Corticosterone standard dilutions are represented by open triangles and pooled feather extracts are represented by open circles. Trend lines are dashed and solid for corticosterone standard and pooled extracts respectively. (b) Expected recovery of exogenous corticosterone added to sample extracts drawn against observed corticosterone recovery (pg/sample).

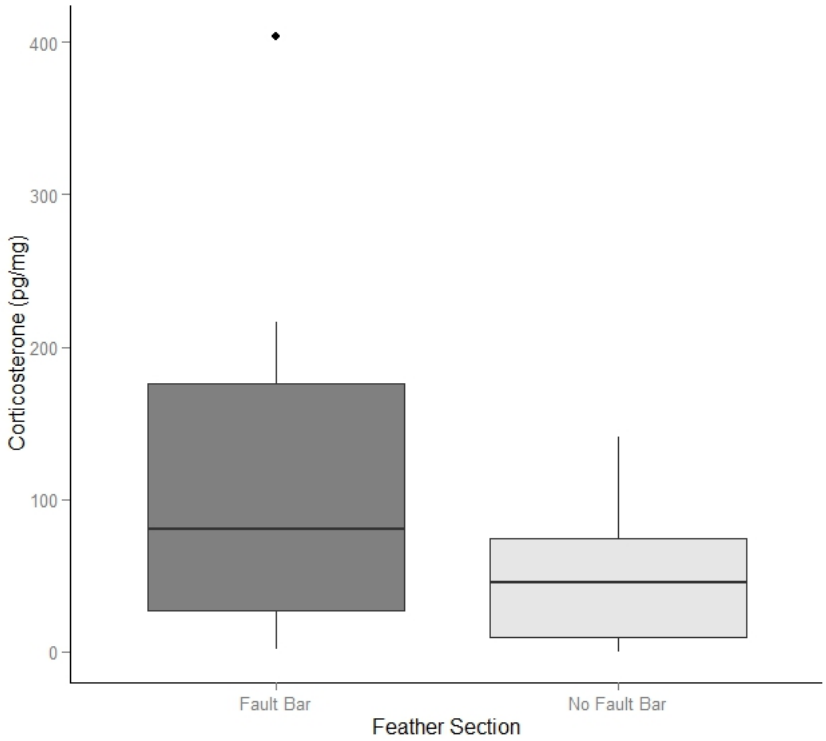


FIGURE 3.2: Corticosterone (pg/mg) in tail feather segments (12 mm) containing fault bars, or no fault bars. Fault bar segments are represented in dark grey, and non-fault bar sections in light grey. Median concentrations of corticosterone are shown with horizontal dark lines in boxes. Whiskers represent ± 1.5 times the interquartile range (distance from the fist to third quartile).

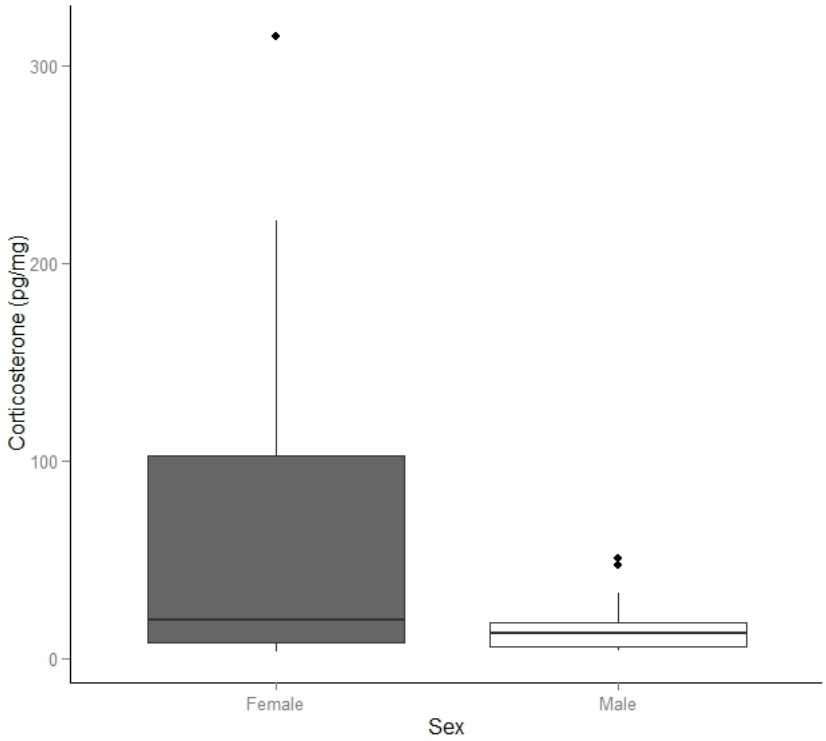


FIGURE 3.3: Corticosterone (pg/mg) deposited in rectrices of female and male Smooth-billed Anis (*Crotophaga ani*) respectively. Females are represented in dark grey, and males in light grey. Median concentrations of corticosterone are shown with horizontal dark lines in boxes. Whiskers represent ± 1.5 times the interquartile range (distance from the fist to third quartile).

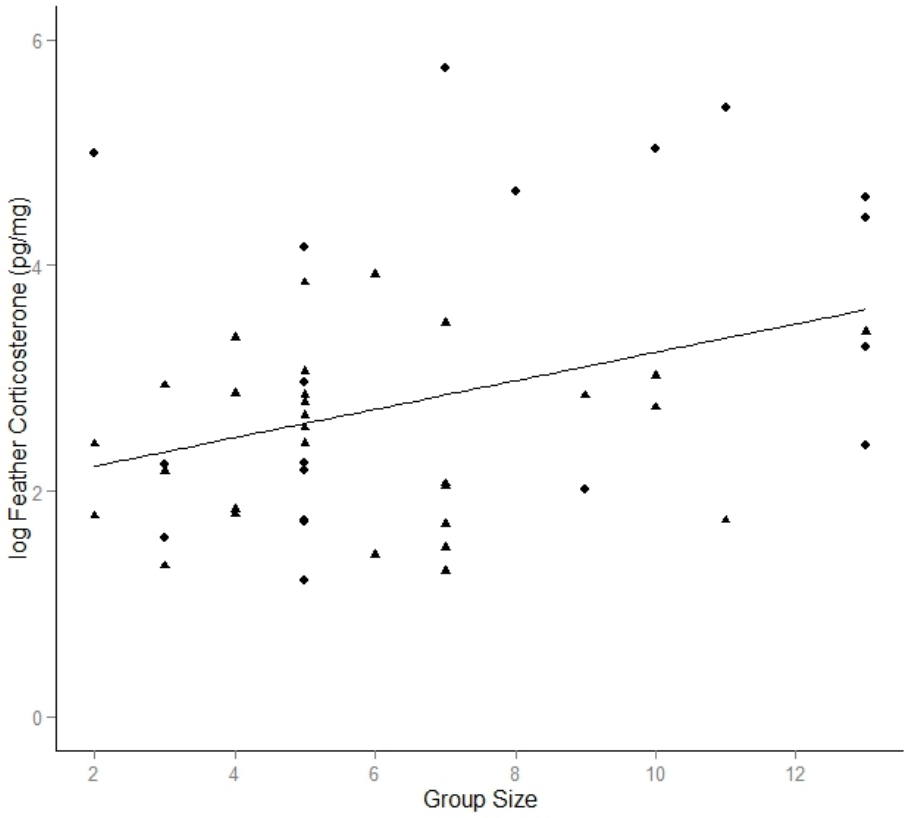


FIGURE 3.4: Log transformed corticosterone (pg/mg) deposited in rectrices of male and female Smooth-billed Anis (*Crotophaga ani*) according to social group size. Individual females are represented by closed circles and males by closed triangles. Trend line irrespective of sex is shown in black.

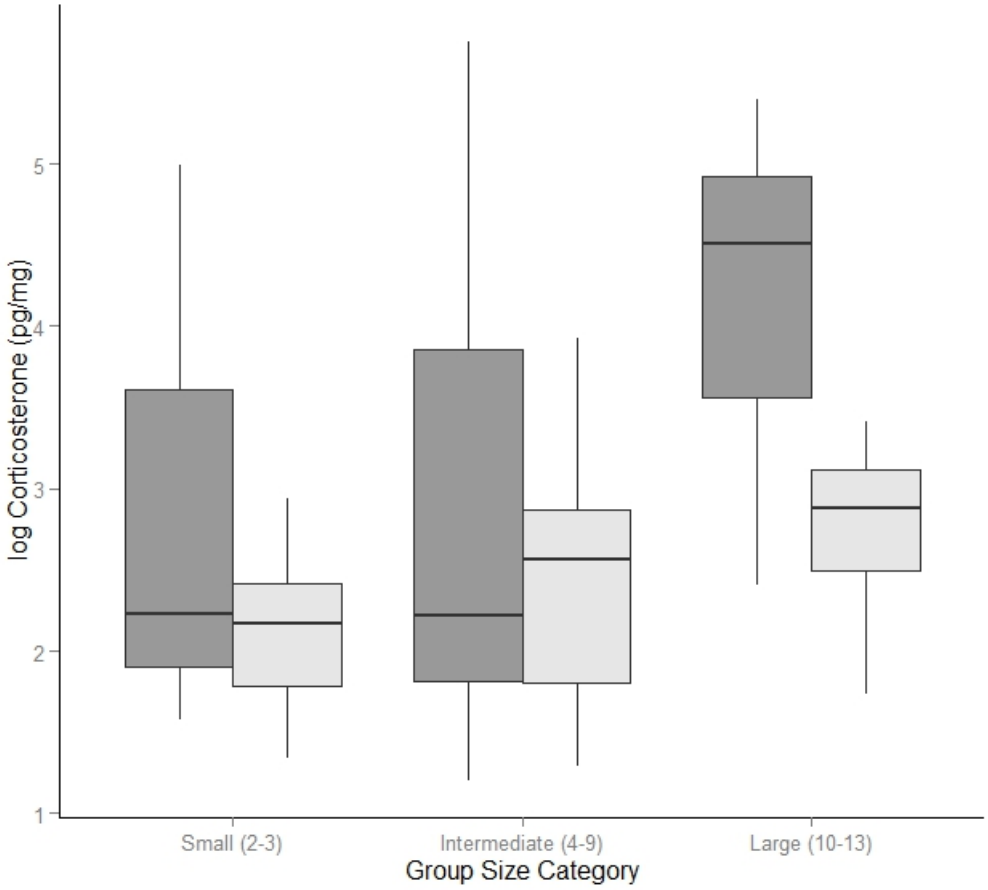


FIGURE 3.5: Log transformed feather corticosterone (pg/mg) of adult Smooth-billed Anis (*Crotophaga ani*) from small, intermediate and large groups respectively. Dark grey bars represent females and light grey bars represent males. Median concentrations of corticosterone are shown with horizontal dark lines in boxes. Whiskers represent ± 1.5 times the interquartile range (distance from the fist to third quartile).

A | Supplementary Figures

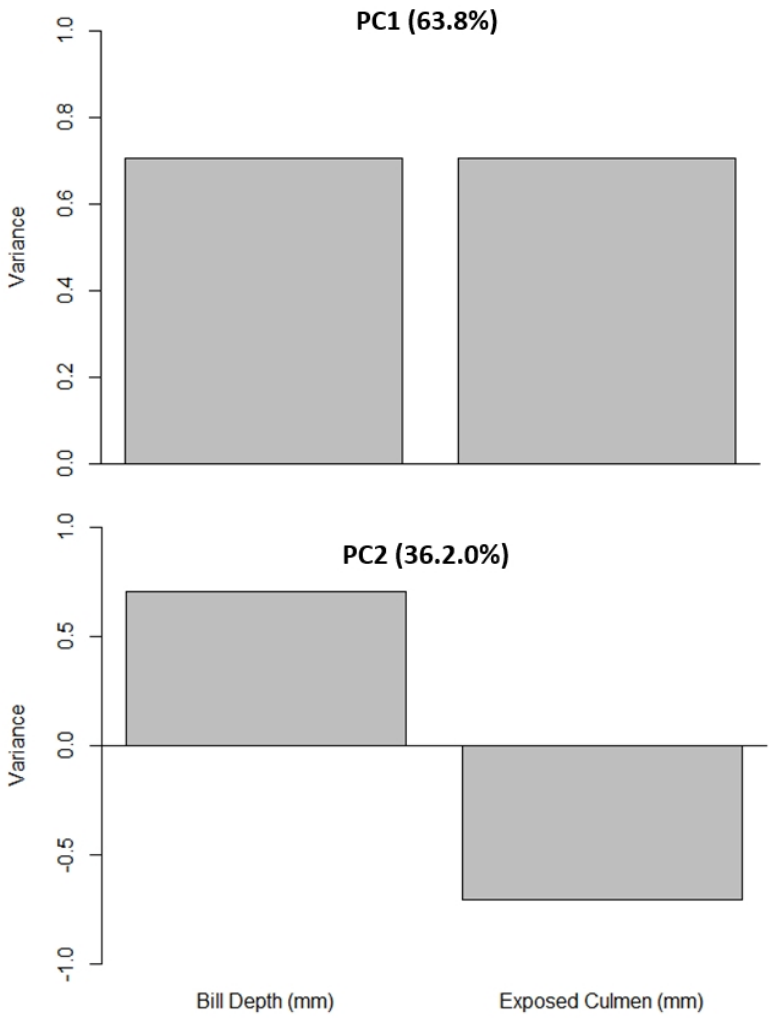


FIGURE A.1: Loading variables and their distributions in primary and secondary components for a principle component analysis (PCA) of Smooth-billed Ani (*Crotophaga ani*) body size. The primary component (PC1) explained 63.8% of loading variance and was used in the development of a multivariate metric of body size (Body Score).

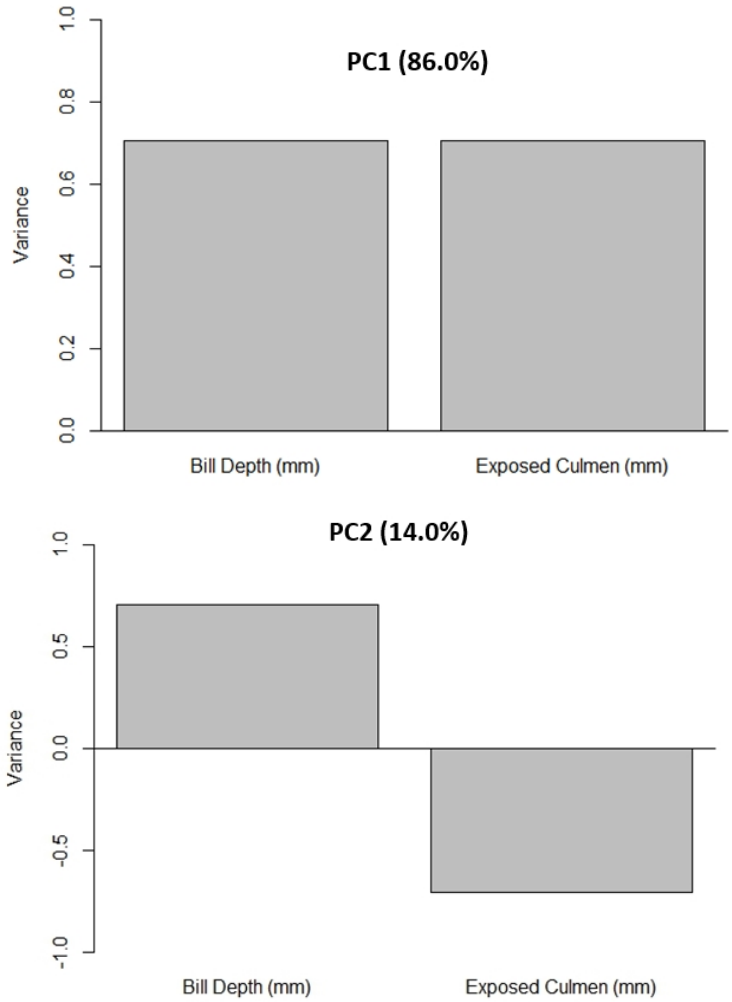


FIGURE A.2: Loading variables and according distributions in primary and secondary components for a principle component analysis (PCA) of Smooth-billed Ani (*Crotophaga ani*) bill size. The primary component (PC1) explained 86.0% of loading variance and was used in the development of a multivariate metric of bill size (Bill Score).

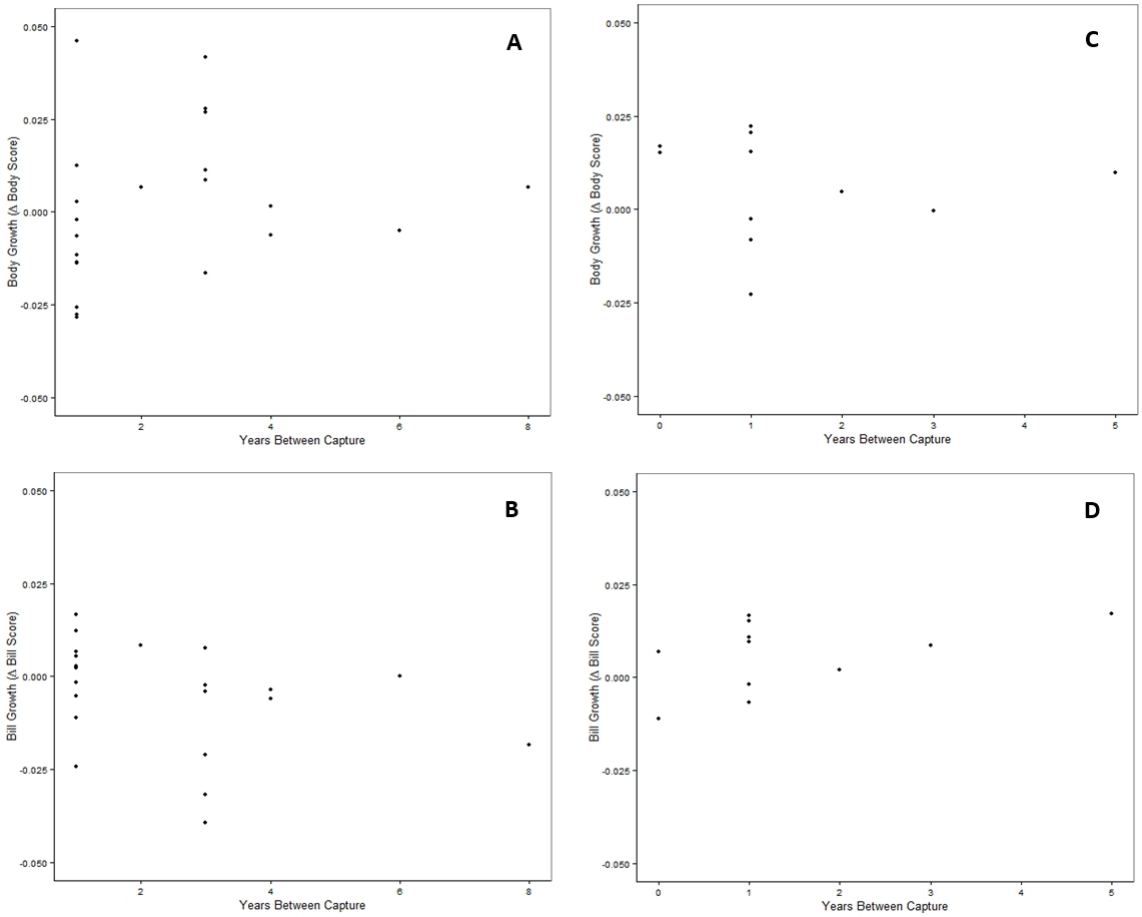


FIGURE A.3: Change in multivariate metrics of bill size (Bill Score) and body size (Body Score) between capture and recapture events of adult Smooth-billed Anis (*Crotophaga ani*). Panels A and B represent morphometric changes in adult females (n = 22), while panels C and D represent morphometric changes in adult males (n = 11).

B | Feather Corticosterone Extraction Recipes and Protocols

Recipes

Plate Coating Buffer

- 50 mM Sodium Carbonate (Na_2CO_3)
- 50 mM Sodium Bicarbonate ($NaHCO_3$)
- 1% Sodium Azide (NaN_3)

Dissolve 5.30 g of Na_2CO_3, 4.20 g $NaHCO_3$ and 10.00 g NaN_3 in 900 mL of ultra-pure H_2O. pH to 9.6 using pure NaOH and/or HCl and top to 1 L with ultra-pure H_2O. Store at 4°C.

10X Wash Buffer

- 1.5 M Sodium Chloride (NaCl)
- 0.5% Tween 20

Dissolve 87.66 g NaCl and 5.0 mL Tween 20 in 1 L ultra-pure H_2O. Store at 4°C. Dilute to 1x in ultra-pure H_2O immediately before use.

ELISA Assay Buffer

- 45.17 mM Sodium Phosphate Monobasic Anhydrous NaH_2PO_4)
- 61 mM Sodium Phosphate Dibasic Anhydrous (Na_2HPO_4)
- 148.87 nM NaCl
- 0.1% Bovine Serum Albumin (BSA)

Dissolve 5.42 grams of NaH_2PO_4 in 195 mL of H_2O and set aside (231.66 mM). In a new sterile beaker, dissolve 8.66 grams of Na_2HPO_4 in 305 mL of H_2O (200 mM). Combine NaH_2PO_4 and Na_2HPO_4 solutions and dilute with 500 mL ultrapure H_2O. Add 8.70 mg NaCl and 1.00 mg dried BSA. Stir at room temperature until dissolved. Adjust pH to 7.0 with HCl and/or NaOH. Store at 4°C for a maximum of one week.

Citrate Buffer

- 50 mM Citric Acid Anhydrous ($C_6H_8O_7$)

Dissolve 9.61 g $C_6H_8O_7$ in 900 mL ultra-pure H_2O. Adjust pH to 4.0 with NaOH and top to 1 L with ultra-pure H_2O. Store at 4°C.

ABTS Solution

- 42.75 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS).

Dissolve 0.55 g ABTS in 25 mL ultra-pure H_2O. pH to 6.0 with HCl and/or NaOH. Store at 4°C. ABTS degrades with light exposure, so wrap storage bottle in aluminum foil to reduce light damage.

Hydrogen Peroxide Solution

- 1.76% H_2O_2

Dilute 500 µl of 30% H_2O_2 in 8.0 mL ultra-pure H_2O. Store at 4°C.

Corticosterone Standard

- 0.1 mg/mL Corticosterone

Dilute 1.0 mg of pure corticosterone anhydrous (Steraloids Inc.) in 10 mL 100% methanol (MeOH). Vortex on high until dissolved. Parafilm cap to reduce evaporation and store at −20°C.

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Extraction Protocol

1. Gently wash feather under ultra-pure H_2O_2 with light rubbing for 1.0 minutes. This step is to remove fecal, blood and environmental contaminants. Tape feather by the calamus to a Kimwipe on a raised, rectangular platform and label. Place in fumehood until dry.
2. Gently wash feather for 15 seconds under a continuous stream of 100% methanol (MeOH) from designated squirt bottle. Ensure even washing. Label and dry as in step 1.
3. Remove the calamus with dissection scissors sterilized in 100% isopropyl alcohol.
4. Measure a 70 mm lateral section of feather beginning from the distal end using a sterile ruler or analogue calipers. Cut the feather laterally at 70 mm with sterile dissection scissors and set aside on Kimwipe. Return remaining proximal feather section to room temperature storage. If the feather is less than 70 mm in length, measure and document the full length of feather.
5. Cut feather sections in thirds with sterile dissection scissors, being careful not to lose barbs or barbules from fragmentation. Place feather sections in a new, sterile scintillation vial.
6. Dice feather sections with sterile dissection scissors inside scintillation vial to approximately 5 mm by 5 mm fragments.
7. Weigh feather sections in scintillation vial to the nearest hundredth of a mg.
8. Add 100% MeOH to scintillation vial at 1.0 mL per 5.0 mg of feather.
9. Seal scintallation vials with parafilm and vortex for 5 seconds on high.
10. Incubate at 50°C for 24 hours.
11. Carefully pipette MeOH-Corticosterone (CORT) extracts into new sterile 20 mL syringes with 22 µm syringe filters. Be careful not to disturb any feather fragments while pipetting. All fragments should remain in original scintillation vials. Rest syringe and filter atop new, sterile 25 mL scintillation vials but do not filter. Place aside.
12. Pipette 1 mL 100% diethyl ether into scintillation vials containing feather fragments. Seal with parafilm and vortex on high for 30 seconds.

13. Add ether-CORT extracts to MeOH-CORT extracts in syringes prepared in step 11. Filter together by slowly pressing syringe plunger into syringe. Discard syringe and filter.

14. Seal extracts with parafilm and store at $-20°$C until further use.

Sample Preparation

1. Place heat block in fumehood and preheat to 50°C.
2. Remove sample from −20°C.
3. Pipette 3.0 mL sample into sterile 8 mL scintillation vial and label.
4. Place scintillation vial with sample on heat block and remove cap. Keep cap in fumehood in labeled weigh-boat for future use.
5. Close fumehood sash, keeping approximately 5 cm space between the sash and base. This will increase face velocity and therefore increase the rate of sample evaporation.
6. Leave samples in fumehood until all liquid has evaporated.
7. Return cap to sample and seal with parafilm. Store at −20°C until use in enzyme-linked immunosorbent assay (ELISA).

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Enzyme-linked Immunosorbent Assay

Day One

Note: Repeater pipette syringes are expense. It is best to designate a single syringe per solution. Label each and wash each carefully before use with ultra-pure H_2O.

1. Pipette 10.0 mL of Plate Coating Buffer into a sterile test tube.
2. Pipette 1.0 µl anti-corticosterone (CORT) (Esoterix B3-163, Calabasas Hills, California, USA) into aliquot of Plate Coating Buffer and mix by pipetting with 12 mL glass pipette.
3. Using a repeater pipette, pipette 50 µl anti-CORT mixture into each well of a new 96 well NUNC maxisorb plate. The basic pH of the Plate Coating Buffer charges the antibody, allowing it to bind to the inside of the wells on the NUNC maxisorb plate.
4. Seal plate with a plastic plate cover and label.
5. Store plate at 4°C overnight (approximately 8-12 hours).

Day Two

1. Remove dried CORT extracts from −20°C storage and warm at room temperature for approximately 5-10 minutes.
2. Pipette 150 µl of ELISA Buffer into each scintillation vial containing dried sample extracts and vortex for 15 seconds. Let rest for at least 1 hour before use in ELISA.
3. Discard anti-CORT solution from plate and using a repeater pipette, wash each well with 100 µl of fresh 1x Wash Buffer. Repeat 5 times and pound dry on kimwipe.
4. With a repeater pipette, add 50 µl ELISA Buffer into each well and incubate for 2 hours. This step is essential to return the antibody to its active state where it is capable of binding to CORT substrate.
5. In a sterile test tube, dilute 10 µl corticosterone standard in 990 µl of 100% pure MeOH.

6. In another sterile test tube, dilute 100 µl 1:100 corticosterone standard in 900 µl of ELISA Buffer (1:1000 total dilution).

7. Serially dilute 1:1000 corticosterone standard sequentially twelve times in sterile test tubes to obtain a concentration gradient from 100 µg/mL to 48.8 µg/mL. Vortex each dilution on high for 5 seconds before continuing to the next.

8. In a sterile test tube, dilute 1.0 µl CORT-Horseradish Peroxidase (HRP) (Creative Diagnostics Inc.) in 10 mL ELISA Buffer and mix by pipetting with a 12 mL glass pipette. Set aside on ice.

9. In the first twenty-four wells (from A1 to H3, loading from top to bottom), pipette 50 µl of corticosterone standard beginning from 100 µg/mL and descending to 48.8pg/mL. Each dilution is run in duplicate to test for accuracy (for example, A1 and B1 will each contain 50 µl of 100 µg/mL corticosterone standard and G3 and H3 will each contain 50 µl of 48.8 pg/mL corticosterone standard.

10. In the remaining seventy-two wells, pipette 50 µl of CORT extract, reconstituted in ELISA Buffer. Again, each samples is run in duplicate, from top to bottom.

11. Using a repeater pipette, immediately pipette 50 µl of diluted CORT-HRP prepared in step 8 to each well.

12. Let the loaded plate stand at room-temperature for 1.5 hours. Here, unlabeled CORT will compete with HRP labeled CORT for binding to the CORT antibody coated to the bottom of the plate.

13. Wash the plate 5 times as per step 3 to remove excess, unbounded CORT. Pound dry on a kimwipe. Here, the plate is relatively stable dry, though some HRP activity will be lost with time. It is best to continue to step 14 as quickly as possible.

14. In a sterile 25 mL scintillation vial, combine 12.5 mL of Citrate Buffer, 40 µl of dilute H_2O_2 and 125 µl of ABTS solution. As ABTS degrades with light exposure, wrap the scintillation vial in foil and mix by vortexing on high for 15 seconds.

15. With a repeater pipette, pipette 100 µl of ABTS solution from step 14 to each well. Cover and shake at low velocity on a plate shaker, ensuring that no sample spills between wells.

16. Watch for colour change across wells. HRP catalyzes oxidation of ABTS, creating a blue-green colour. Higher quantities of CORT-HRP bound to anti-CORT will therefore generate a deeper colour than lower quantities of bound CORT-HRP. It is essential that optical density is measured when expected differences between wells are distinguishable. ABTS, however, is oxidized without HRP, though much more slowly. It is therefore equally essential that optical density is measured before oxidation is completed in all wells (when ambient ABTS oxidization overwhelms that catalyzed by CORT-HRP). Typically, the optimal time of plate shaking lies between 10-30 minutes.

17. Using a micoplate reader, measure optical density in each well at 405 nm.

18. Calculate average optical density between duplicate samples.

19. Plot concentrations of standard samples as a function of mean optical density and fit a logarithmic or exponential curve. Calculate an r^2 to interpret the accuracy of your fitted curve in predicting concentration by observed optical density. If the r^2 value of the curve exceeds 0.9, the equation of this line will be used to calculated CORT concentrations within each sample according to their measured optical density. If the r^2 value of the curve does not exceed 0.9, accurate interpolation is limited and it is best to return to day one.