MATERNAL EFFECTS IN A JOINT-NESTING BIRD

EGG-LAYING COMPETITION AND MATERNAL EFFECTS IN A PLURAL-BREEDING JOINT-NESTING BIRD

By

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THESIS ABSTRACT

I investigated the maternal effects that take place in a joint-nesting bird: the smooth-billed ani. Female anis were shown to respond to increasing group size by increasing the number of eggs produced per capita, by tossing and burying more eggs per capita, and by taking longer to reach the dedicated incubation phase. These results support the hypothesis that females respond to increased egg laying competition by trying to skew the contents of the final incubated clutch of eggs in their own favor.

I showed that in ani groups, yolk testosterone and estradiol deposited by females in eggs increased from early- to late-laid eggs. Increases in yolk steroid levels over the laying sequence may function to mitigate the disadvantage of being a later-hatched chick. This maternal influence may not be a mere reflection of a female's hormonal status as female plasma circulating levels of testosterone and estradiol did not vary in the same direction as yolk hormone profiles.

I showed that yolk corticosterone levels, an indicator of maternal physiological stress, increased with laying order in multi-female groups, but not in single-female groups. Results suggest that laying females experience higher levels of stress in multi-female groups. The above results suggest that communal life in anis generates competition and egg production waste that likely reduces short-term per capita reproductive benefits.

Female anis can vary egg quality via deposition of hormones in eggs, and also lay eggs of different sizes. I showed that circulating plasma testosterone levels were higher in

nestlings with better begging abilities. Furthermore, nestlings hatched from eggs laid late in the laying sequence had better begging abilities. These results suggest that testosterone is an important controlling mechanism of begging behaviour, and that female testosterone depositions in eggs have long lasting effects on offspring development and behavior.

PREFACE

This thesis consists of seven chapters. The first chapter is a general introduction, and the seventh is a synthesis and suggestions for future research. Chapters 2 through 6 have been written as manuscripts for publication in peer-reviewed scientific journals. At the time that this thesis was submitted, Chapters 2 and 3 were previously published (reprinted with permission). Chapters 4, 5, and 6 are in preparation for submission to the journals *General and Comparative Endocrinology*, *Journal of experimental zoology*, and *Animal Behaviour*, respectively. Information about the title, authors, and individual contributions to each of the chapters is detailed below:

Chapter 2: "Competition and waste in the communally breeding smooth-billed ani: effects of group size on egg-laying behaviour."

> Authors: G. Schmaltz, J. S. Quinn, and C. Lentz.
> Reference: Animal Behaviour, 2008, 76, 153 – 162.
> Contribution: field work was performed by the candidate under the guidance of Dr. J.S. Quinn. The statistical analyses and writing of the manuscript were conducted by the candidate with guidance and suggestions from the co-authors and Drs. S. Balshine, S.A. Dudley, and B.J. Stutchbury.

Chapter 3: "Do group-size and laying order influence maternal deposition of testosterone in smooth-billed ani eggs?"

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Chapter 4: "Maternal estrogens in eggs of a communal breeder: the smooth-billed ani (*Crotophaga ani*)."

Authors: G. Schmaltz, J. S. Quinn, and S.J. Schoech.
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Chapter 5: "Maternal corticosterone deposition in avian yolk: influence of clutch laying order and group size in a joint-nesting cooperatively breeding species."

Authors: G. Schmaltz, J. S. Quinn, and S.J. Schoech.
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Chapter 6: "Effect of relatedness and nestling testosterone on begging behaviour in the communally breeding smooth-billed ani."

Authors: G. Schmaltz, J. S. Quinn, S.J. Schoech, and J. Wright.
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I started). Hopefully, I have added a few pieces of my own to help solve the puzzle of the unusual ani mating system. There were so many people who helped with field and lab work that I do not know where to start. Among many others, I would like to thank C. Musters, S. Gregory, Luis Rosado, Ben Bravery, Manuel Cruz, Heather Darrow, Alana Demko, Jessica Eyster, and Robin Land for their help in the field. P. Sharma, Derek Ho, M. Sztajnmec, R.J. De Catanzaro, C.L. Carew, M. Spudic, and M. Beaucreux provided much appreciated help in the lab. Special thanks to all the folks in Puerto Rico at the Cabo Rojo National Wildlife Refuge for logistical support: Susan Silander, Joseph Schwagerl, Fernando Rodriguez, Mariano Gonzales, and James Padilla. Also thanks to Fred Schaffner for being such a reliable and knowledgable resource person in Puerto Rico.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Maternal Effects

The causes and consequences of individual phenotypic variation are of fundamental interest to biologists because these variations in physiological, morphological, and behavioural traits are the raw materials upon which natural selection operates. All individuals are a combination of a unique set of physiological, morphological, and behavioural traits. These unique combinations are the result of the interplay between the set of genes the individual inherited from its parents, and the environment in which the individual develops. Quantitative genetics studies demonstrate that an individual's phenotype is also influenced by the environmental condition experienced by conspecifics (Groothuis et al. 1995; Sinervo and Doughty 1996; Mousseau and Fox 1998). Such indirect genetic effects (IGEs) happen when the phenotype of one individual is influenced by the expression of genes in a different individual, which often happens to be the mother (Wolf et al. 1998).

Mothers can influence offspring phenotype in many different ways. First, mothers, through mate choice can select the best potential partner to transfer the best possible genes in a given environment to its offspring. In birds, parents then select the best potential nest site and mothers can delay egg-laying until offspring survival probabilities are high. Mothers can also determine the number of eggs that will be laid (for indeterminate laying species), the size of each egg, the amount of time that will be devoted to incubation, and the nestlings provisioning rate. Mothers can therefore strongly influence the environmental conditions its offspring will experience. If such maternal

effects influence offspring development and survival, they will be shaped by the action of natural selection (Price 1998; Mousseau and Fox 1998).

Maternal effects occur when the expression of genes from the mother, or the environmental conditions she experiences, are passed on to her offspring, and affects the phenotype of such offspring (Groothuis et al. 1995; Mousseau and Fox 1998). Maternal effects are important than changes later on in life because early maternal influences are more likely to lead to irreversible modifications on offspring phenotype. Furthermore, in oviparous species, embryos rely heavily on maternal information about environmental conditions to adjust their development since they cannot easily register these outside conditions. Maternal effects have received considerable attention in recent years and many studies are aiming at getting a better understanding of the adaptive significance of such maternal effects on offspring phenotype and fitness (the term: "adaptive transgenerational phenotypic plasticity" is sometime used to refer to these effects). Such effects are advantageous (adaptive) from the mother's perspective if she can increase her fitness by influencing the fitness of her offspring. Maternal effects in birds can be grouped into different categories: 1) prenatal effects on mate choice of offspring fitness, 2) prenatal effects of maternal egg-laying behaviour on offspring development and fitness, 3) prenatal maternal differential investment in eggs, 4) prenatal incubation behaviour, and 5) postnatal parental care.

1.2 Effects of Maternal Egg-Laying Behaviour in Joint-Nesting Birds

In many animals such as insects, fish, and birds, the number of eggs a female lays and the timing of egg-laying will influence the intensity of resource competition offspring face, and thus influences the growth and survival of such offspring (Wilson and Lessells 1994; Price 1998). In many species, females can vary the number of eggs laid to an optimum as predicted by optimality mathematical models (Wilson and Lessells 1994). For instance, game theoretical and other mathematical models predict that in avian species with intra-specific brood parasitism, parasites can achieve optimal fitness at a given number of eggs laid for a given host species (Maruyama and Seno 1999; Broom and Ruxton 2002). This maternal effect is pronounced in joint-nesting species where multiple females lay eggs in the same nest (Brown 1987; Vehrencamp & Quinn 2004). In most avian communal joint-nesters, and certain communally breeding mammals, life in groups creates competition among adults (Koford et al. 1990; Macedo 1992; Koenig et al. 1995; Gilchrist 2006). In birds, since more eggs are laid than can be successfully incubated to produce hatchlings, adults compete over which eggs will be incubated (Vehrencamp & Quinn 2004). In such plural-breeding, joint-nesting systems females should respond to increased group size and egg-laying competition by trying to skew the contents of the final incubated clutch of eggs in their own favor by tossing and/or burying competitors' eggs and by producing more of their own eggs.

1.3 Maternal Effects: Egg Size

Maternal effects on offspring development can take many forms. For instance, environmental maternal effects on seed dormancy in plants or probability of diapause in

insects are common (Roach and Wulff 1987; Mousseau and Dingle 1991). In many animals, offspring size at hatching is an important life history trait that influences an individual's survival probability and overall fitness (Clutton-Brock 1991; Roff 1992). Maternal effects on egg size have important fitness consequence for both mother and offspring. Since female fitness is a function of her offspring's reproductive success and survival, it pays females to invest resources in offspring (Bernardo 1996; Chambers and Leggett 1996; Sinervo and Doughty 1996), especially as increased investment has been showed to positively influence offspring fitness (Roff 1992; Williams 1994; Price 1998; Chambers and Leggett 1996; Mousseau and Fox 1998). As with the number of egg laid by a female, egg size should be an important maternal effect in joint-nesting species where nestmates of mixed parentage compete for survival. In such joint-nesting systems females may respond to increased group size and egg-laying competition by investing more in eggs to provide a competitive edge to their own offspring.

1.4 Maternal Effects: Hormone Deposition as an Adaptive Maternal Effect

Just as egg size, egg quality is an important maternal effect that can profoundly influence fitness in both mother and offspring (Bernardo 1996; Mousseau and Fox 1998). As in other oviparous animals, females in avian species must enclose all resources necessary for embryonic development in a self-contained package. Studies from several avian species indicate that egg yolks contain significant amounts of maternally derived hormones (Gil 2003; Groothuis et al. 2005; Schwabl 1993). Mothers can affect offspring phenotype and, therefore, influence the development of behavioral and life-history

strategies through this transmission of hormones from mother to offspring (Ketterson and Nolan 1999; West-Eberhard 2003). Testosterone, the best studied hormone deposited in eggs, has been found to vary with laying order and to affect chick begging behavior, growth, and social dominance among nestmates in many species (Gil 2003; Groothuis et al. 2005; Schwabl 1993, 1996a). For instance, increased testosterone levels in later-laid eggs resulted in increased mass of the hatching muscle (*musculus complexus*), which allows breaking out of the shell and subsequent flexion of the neck during begging, in red-winged blackbirds (*Agelaius phoeniceus*, Lipar and Ketterson 2000), as well as higher begging levels and growth rates in canaries (*Serinus canaria*, Schwabl 1996a) and black-headed gulls (*Larus fuscus*, Eising et al. 2001, 2003a).

Estradiol is another steroid hormone deposited in eggs that may be very important for both mothers and offspring. Estradiol can have a number of effects upon development, including sexual differentiation (Adkins-Regan et al. 1995; Balthazart & Adkins-Regan 2002), embryonic development (Balthazart & Adkins-Regan 2002; von Engelhardt et al. 2004), male embryonic mortality (Williams 1999; von Engelhardt et al. 2004), and nestling growth (von Engelhardt et al. 2004). Most studies on estradiol conducted to date suggest that elevated levels of yolk estrogen levels may be detrimental to the developing embryo (Adkins-Regan et al. 1995; Balthazart & Adkins-Regan 2002; Williams 1999); however, this does not appear to always be the case. In a recent study, when compared with untreated controls, estrogen-treated female zebra finches (*Taeniopygia guttata*), laid eggs that 1) were characterized by shorter incubation periods (suggesting increased rates of embryonic development) and 2) produced nestlings that

were heavier at day 7 (von Engelhardt et al. 2004). Although yolk estrogen levels were not assayed in the study, the authors argue that the above results were likely due to the transfer of estrogens to the egg as parental incubation behaviour was not affected by estrogen treatment (von Engelhardt et al. 2004). These results suggest that estrogen in egg yolks has anabolic effects upon offspring development that are similar to those of androgens (Lipar & Ketterson 2000). If so, females could influence offspring phenotype, nestling competition, and nestling survival via differential allocation of both androgens and estrogens to eggs.

Patterns of hormone deposition could provide females with a means to manipulate sibling competition within the clutch, thereby increasing offspring survival and their own fitness (Gil 2003; Groothuis et al. 2005). Specifically, females might adjust androgen and estrogen deposition depending on the magnitude of hatching asynchrony, a factor that often influences sibling competition and subsequent chick survival (Schwabl et al. 1997; Stoleson and Beissinger 1995). In species without adaptive brood reduction, increases in yolk hormone levels over the laying sequence may function to mitigate the disadvantage to later-hatched chicks (Groothuis et al. 2005; Lipar et al. 1999; Schwabl, 1993, 1996a). Because hatching asynchrony produces a size and motor-skills disadvantage for the later-hatched chicks, increases in steroid hormone levels with laying order would enable later-hatched chicks to make up their growth deficit. As with egg size, hormone deposition in eggs should be an important maternal effect in joint-nesting species where nestmates of mixed parentage compete for survival. In such joint-nesting systems females should

respond to increased group size and egg-laying competition by investing more in eggs to enhance the competitive abilities of their own offspring.

1.5 Maternal Effects: Detrimental Effects of Hormone Deposition

As highlighted above, maternal steroid deposition in eggs may give a competitive edge to offspring. For instance, hormone deposition may result in more intense begging later on in life (Eising et al. 2001, 2003a; Schwabl, 1996a). Recent studies suggest that these maternal effects may not be restricted to the period around hatching but influence offspring behavior and fitness during the entire lifespan. In black-headed gulls, prenatal testosterone injections resulted in offspring with higher levels of threat and courtship displays, aggression, and number of interactions won ten months after hatching (Eising et al. 2003b; Groothuis et al. 2005). However, hormone depositions are not always beneficial as prenatal testosterone injections in the same species resulted in lowered juvenile and adult survival (Groothuis et al. 2005). It is likely that there are costs associated with the production and/or exposure to high maternal hormone levels. If no costs were present, one would expect high levels of hormones in all laid eggs, and this is not the case (Groothuis et al. 2005). For instance, high physiological levels of testosterone have been associated with decreased immune function (Saino et al. 1995) and survival (Dufty 1989).

Since the environmental conditions experienced by a mother affects her physiology and in turn affects the offspring when factors are transferred into the egg (Gil 2003; Groothuis et al. 2005), it is important to track the internal physiological condition

of the mother during the egg production period. One hormone that enables us to do so is corticosterone. Various environmental perturbations, such as food shortage (Lynn et al. 2003), adverse weather conditions (Wingfield et al. 1983; Astheimer et al. 1995), human disturbance (Wasser 1997; Creel et al. 2002), and predatory encounters (Boonstra et al. 1998; Silverin 1998; Scheuerlein et al. 2001), can elevate plasma glucocorticoid levels in vertebrates. Individuals with high parasitic loads or low body condition also experience high baseline levels of glucocorticoids, such as corticosterone in birds, reptiles, and amphibians, or cortisol in fish and mammals (Breuner & Hahn 2003; Kitaysky et al. 1999 a,b; Love et al. 2005). Glucocorticoids trigger what are considered to be adaptive physiological and behavioral responses to stressful events that serve to bring the internal environment of the organism back to homeostasis (Silverin 1998). These hormones are, therefore, widely used to measure the response of an individual to stress (Broom & Johnson 1993; Von Holst 1998; Sapolsky et al. 2000; Downing & Bryden 2002). High levels of plasma glucocorticoids have been found to suppress reproduction, and affect both foraging and territorial behaviors (Wingfield et al. 1997; 1998; Wingfield & Kitaysky 2002; Salvante & Williams 2003). If the environmental conditions are not harsh to the point where an animal altogether abandons its reproductive effort, but are severe enough to result in elevated corticosterone levels, for example in a laying female, corticosterone transferred to yolk may influence offspring phenotype in a number of ways.

Prenatal exposure to maternal glucocorticoids can have negative effects both upon embryonic development (Mashaly 1991; Heiblum et al. 2001; Eriksen et al. 2003), as

well as longer-term effects upon other traits, such as offspring body mass (Love et al. 2005; Saino et al. 2005), growth (Hayward & Wingfield 2004; Heiblum et al. 2001; Love et al. 2005), sensitivity to stressors (Welberg & Seckl 2001; Hayward & Wingfield 2004), and behaviour (Lesage et al. 2004; Rubolini et al. 2005). Corticosterone deposition in the egg may link offspring development to maternal environmental conditions (Love et al. 2005; Saino et al. 2005). For example, female barn swallows (*Hirundo rustica*) exposed to a predator during egg-laying produced eggs with higher concentrations of corticosterone than controls (Saino et al. 2005). Further, embryos exposed to higher corticosterone levels were less likely to hatch, and eggs that did hatch produced young that at fledging were smaller (Saino et al. 2005). Therefore, stressful conditions experienced by mothers may influence egg quality and offspring phenotype, which, in turn, may negatively affect maternal fitness (Sinervo 1998; Rubolini et al. 2005; Saino et al. 2005). This is potentially very important in joint-nesting species as the increased egg-laying competition experienced by females in multi-female groups is likely to induce social stress (Abbott et al. 2003; Goymann & Wingfield 2004; Sapolsky et al. 2000; Wingfield et al. 1997).

1.6 Maternal Effects: Active Investment or Passive Diffusion?

There are many different lines of evidence (see above) that suggest that maternal effects are adaptive processes. However, to get a better understanding of the mechanisms involved in maternal effects, we need to learn how steroid hormones get into the egg. Such understanding is currently lacking. For instance, little is definitively known about

how corticosterone gets into the egg yolk and subsequently affects chick development (Gil 2003; Rettenbacher et al. 2005). Corticosterone is produced by the adrenals and likely enters the egg yolk from the maternal blood circulation. Yolk corticosterone levels should, to some degree reflect maternal plasma corticosterone levels as corticosterone, a lipid-soluble hormone, likely enters the yolk by passive diffusion (Hayward & Wingfield 2004; Groothuis et al. 2005; but see Rettenbacher et al. 2005).

Our understanding on how androgens and estrogens get into the egg is also very limited. In canaries, female plasma testosterone levels peak the day before the first egg is laid and then decrease until reaching a minimum about four days after the first egg is laid (Schwabl 1996b). However, in canaries, yolk testosterone levels increase with laying order (Schwabl 1993). Female plasma testosterone variations cannot therefore easily explain variations in yolk testosterone levels in that species. In other species, such as the black-headed gull, maternal blood testosterone levels correlate positively with testosterone egg yolk levels (Groothuis et al. 2005). As for androgens, we currently do not know whether females can adjust yolk estrogen levels independently of their own plasma levels as the handful of studies on the topic show opposite trends (Pilz et al. 2003; Groothuis et al. 2005; Williams et al. 2004).

1.7 Maternal Effects on Offspring Begging Behaviour

Begging displays of nestling birds are often seen as selfish attempts to influence parental food allocation. Models for the evolution and maintenance of begging behaviour predict that this parent-offspring conflict may be resolved by scramble competition

between nestlings, or by costly honest signalling of offspring need to parents (Godfray 1995; Mock and Parker 1997; Johnstone and Godfray 2002). Despite much research in this area, our understanding of the mechanisms that control begging behaviour during nestling competition remains limited. Such information may improve our understanding of the selective pressures shaping begging behaviour. Studies of maternal effects provide new insight, suggesting that steroid hormones are involved in the regulation of begging behaviour.

Studies from several avian species indicate that mothers can affect offspring begging through hormone deposition into eggs (Gil 2003; Groothuis et al. 2005; Schwabl, 1993; see above in section 1.4). For instance, increased testosterone levels in eggs have been linked to increased mass of the hatching muscle in red-winged blackbirds (Lipar and Ketterson 2000) and to higher begging levels and growth rates in canary (Schwabl, 1996a) and black-headed gull chicks (Eising et al. 2001, 2003). Results from these studies suggest that maternal testosterone depositions control testosterone production or some other regulatory mechanism of begging in nestlings. Patterns of yolk androgen deposition could therefore provide females with a means to manipulate nestling competition within the brood, thereby increasing offspring survival and maternal fitness (Gil, 2003; Groothuis et al., 2005). If adaptive, such investment should be especially relevant in many joint-nesting species, where brood reduction would not serve an adaptive function, and typically unrelated females should favour their own offspring.

Many studies have looked at the effect of maternal testosterone on offspring phenotype, but very little work has been conducted on the effect of maternal testosterone

on endogenous chick testosterone production, or on the effect of circulating plasma chick testosterone levels on begging behavior. Results differ in the few studies that have investigated the potential role of nestling circulating plasma testosterone as a control mechanism of nestling begging behavior. In white storks (*Ciconia ciconia)*, the first-hatched chicks, which had higher plasma testosterone levels, responded faster to feeding events and received more food (Sasvári et al. 1999). In pied flycatchers (*Ficedula hypoleuca*) and thin-billed prions (*Pachyptila belcherie*), nestlings with the most intense begging had the highest plasma testosterone levels (Goodship & Buchanan 2006; Quillfeldt et al. 2006). However, in black-headed gulls, testosterone-implanted chicks had suppressed begging compared to controls (Groothuis & Ros 2005).

1.8 Plural-Breeding Joint-Nesters and Study Species

In this study, I will investigate different maternal effects in the smooth-billed ani (*Crotophaga ani*), a member of the cuckoo family. Anis are plural-breeding joint-nesters that are socially monogamous and maintain territories during the breeding season (Quinn & Startek-Foote 2000). I studied smooth-billed anis at the Cabo Rojo and Laguna Cartagena National Wildlife refuges in south-western Puerto Rico (17°59'N, 67°10'W elevation from 2 to 42 m, and 18°01'N, 67°06'W elevation from 55 to 71 m, respectively) during the rainy season (September-January), when most ani breeding occurs. The south-western portion of Puerto Rico has a dry tropical climate. Both Cabo Rojo (587 acres) and Laguna Cartagena (794 acres) refuges contain second-growth dry

scrubland, open, and disturbed habitats that attract breeding anis (Loflin, 1983; Quinn and Startek-Foote, 2000). Anis breed at high enough densities at the two field sites (about one bird per 2 acres) to make it possible to obtain meaningful sample sizes. Ani group sizes can range from two to seventeen adults with up to five females laying eggs in the same nest (Quinn & Startek-Foote 2000). Group size is known to average 6.7 individuals in Florida (Loflin 1983) and females can lay from 3 to 7 eggs with an average of 5 eggs per female (Loflin 1983). Adult group members are generally not related to each other and cooperate in defending the territory and rearing young (Quinn & Startek-Foote 2000). As in other joint-nesters like guira cuckoos, acorn woodpeckers (*Melanerpes formicivorus*), and groove-billed anis (*Crotophaga sulcirostris*), smooth-billed anis compete for access to the incubated clutch of eggs by tossing eggs out of the nest (Loflin 1983; Quinn & Startek-Foote 2000; Haydock & Koenig 2002; Macedo et al. 2004; Vehrencamp & Quinn 2004). However, smooth-billed anis also compete by burying many eggs into the nest structure (Quinn & Startek-Foote 2000). In a study of 41 communal nests in Florida, 37% of eggs laid were tossed, while 38% of eggs laid were buried (Loflin 1983). Detailed information about the egg-laying, mating, social, and parental behaviour in the smooth-billed ani is not currently available. This thesis will provide additional information on the natural history of the species in order to build hypotheses that are biologically meaningful.

1.9 Thesis Objectives

Long-term field studies of maternal effects in vertebrates remain rare, and this is especially true in the study of cooperatively breeding birds. To date, various studies have looked at egg-laying competition in some species, and maternal hormone depositions in others, but more studies focussing on the different forms of maternal effects in a given species are required to get a better understanding of the adaptive significance of such maternal effects on offspring phenotype and fitness. Furthermore, we do have to focus not only on the short-term consequences of maternal effects, as most studies did, but also on the long-term consequences of maternal effects.

The primary objective of my thesis was to investigate the egg-laying competition and maternal effects that take place in a plural-breeding joint-nesting bird. Most of my experiments and observations were carried out for four consecutive years (2001-2004) under natural field conditions in order to draw general conclusions about the questions being tested. I have also tried to minimize the confounding effects of various environmental variables and year effects by conducting controlled experiments on nestling begging behaviour and by applying various statistical tools to control such effects.

As a common theme throughout this thesis, I focused on the potential of mothers to adjust **adaptively** the development of their offspring as this is a key topic of interest currently being investigated in this field of research. I also paid special attention to two important variables throughout this thesis: laying order and group size. Since anis are plural breeders that live in groups of various sizes, group size is likely to be a very important variable that will affect many other variables under study. Similarly, laying

order effects are likely to be very important in anis since many eggs, especially the ones laid early, are likely to be tossed or buried. Below, I will provide a brief introduction for each chapter. More specific introductory material is included in each data chapter.

In **chapter 2**, I investigated the egg-laying behavior in anis. Specifically, I examined the influence of group size on egg-laying behaviour. I proposed a new hypothesis, the competitive female egg-investment hypothesis, to try to explain such potential effect of group size on egg-laying behaviour. This hypothesis states that females in multi-female groups respond to increased group size and competition by trying to skew the contents of the final incubated clutch of eggs in their favour by tossing and/or burying competitors' eggs while producing more of their own eggs. As such, I predicted that both the number of eggs produced per capita and the number of eggs lost per capita should increase with increasing group size. There are two obvious reasons as to why I needed to investigate egg-laying behaviour first: 1) I needed to get a better understanding of the natural history of the study species in order to make testable predictions in the following chapters, and 2) number of eggs laid and timing of egg-laying are important factors under maternal control that influence both maternal and offspring fitness.

In **chapters 3 and 4**, I examined whether egg size and egg quality are important maternal effects in anis. Specifically, I investigated whether patterns of hormone deposition could provide females with a means to manipulate sibling competition within the clutch, thereby increasing offspring survival and their own fitness. I tested the predictions of the hatching asynchrony adjustment hypothesis which states that increases in yolk hormone levels over the laying sequence function to mitigate the disadvantage to

later-hatched chicks in species without adaptive brood reduction. I predicted that testosterone and estradiol levels should increase with egg-laying order to mitigate the disadvantage of being a later-laid egg. I also tested the predictions from a second hypothesis, the competitive female hormone investment hypothesis. This hypothesis states that competition among unrelated nestlings in multi-female groups should select for increased hormone deposition in eggs to enhance the competitive abilities of an individual's offspring. I predicted that yolk hormone levels should be higher in multi-female groups when compared to single-female groups. Additionally, in chapter 4, I looked at the relationship between egg size and estradiol levels in females to evaluate whether females could potentially adjust egg size with laying order. Finally, in chapters 3 and 4, I aimed at getting a better understanding as to whether maternal sex steroid hormone deposition in eggs is a passive or active process. I did so by comparing egg hormonal data to hormonal profiles in females.

As highlighted above, maternal steroid deposition in eggs may give a competitive edge to offspring. It is likely that there are costs associated with the production and/or exposure to high maternal hormone levels. **Chapter 5** examines some of the maternal physiological costs that might be related to the intense competition mothers face during egg-laying. Maternal physiological status is important to the offspring because stressful conditions experienced by mothers may influence egg quality and offspring phenotype (Sinervo 1998; Rubolini et al. 2005; Saino et al. 2005). Here, I hypothesize that the increased egg-laying competition experienced by females in multi-female groups creates a physiological stress that is reflected in the corticosterone profiles of their eggs. I

therefore predicted an increase in yolk corticosterone levels from single female to multi-female groups. Furthermore, because reproductive costs and physiological stress likely increase during egg production (Salvante & Williams 2003; Williams 2005), I predicted to find an increase in yolk corticosterone levels from the early to the late egg-laying period.

Results from different studies suggest that yolk androgen depositions influence offspring begging behaviour (Gil, 2003; Groothuis et al., 2005). If so, maternal effects would have long lasting effects on offspring phenotype. However, it remains unclear how prenatal maternal exposure to androgens positively affects begging behavior within broods. **Chapter 6** describes an experiment under controlled single-chick conditions to determine the influence of circulating plasma testosterone levels on begging behaviour. If testosterone is an important controlling mechanism of begging behaviour, I predicted that nestlings with higher testosterone levels should have better begging abilities than nestlings with low testosterone levels. Additionally, I investigated for the first time the importance of nestling relatedness levels on nestling begging behaviour. Given that group members are typically unrelated to each other in multi-female nests, I expected to see high levels of nestmate competition and low levels of relatedness among nestlings in those groups. I predicted that nestling begging abilities should be highest in such nests where average relatedness values are low.

Lastly, in **chapter 7**, I provided a synthesis of the results described in this thesis, and sought to put these findings in perspective to results obtained in other studies. I also provided further insight into issues remaining for future research.

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CHAPTER 2

Competition and waste in the communally breeding smooth-billed ani: effects of group size on egg-laying behaviour

Running headline: Competition in a communally breeding bird, Schmaltz et al.

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We investigated the effects of group size on egg-laying behaviour in the communally breeding joint-nesting smooth-billed ani, *Crotophaga ani*. We tested the predictions of the competitive female egg-investment hypothesis, which states that females in plural-breeding, joint-nesting systems respond to increased group size and egg-laying competition by trying to skew the contents of the final incubated clutch of eggs in their own favor by tossing and/or burying competitors' eggs and by producing more of their own eggs. Results supported the predictions of the hypothesis as both the number of eggs produced per capita and the number of eggs lost per capita increased with increasing group size. Egg tossing and burial behaviors were almost entirely restricted to multifemale groups and 56% of the 829 eggs laid in communal nests were lost to either egg tossing or burial. As a consequence of this egg-laying competition, the number of eggs incubated per capita decreased with increasing group size. Large groups laid more eggs and took more time to synchronize laying compared to smaller groups. Finally, we found that chicks hatched late within a communal clutch were more likely than earlier hatching chicks to die during the first 5 days of life. We conclude that communal life in anis generates competition and egg production waste that reduces short-term per-capita reproductive benefits. Long-term data are needed to clarify individual benefits associated with communal breeding in this species.

Keywords: asynchrony; communal breeding; competition; *Crotophaga ani*; group living; laying synchrony; offspring mortality; smooth-billed ani

The determinants of clutch size in animals have fascinated ecologists for over six decades and the theory of optimal clutch size has generated much interest and research in behavioural ecology since its inception. Lack (1947, 1954) proposed that clutch size should reflect the maximum number of young that adults can raise. In certain communally breeding mammals, communal rearing allows mothers to invest in pups at a reduced cost since rearing is shared (Hayes & Solomon 2004). In birds, most questions related to optimal clutch size theory have centered on productivity of a single clutch of eggs, but some have looked at the influence of clutch size on lifetime reproductive success (Monaghan & Nager 1997). Clutch or litter size has been found to vary with environmental factors such as food availability (Hussell & Quinney 1987; Bolton et al. 1993; Hayes & Solomon 2004), latitude (Young 1994; Fargallo & Johnston 1997), seasonality (Lack 1954; Crick et al. 1993), predation (Lima 1987), and female quality (age: Afton 1984; body condition: Bolton et al. 1993; breeding experience: Klomp 1970). Most studies of avian clutch size to date have focused on species in which females nest singly or with their mate. In communal joint-nesting species, two or more females lay eggs and raise young in the same nest (Brown 1987; Vehrencamp & Quinn 2004). In most communal joint-nesters, and certain communally breeding mammals, life in groups creates competition among adults (Koford et al. 1990; Macedo 1992; Koenig et al. 1995; Gilchrist 2006). Since more eggs are laid than can be successfully incubated to produce hatchlings, adults compete over which eggs will be incubated (Vehrencamp & Quinn 2004). In such groups, females benefit by adjusting clutch size and egg-laying behaviour to maximize their own fitness. In guira cuckoos, *Guira guira*, where up to seven females

share a nest, competition is manifested through egg tossing and nestling infanticide (Macedo & Melo 1999; Cariello et al. 2002). Eggs laid early during the nesting effort are typically tossed, but egg loss also occurs throughout egg-laying and incubation (Macedo 1992; Macedo et al. 2004a). Such egg losses and infanticide can occur when females breed alone, although the probability is low (Macedo 1992; Macedo et al. 2004a).

Smooth-billed anis, *Crotophaga ani*, are communal joint-nesters that are socially monogamous (Quinn & Startek-Foote 2000). Ani group sizes can range from two to 17 adults with up to five females laying eggs in the same nest (Quinn & Startek-Foote 2000). Group size is known to average 6.7 individuals in Florida (Loflin 1983) and females can lay from 3 to 7 eggs with an average of 5 eggs per female (Loflin 1983). Adult group members are generally not related to each other and cooperate in defending the territory and rearing young (Quinn & Startek-Foote 2000). As in guira cuckoos, acorn woodpeckers (*Melanerpes formicivorus*), and groove-billed anis (*Crotophaga sulcirostris*), smooth-billed anis compete for access to the incubated clutch of eggs by tossing eggs out of the nest (Loflin 1983; Quinn & Startek-Foote 2000; Haydock & Koenig 2002; Macedo et al. 2004a; Vehrencamp & Quinn 2004). However, smooth-billed anis also compete by burying many eggs into the nest structure (Quinn & Startek-Foote 2000). In a study of 41 communal nests in Florida, 37% of eggs laid were tossed, while 38% of eggs laid were buried (Loflin 1983). In groove-billed anis, communal joint-nesting females are thought to perform most of the egg tossing/burial (Vehrencamp 1977) although the contribution of each sex has yet to be quantified. Apparently, in Crotophaginae, individual females stop destroying eggs when they start laying, possibly

to avoid mistakenly destroying their own because they do not seem to recognize their own eggs (Vehrencamp 1977; Cariello et al. 2004).

In this study, we examine smooth-billed ani group size influences on egg-laying behaviour and competition. The main objective of this paper is to determine if the number of laying females i.e., female group size significantly affects nesting variables such as breeding synchrony, number of eggs laid, and egg loss probabilities within the laying sequence. Given such effects we examine how individuals adjust their egg-laying behaviour in response to the heightened competition and conflict.

We propose a new hypothesis, the competitive female egg-investment hypothesis, to try to explain the effect of group size on egg-laying behaviour. This hypothesis states that females in multi-female groups respond to increased group size and competition by trying to skew the contents of the final incubated clutch of eggs in their favour by tossing and/or burying competitors' eggs while producing more of their own eggs. This hypothesis predicts different female strategies in single versus multi-female groups:

1) The number of eggs produced per capita should increase with the number of females per group (female group size).
2) The number of eggs lost to tossing and burial per capita should increase with female group size.
3) Egg tossing and burial should be restricted to multi-female groups.

METHODS

Study sites

We studied smooth-billed anis at the Cabo Rojo and Laguna Cartagena National Wildlife refuges in south-western Puerto Rico (17°59'N, 67°10'W elevation from 2 to 42 m, and 18°01'N, 67°06'W elevation from 55 to 71 m, respectively) from 2000 to 2004 during the rainy season when most ani breeding occurs (September-January). The south-western portion of Puerto Rico has a dry tropical climate and both Cabo Rojo (587 acres) and Laguna Cartagena (794 acres) refuges contain second-growth dry scrubland, open, and disturbed habitats that attract breeding anis (Loflin 1983, Quinn & Startek-Foote 2000).

During the five year study, we followed the fate of 41 nests at Cabo Rojo and 18 nests at Laguna Cartagena. Those 59 nest-years represent a total of 38 different breeding locations. The majority of nests under study were found in 2003 and 2004 (21 and 18 nests respectively). These two field sites are ideally suited to test the predictions of the female egg-investment hypothesis since both single and joint-nesting females breed within the same population at our two locations.

Banding and group size determination

Adults were captured using two 1.9 cm mesh mist nets (18 m length) set one directly above the other on 7.2m telescoping poles (Meyers & Pardieck 1993) or using a nest trap (Mock et al. 1999), or using a hardware cloth funnel trap containing a caged hand-reared lure bird placed within a group's territory before or during nesting (Vehrencamp 1977).

After capture, birds were blood sampled, measured, and banded with a unique combination of one numbered metal and three colour bands. For the 59 groups under study, adult group size was counted before, during, and after nesting effort, recording both total number of birds and number of banded and unbanded adults whenever visible during each visit to the group's territory (typically every other day). For ani groups where all group members were caught (n= 16), female group size was determined by the analysis of blood samples that were sexed by amplifying an intron in the Chromo-Helicase-DNA (CHD) binding gene (Griffiths et al. 1998). For the other groups (n= 43), female group size was calculated by dividing adult group size by two since anis exhibit pairing behaviour and are socially monogamous within groups (Quinn & Startek-Foote 2000). In case of odd group sizes, female group size was assigned to the lower round number (i.e group size of 11 birds contained an estimate of 5 females). This approach matched the data for fully sampled groups (n= 16) with a slight male bias 1.19:1 (M:F) also found elsewhere for anis (Davis 1940; Skutch 1959). Four groups under study had two nests with a full clutch within a breeding season. Since group composition did not change within the breeding season for those four groups we averaged the data from the two nests (i.e. number of egg laid, buried, tossed) for those groups. Throughout this paper, adult female group size refers to the estimated or determined number of laying females in the group. Each adult female group member was considered to be a breeder, as nonbreeding individuals (helpers) are typically restricted to juveniles (Loflin 1983; Quinn & Startek-Foote 2000). The fact that communal clutch size is highly correlated with the number of laying females (see results) further suggests that most, if not all, adult females

are breeders. For any given smooth-billed ani nesting group, membership and group sizes were different from year to year. As well, when the same general area was used from one year to the next, territory size and shape changed (C. Lentz, J. Quinn & G. Schmaltz, unpublished data). To confirm that groups could be treated as independent units, the proportion of dyads that remained together was calculated at 12 different locations in two years at the Cabo Rojo refuge. We took into consideration the change in group size as well as adult composition. We calculated this proportion of shared dyads for the transition from 2003 to 2004 as these two years represent the majority of nests under study (66% of all nests). An average of 62% of all birds present at the Cabo Rojo site during the 5 year study were banded in any given study year. Between the 2003 and 2004 breeding seasons, the proportion of shared dyads that remained together at Cabo Rojo ranged from a minimum of 7% (if we consider that all unbanded birds in a group dispersed from one year to the next) to a maximum of 20.4% (if we consider that all unbanded birds in a group remained from one year to the next) (See appendix for details on the calculations). Groups were considered as independent data points as the above estimates were low and likely did not influence the findings in this study.

Nest checks

In south-western Puerto Rico, anis use predominantly two species of thorned trees, mesquite (*Prosopis pallida*) and rólon (*Pithecellobium dulce*), for nesting, and do not reuse old nests (Quinn & Startek-Foote 2000). Nests were found by following adult group members and by checking trees for recent nest structures. Each nest under study was

checked regularly (typically every third day, range 1-5) until egg-laying started. Eggs were sequentially marked as laid with non-toxic markers to indicate egg number in the laying sequence. Nest contents were checked daily until egg-laying stopped and eggs felt warm (i.e. incubation started). In cases where two or more new eggs were discovered the same day, eggs were assigned the same numbers in the laying sequence. Nest checks were resumed two days prior to the anticipated first hatching date until the youngest (latest hatched) chick reached 5 d old. During each visit, we recorded the number of each egg present in the nest as well as the identity of newly tossed eggs. At the end of the nesting effort (nestlings fledged or nest was predated after incubation started), we took apart each nest to recover and record eggs that had been buried in the nest structure. In this study, we only included nests that were not predated before egg-laying stopped and incubation started. We were able to follow the fate of each marked egg as tossed, buried, or incubated as no eggs disappeared from the nest vicinity after eggs were marked.

Data and statistical analysis

We calculated an order index for each egg within the communal clutch to determine the effect of laying order on egg loss probabilities independent of group size. To do so, we divided the position of a given egg in the clutch by the total number of eggs laid (after Macedo et al. 2004a). For instance, the second egg laid in a clutch of 10 eggs would have an index score of 0.2. Therefore, this order index gives us an estimation of how early (index values closer to 0) or how late (index values closer to 1) an egg was laid within the clutch independent of clutch size. The probability of loss of eggs through tossing was

calculated for each 0.1 increment of this order index by dividing the number of eggs tossed by the total number of eggs laid in that increment for all clutches for which we knew each egg's position in the laying order. The probability of loss through burial was calculated similarly. We excluded single breeding female groups from this analysis, as both tossing and burial events are rare to nonexistent in those groups (see results). For 23 groups (446 eggs), we were able to determine the date of laying for each egg (including first egg laid in the clutch) and to calculate the total duration of egg-laying. For each of these 23 groups, we calculated the standard deviation of egg-laying based on laying dates as an estimate of hatching asynchrony, lower standard deviation values indicating more synchronous egg-laying (Emlen & Demong 1975). For joint-nests from 2003 and 2004 (17 of the 23 nests), we also monitored nests during incubation (nests visited every 4 days on average) to determine whether egg burial also occurred after incubation started and egg-laying ended. We also calculated an order index for chicks using a similar approach as above for the eggs by dividing the position of a given chick in the clutch by the total number of hatched chicks. For instance, the second chick hatched in a brood of 10 chicks would have an index score of 0.2. We calculated this index to determine the effect of hatching order on daily chick mortality probabilities. These daily mortalities were calculated based on the Mayfield method (Mayfield 1975) from daily nest checks from the time the first chick hatched until the time the last hatched chick was about 5 d old. For this analysis, we included only the 23 study nests in the sample in which at least 5 chicks hatched. This sub sample of all nests is different from the 23 nests selected for the calculation of the standard deviation of egg-laying (see above) as nest selection is based

on different criteria for these two analyses. Finally, to determine whether late-hatched chicks came predominantly from late-laid eggs we analyzed the relationship between egg-laying order and chick-hatching order in 13 nests (101 chicks) where at least 5 chicks hatched and where we could assign the provenance of each chick to its respective egg.

The number of eggs laid per female and the number of eggs tossed and buried per female did not differ significantly between groups of 4 and 5 females. These two group sizes were pooled for increased statistical power as sample sizes for those two categories were low (n = 6 and 5 respectively). We used nonparametric statistics as some important variables were not normally distributed (number of females, number of eggs tossed and buried). We used Spearman rank correlations to examine relationships between female group size and different nesting variables including nesting asynchrony, number of days laying, eggs laid, tossed, and buried. We used Mann-Whitney U tests to look for differences between single and multi-female groups and Kruskal-Wallis tests to determine whether different group size classes differed from one another for a given egg-laying variable. When such differences were found, we used the multiple comparisons between treatments method (Siegel & Castellan 1988) as a nonparametric post-hoc test to detect which group size classes differed significantly. All tests were two-tailed with an α of 0.05 and were performed in SPSS 10.0 (SPSS Inc.). Averages are provided with standard errors (SE).

RESULTS

The number of eggs per female varied from 3 to 13 eggs with an average of 6.4 eggs per female ($N = 59$; ± 0.3). Females in single female groups laid 3 to 7 eggs (5.3 ± 0.3; $N = 19$) eggs whereas females in multi-female groups laid from 3 to 13 eggs (6.8 ± 0.4; $N = 40$). This female per capita number of eggs laid was significantly different in single *versus* multi-females groups (Mann-Whitney U test, $N = 59$, $Z = -2.46$, $P = 0.01$) and in the different group size categories which ranged from 1 to 5 females with an average of 2.3 ± 0.2 females per nest: (Kruskal-Wallis test: $H_3 = 9.1$, $P = 0.03$; Fig. 1). The per capita number of eggs laid increased with number of females ($r_s = 0.39$, $N = 59$, $P = 0.002$) until reaching a plateau at 4-5 females (multiple comparisons between treatments method conducted for all category combinations, with the only significant difference being between group sizes of 1 female Vs 4-5 females; Fig.1). Females in multi-female groups laid on average 1.5 more eggs than their singly nesting counterparts (Fig.1). However, we also found that in 10% of all communal nests (4/40), some females likely double brooded as the number of eggs per capita (10-13 eggs) and the total number of days laying in those nests were high (24 days on average compared to 16 days on average in other multi-female nests of similar group sizes). Excluding those 4 nests from the analysis did not change our conclusions as the female per capita number of eggs laid was still significantly different in single *versus* multi-females groups (Mann-Whitney U test, $N = 55$, $Z = -2.02$, $P = 0.04$) and the per capita (female) number of eggs laid increased with female group size ($r_s = 0.33$, $N = 55$, $P = 0.01$). Communal clutch size was highly

correlated with the number of laying females (Spearman rank test: r_s= 0.91, N= 59, P < 0.001; Fig.2) and the number of eggs present in a nest increased by an average of 9.2 eggs (± 1.1) for each group size increment of one female (Fig.2).

During this study, 68% (40/59) of the groups contained two or more joint-nesting females. In the 40 communal nests, a total of 829 eggs were laid, 462 of which ended up being tossed or buried (56% of all eggs laid). The average loss of eggs per capita in multi-female nests was 3.4 eggs due to tossing and burial. As a result, the per capita number of eggs incubated in multi-female groups was 3.4 (± 0.2) versus 5.2 (± 0.3) eggs incubated per capita in single female groups. This female per capita number of eggs incubated was significantly different in single *versus* multi-females groups (Mann-Whitney U test, N = 59, Z = -4.04, P < 0.001). The number of eggs incubated per capita significantly decreased with increasing group size (r_s= -0.71, N= 59, P < 0.001). As number of females increased, so did the number of tossed and buried eggs per female (r_s= 0.80, N= 59, P < 0.001). The probability of egg burial differed significantly by group size categories (Kruskal-Wallis test: H_3 = 146.7, P < 0.001; Fig.3). The probability of egg burial increased with group size and all groups were significantly different from one another (multiple comparison between treatments method conducted for all category combinations, with all pairwise comparisons being significant; Fig.3). We observed no tossing and very low levels of burial in single female groups (Fig.3); burial only occurred in one of 19 single female nests. When tossing occurred, there were no significant differences in tossing probabilities in the different multi-female group sizes (Kruskal-Wallis test: H_2 = 2.0, P = 0.36; Fig.3). For 39 nests in which chicks survived until they

were old enough to be banded (i.e. 4-5 d old chicks), the per capita number of chicks in multi-female groups was 2.1 (± 0.2) versus 3.5 (± 0.5) chicks in single female groups. This female per capita number of chicks was significantly different in single *versus* multi-females groups (Mann-Whitney U test, $N = 39$, $Z = -2.76$, $P = 0.006$).

Egg tossing in multi-female groups happened predominantly early during egg-laying (independent of clutch size) with the very first eggs laid being more likely to be tossed (Fig.4a). Egg tossing probabilities dramatically decreased and no eggs were tossed during the second half of egg-laying (Fig.4a) or during the 14 d incubation period. Egg burial probabilities were high for most of the egg-laying sequence (ranging from 30 to 95%) only tailing off to about 15% for the very last laid eggs (Fig.4b). Egg burial probabilities were highest for larger female group sizes throughout egg-laying ((Kruskal-Wallis test: $H_2 = 11.27$, $P = 0.004$; multiple comparison between treatments method conducted for all three category combinations, with all pairwise comparisons being significant; Fig.4b). Even toward the very end of egg-laying, the egg burial probabilities were high in 4-5 female groups (Fig.4b). Egg burial rarely occurred during incubation. In four of 17 nests monitored, one egg ended up buried after the beginning of incubation. In communal groups, the first eggs laid in a nest were almost certain to be lost through egg tossing or burial. Probabilities of loss are almost 100% for eggs laid early in the egg laying sequence (Fig.4c). This effect is even more pronounced and lasts longer in the laying sequence in larger groups. For example, the probabilities of loss are close to 100% for the first 40% of eggs laid in groups of 4-5 females (Fig.4c).

Egg-laying competition increased with female group size, affecting timing and duration of egg-laying. The number of buried and tossed eggs significantly increased with female group size (see above) but also with the degree of hatching asynchrony (higher standard deviation values) (r_s= 0.69, N= 23, $P < 0.001$). The total laying duration also increased with female group size (r_s= 0.72, N= 23, $P < 0.001$). Daily chick mortality probabilities were affected by hatching order (Kruskal-Wallis test: $H_4 = 9.832$, $P = 0.043$; Fig.5). Chicks hatched late within the hatching sequence were more likely to die than older brood members (Fig.5). Late hatched chicks came predominantly from late laid eggs as there was a positive and significant relationship between egg-laying order and chick hatching order (r_s= 0.5, N= 101, $P < 0.001$).

DISCUSSION

In this study, females responded to the increased competition in multi-female groups and adjusted their egg-laying behaviour according to female group size. The three predictions of the competitive female egg-investment hypothesis were supported. The numbers of eggs laid per capita were greater in multi-female compared to single female groups, as well as with communal group size (prediction 1; Fig.1), apparently as a response to increased reproductive competition and egg loss. In guira cuckoos and anis (subfamily Crotophaginae, Cuculidae), this increase in egg-laying appears to be a strategy to negate the detrimental effects of egg tossing and burial (Vehrencamp 1977; Macedo 1992; Macedo et al. 2004b). In the only passerine joint-nesting species discovered to date, the

Taiwan yuhina (*Yuhina brunneiceps*), the number of eggs in communal nests also increases with group size, however, the number of eggs per female decreases with group size (Yuan et al. 2004). Egg loss through tossing or burial has never been observed in this species (Yuan et al. 2004). This finding supports the idea that the increase in the number of eggs laid per capita found in the majority of joint-nesting species studied to date mitigates the detrimental effects of egg destruction. Egg-laying strategies seem to take two forms in multi-female ani groups. On average, females in multi-female groups laid 1.5 more eggs than their singly nesting counterparts (Fig.1). In a few nests, some females likely laid two clutches as the number of eggs per capita and the total number of days laying was high. Future studies should investigate whether this double brooding is a rare event and whether it functions as an adaptive strategy to increase a female's representation in the final incubated clutch of eggs. If this behaviour is adaptive, we expect that females should invest differentially in their two clutches as the second clutch contains eggs that are more likely to hatch.

Egg tossing and burial were clear manifestations of competitive behaviours in multi-female groups. These behaviours were rare or nonexistent in single female groups where females dealt only with their own eggs (prediction 3). Tossing did not increase with group size in multi-female groups, probably because this behaviour is restricted to the early stages of egg-laying (Fig.4a). As eggs begin to accumulate in the communal nest and visitation to the nest becomes more frequent, it may be easier and less detectable for group members to bury rather than toss eggs. Unlike egg tossing, egg burial and overall egg loss did increase with increasing numbers of females (prediction 2). This result

highlights the fact that egg burial plays a major role in explaining the group size effect found in this study. This contrasts with findings in a closely related species, the guira cuckoo, where there is no difference in egg loss probabilities in multi-female groups of different sizes (Macedo 1992; Macedo et al. 2004a). Different life history characteristics may help explain such differences. In guira cuckoos, females lay an average of 2 to 3 eggs, groups often have multiple nests within a breeding season, and group members commit infanticide once chicks hatch (Macedo 1992; Macedo et al. 2004a). In smooth-billed anis, females invest more resources for each nesting attempt (6.3 eggs per female in this study), possibly because renesting in smooth-billed anis is uncommon (only 4 groups laid a second full communal clutch in this study during a given breeding season). Since adult group sizes are similar in these two species (5-6 adults per group on average: Macedo 1992; Quinn & Startek-Foote 2000), the increased number of eggs laid per female in anis translates into larger communal clutch sizes than in guira cuckoos. As a result, competition for access to the incubated layer of eggs is intense in anis, and infanticide (through tossing and burial) is common during the egg stage. Such reproductive competition seems to decrease drastically during the chick rearing stage as anis do not typically commit chick infanticide but instead cooperate to feed nestlings (Quinn & Startek-Foote 2000), similar to some communally breeding mammals (Gilchrist 2006).

Rates of egg burial were high for most of the egg-laying sequence, especially in larger communal groups. Burial was not confined to the early stages of egg-laying (Fig.4b) as previously found in groove-billed anis (Vehrencamp 1977). In guira cuckoos, eggs are

also typically lost early, but lower levels of egg destruction also occur throughout egg-laying and incubation (Macedo 1992; Macedo et al. 2004a). Here, the higher rates of egg loss found for most of the egg-laying sequence highlight the intense egg-laying competition in smooth-billed ani groups when compared to other joint-nesters. Furthermore, we see that late egg burial is not uncommon as 30-40 % of all eggs laid in the last 20% of the clutch are buried in 4-5 female groups (Fig.4b). These burial rates are much higher than what we would expect from accidental burial (only one burial event in 19 single female nests). The finding that egg burial persists in multi-female groups late in the laying sequence suggests that egg burial continues after all females have laid at least one egg. This result calls into question suggestions that Crotophagidae females stop egg tossing/burying once they start laying (lack of own egg recognition) and weakens the argument that late laying females are in the best position since they benefit from no egg loss (Vehrencamp 1977). In smooth-billed anis, eggs laid late in the laying cycle tend to hatch late in the communal nest. Since late hatched chicks have increased daily mortality probabilities, late laying females suffer chick losses not experienced by earlier laying females. It is very likely that late hatched chicks were at a competitive disadvantage against older and heavier nest mates. Relative timing of laying within smooth-billed anis is therefore very important as both egg loss and chick mortality probabilities vary with egg-laying order, though in opposite directions.

Egg destruction is an extreme manifestation of reproductive competition and affected 56% of all eggs laid communally in this study. Group living therefore appears to come with short-term costs to females. First, the number of incubated eggs per capita decreases

with increasing female group size. Second, laying extra eggs is likely very energetically costly in the short term (Monaghan & Nager 1997; Vézina & Williams 2002; Williams 2005), but may also reduce the female's survival probability (Nager at al. 2001), or efficiency at incubating eggs and rearing chicks (Monaghan & Nager 1997; Monaghan et al. 1998). Egg production in Crotophaginae seems especially costly since eggs are relatively large compared to a female's body weight (15% on average in anis: Quinn & Startek-Foote 2000; 16% in guira cuckoos: Macedo 1992).

Groove-billed ani females benefit from a male-biased nocturnal incubation and gain weight during the second half of the incubation period (Vehrencamp 2000). This male nocturnal incubation may be an important feature in the evolution and maintenance of joint-nesting systems by freeing females from nocturnal incubation duties, thus allowing them to spend more energy during egg-laying and less during incubation (Vehrencamp 2000). Similarly, other characteristics of joint-nesting species such as the group participation in chick rearing and foraging sentinel duties may also be important factors explaining how females can invest heavily in egg-laying.

Egg destruction may enable communal groups to coordinate the completion of a large shared clutch without compromising the nesting effort. In acorn woodpeckers, egg destruction synchronizes egg-laying and equalizes the number of eggs each female has in the nest (Koenig et al. 1995). Results from our study support this view, though this may be incidental to reproductive competitiveness. In the later stages of the egg-laying period, rates of egg burial and egg tossing decrease, likely because all females have laid eggs at that point as in the case in groove-billed anis (Vehrencamp 1977). As a consequence of

this reduced egg destruction, synchronization occurs. Larger communal groups take more time to synchronize egg-laying since more females need to lay eggs at roughly the same time for this synchronization to occur. Group membership in larger communal groups may therefore come with additional costs when compared to smaller communal groups. A similar result was found in greater rheas (*Rhea americana*) where nests with over 30 eggs had longer laying periods and had lower hatchability (Fernández & Reboreda 1998). This cost contributed to lower reproductive success per capita with increasing group size in anis and other crotophagids (Koford et al. 1990; Macedo 1992; C. Lentz, J. Quinn & G. Schmaltz, unpublished data). Current published evidence suggests that females are thought to perform most of the egg tossing/burial (Vehrencamp 1977). Future studies should try to quantify the contribution of each sex to egg destruction behaviors. Ongoing work is aimed at trying to link behaviours associated with nest building, egg destruction, and egg-laying timing with parentage of young .

Communal life in anis and other joint-nesters generates competition and egg production waste as predicted by "tug-of-war" skew models (Johnstone 2000; Reeve & Shen 2006). In joint-nesting species, this egg-laying competition is unlikely to lead to high short-term per capita reproductive benefits (Vehrencamp 1977; Koenig 1981; Macedo 1992). Furthermore, in smooth-billed anis and guira cuckoos, dispersal options do not seem to be constrained by a lack of suitable breeding territories (Macedo & Bianchi 1997; C. Lentz, J. Quinn & G. Schmaltz, unpublished data; but see Koford et al. 1986). Given this lack of habitat saturation and the reproductive costs highlighted in this study, it seems that lifetime fitness benefits are required to explain the maintenance of

this social system. Long-term field studies are therefore needed to clarify individual lifetime benefits associated with communal breeding in joint-nesting species.

Avian plural breeding systems are not alone in the apparent short-term reproductive costs of living in larger groups. In a review of the adaptive value of sociality in mammals, Silk (2007) cites studies of some communal breeding mammals in which seasonal reproductive success per female declines with group size (yellow-bellied marmots, *Marmota flaviventris;* Tuco-tucos, *Ctenomys sociabilis*, and black-tailed prairie dogs, *Cynomys ludovicianus*), or in relative large groups (red deer, *Cervus elaphus*; long-tailed macaques,*Macaca fascicularis*). Increased group size may impose costs associated with competition for food and associated energetic costs of increased travel in some mammalian species. Silk suggests that survivorship improvements may occur as group size increases. This may be at play in some joint-nesting plural breeders such as smooth-billed anis.

Most clutch size studies have focused on species that breed singly as socially monogamous pairs. Here, we have shown that in a communally breeding joint-nesting species there is intense egg-laying competition and egg loss. Joint-nesting individuals try to skew the contents of the final incubated clutch of eggs in their own favour by tossing and/or burying competitors' eggs and by producing more of their own eggs. It therefore seems that group living heavily influences egg-laying strategies and that the selective pressures facing pair-nesting socially monogamous species and communal joint-nesters are very different. If group-living influences processes such as individual clutch size, it also likely influences other egg-laying variables under maternal control such as egg mass,

egg size, and egg content. Future studies focussing on egg-laying in communal breeders are required to highlight potential behavioural and physiological differences between socially monogamous and joint-nesting species. These maternal effects on egg mass and hormonal egg content in joint-nesting anis are the subject of ongoing studies.

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APPENDIX

Proportion of shared dyads (% SDY) using three different estimates

Location	Group size in 2003 (number of birds banded within the group)	Group size in 2004 (number of birds banded within the group)	Number of birds banded present in both 2003 and 2004	% SDY if all unbanded birds in 2003 remained in their group in 2004	% SDY if all unbanded birds in 2003 dispersed in 2004	% SDY calculated using banded birds only
4-Way	3 (2)	4 (4)	2	0.222	0.222	0.222
Casablanca	3 (1)	3 (3)	0	0	0	0
Congrejos	3 (2)	6 (1)	1	0.111	0	0
Finca Newfie	2 (1)	4 (2)	0	0	0	0
Gully	3 (3)	5 (0)	0	0	0	0
Home	3 (3)	3 (3)	2	0.333	0.333	0.333
North East	5 (3)	7 (1)	1	0.194	0	0
North Farm	5 (3)	2 (0)	0	0.182	0	0
North House	12 (10)	5 (5)	5	0.263	0.263	0.263
North West	13 (8)	7 (2)	2	0.424	0.02	0.069
South Fence	7 (2)	7 (1)	1	0.714	0	0
US Flag	3 (3)	16 (0)	0	0	0	0
Average				0.204	0.0698	0.074

Proportion of shared dyads (% SDY) for 12 groups at the Cabo Rojo wildlife refuge during the transition from 2003 to 2004. The proportion of shared dyads (% SDY) was calculated as follows:

$$\% \text{ SDY} = \frac{2\left(\sum_{x=1}^{S}(s-x)\right)}{\left(\sum_{x=1}^{A}(A-x)\right)+\left(\sum_{x=1}^{B}(B-x)\right)}$$

Where S is the number of birds that remained together between seasons (shared dyads), A is the total number of birds in the group for 2003 and B is the total number of dyads in the group for 2004. For instance, a group that had 3 birds in 2003 (A = 3) and 6 birds in 2004 (B = 6) with two birds staying in the group from 2003 to 2004 (S = 2) would have a proportion of shared dyads of: SDY = (2 * (2-1)) / ((2+1) + (5+4+3+2+1)) = 11.1%. The estimated proportion of shared dyads that remained together from 2003 to 2004 ranged from a minimum of 7% (if we consider that all unbanded birds in a group dispersed from one year to the next), to 7.4% (if we calculated the % SDY from banded birds only), to a maximum of 20.4% (if we consider that all unbanded birds in a group remained from one year to the next). It is important to note that 20.4% probably overestimates the proportion of shared dyads in a group as this value is inflated by a few groups (North West, South Fence) in which many unbanded birds were present in both 2003 and 2004.

Figure legends

Figure 1. Distribution of number of eggs laid per female (± SE) as a function of female group sizes. The number of eggs laid per female increased in the different female group size categories (Kruskal-Wallis test: $H_3 = 9.1$, $P = 0.03$). Female group sizes having significant differences (multiple comparisons between treatments method, $P < 0.05$) in mean number of eggs laid per female are indicated by different letters above the bars. n = number of nests.

Figure 2. Number of eggs laid in a nest (i.e. communal clutch size) as a function of female group size. Data points in the figure represent individual nests. Communal clutch size was highly correlated with the number of laying females ($r_s = 0.91$, N= 59, $P < 0.001$). n = number of nests.

Figure 3. Distribution of egg loss probabilities (± SE) through tossing (solid bars) or burial (striped bars) among different female group sizes. The probability of egg burial differed significantly by group size categories (Kruskal-Wallis test: $H_3 = 146.7$, $P < 0.001$). Burial probabilities were significantly different in the female group size categories as indicated by different letters above the bars (multiple comparisons between

treatments method, $P < 0.05$). There were no significant differences for tossing probabilities in multi-female groups. n = number of nests.

Figure 4. Distribution of egg tossing probabilities (± SE) (panel A), burial probabilities (± SE) (panel B), or egg loss probabilities (± SE) (panel C) among different egg order indices and female group sizes calculated from 23 joint-nests.

Figure 5. Distribution of daily chick mortality probabilities (± SE) among different chick order indices calculated from 23 nests. Daily chick mortality probabilities were affected by hatching order (Kruskal-Wallis test: $H_4 = 9.832$, $P = 0.043$). Chicks hatched late within the hatching sequence were more likely to die than older, previously hatched chicks. Chick order indices having significant differences (multiple comparisons between treatments method, $P < 0.05$) in daily mortality probabilities are indicated by different letters above the bars.

Figure 1.

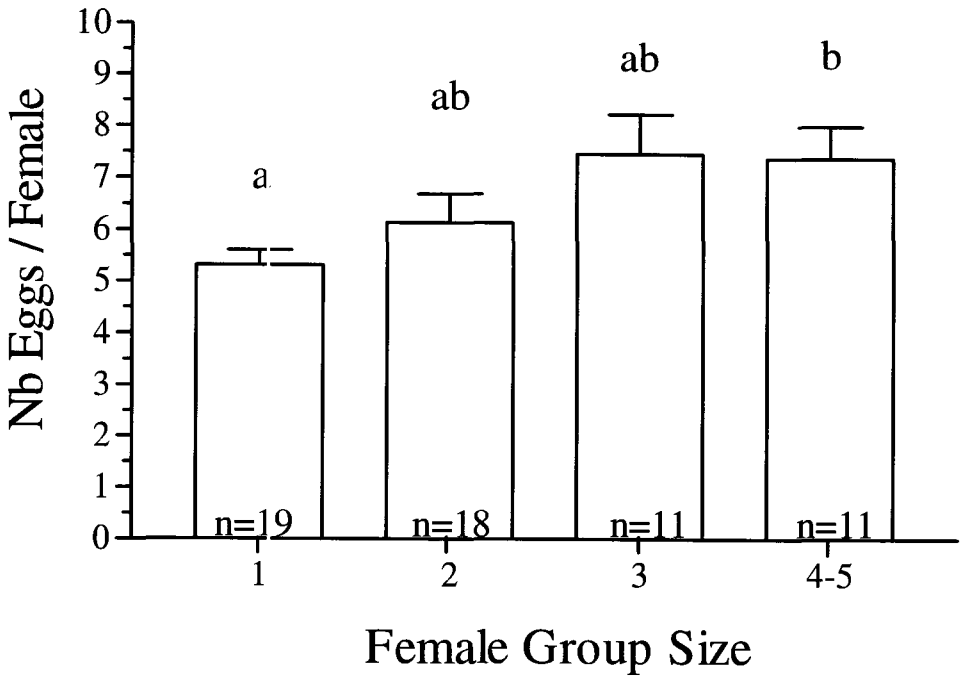

Nb Eggs / Female
10
9
8
7
6
5
4
3
2
1
0
a
ab
ab
b
n=19
n=18
n=11
n=11
1
2
3
4-5
Female Group Size

Figure 2.

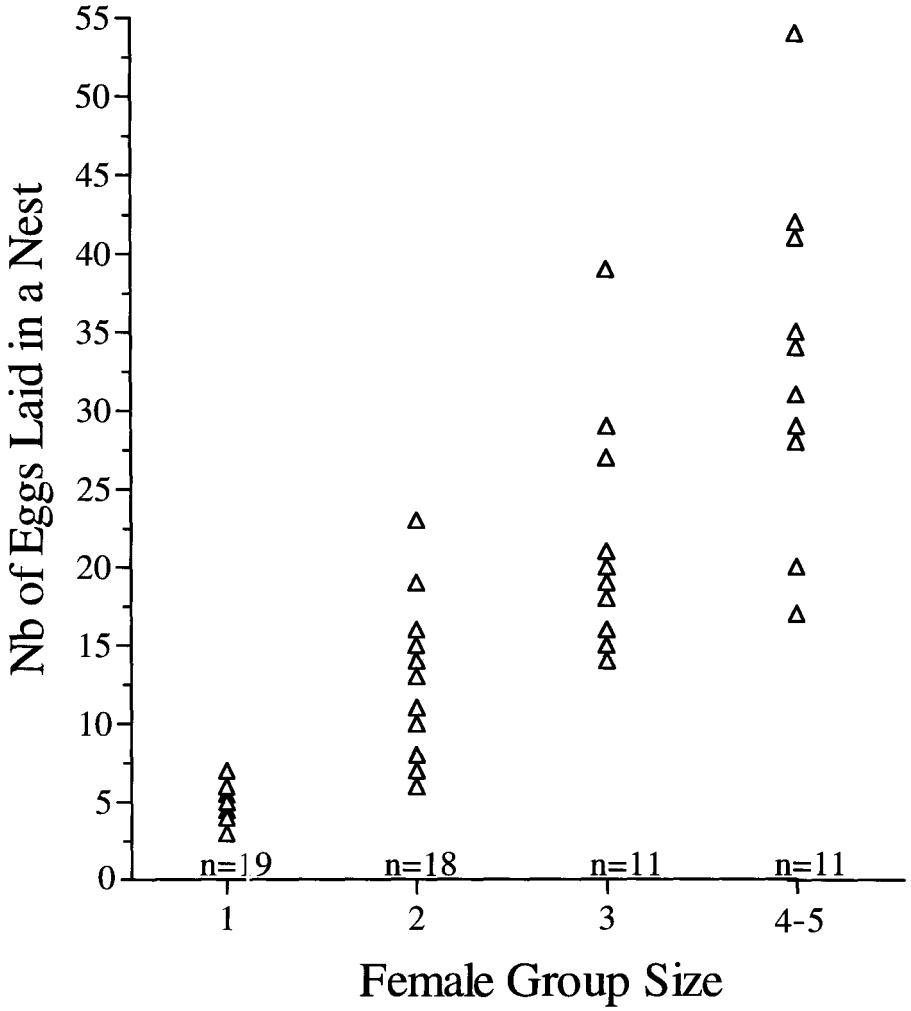

Nb of Eggs Laid in a Nest
55
50
45
40
35
30
25
20
15
10
5
0
n=19
n=18
n=11
n=11
1
2
3
4-5
Female Group Size

Figure 3.

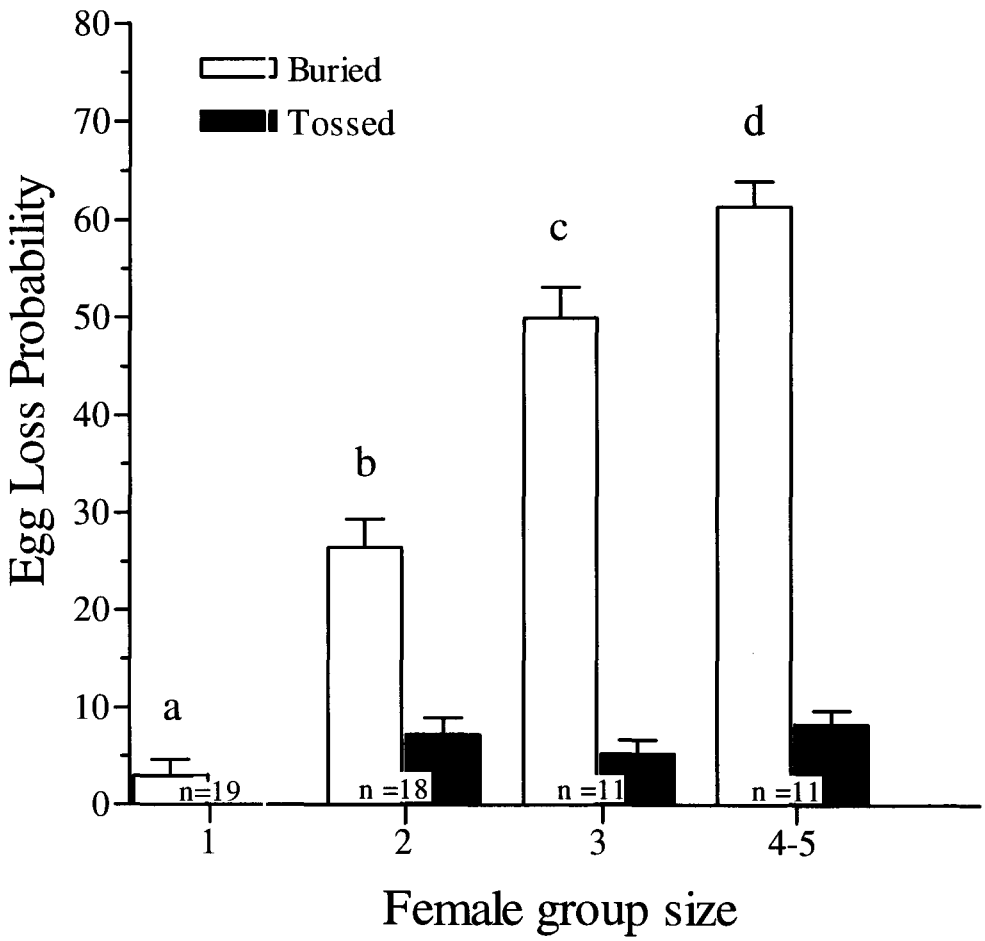
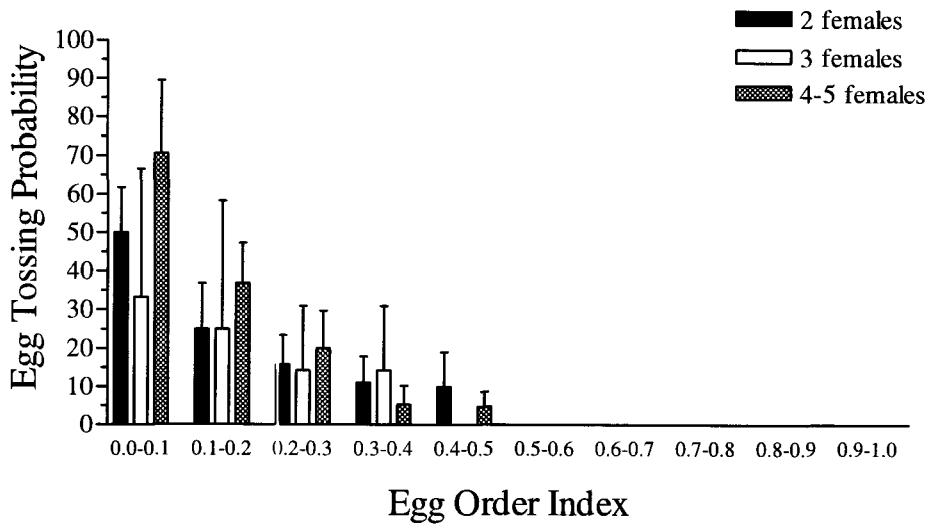
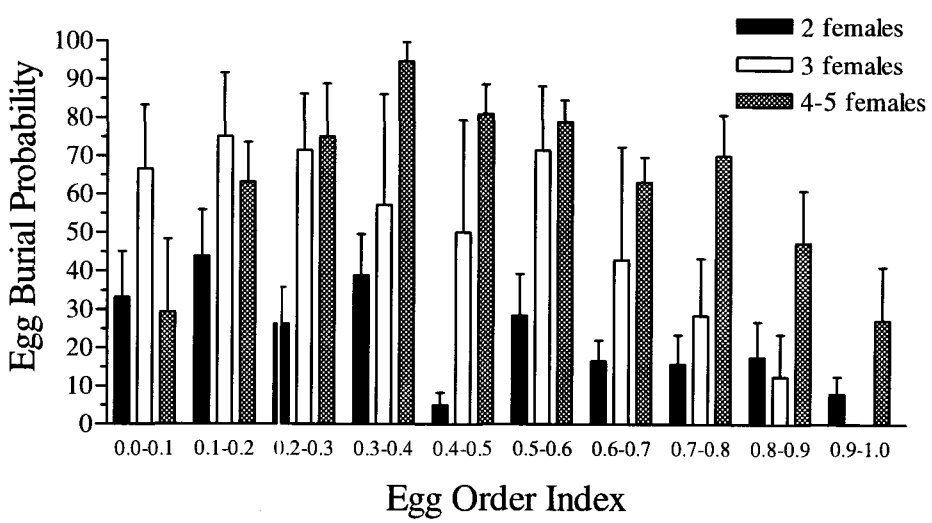

Buried
Tossed
Egg Loss Probability
80
70
60
50
40
30
20
10
0
a
b
c
d
n=19
n =18
n =11
n =11
1
2
3
4-5
Female group size

Figure 4.

Panel A



Panel B



Panel C

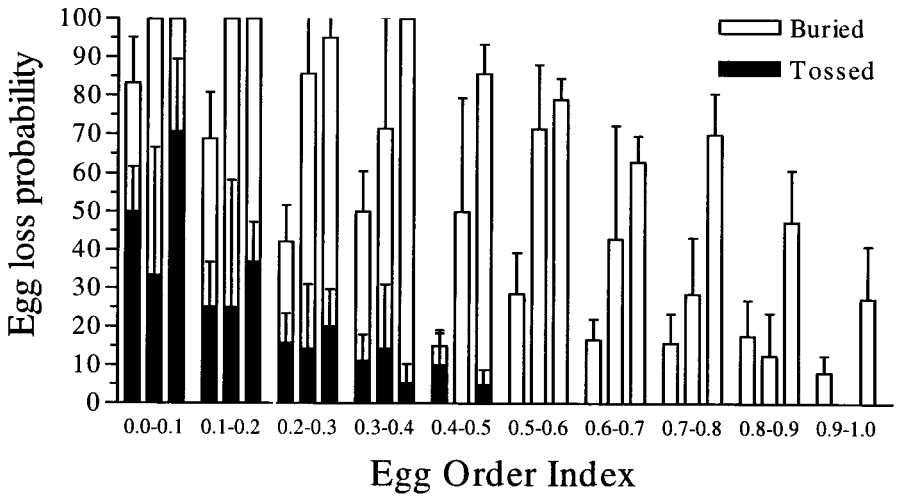
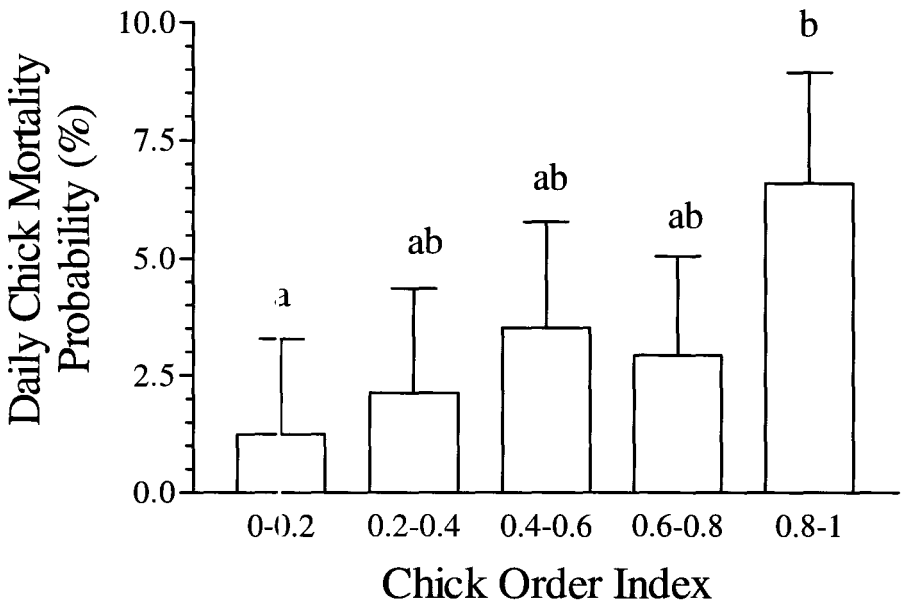

Buried
Tossed
Egg loss probability
100
90
80
70
60
50
40
30
20
10
0
0.0-0.1
0.1-0.2
0.2-0.3
0.3-0.4
0.4-0.5
0.5-0.6
0.6-0.7
0.7-0.8
0.8-0.9
0.9-1.0
Egg Order Index

Figure 5.


Daily Chick Mortality Probability (%)
10.0
7.5
5.0
2.5
0.0
a
ab
ab
ab
b
0-0.2
0.2-0.4
0.4-0.6
0.6-0.8
0.8-1
Chick Order Index

CHAPTER 3

Do group-size and laying order influence maternal deposition of testosterone in smooth-billed ani eggs?

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Abstract

The avian egg contains maternal hormones that affect behavior, growth, morphology, and offspring survival. Evidence to date suggests that patterns of yolk androgen deposition could provide females with a means to manipulate sibling competition and, thereby, increase their fitness. We examined yolk testosterone (T) concentrations in eggs of the smooth billed ani (*Crotophaga ani*) to understand patterns of androgen deposition in eggs of this plural-breeding joint-nesting cooperatively breeding species. We tested the hatching asynchrony adjustment hypothesis, which states that increases in yolk androgen levels over the laying sequence function to mitigate the disadvantage of being a later-hatched chick in species without adaptive brood reduction. We also investigated the effect of group size on yolk T deposition to test the hypothesis that females in multi-female groups could give a competitive edge to their own chicks by depositing higher T levels in their eggs. Predictions of the hatching asynchrony adjustment hypothesis were supported in both single and multi-female groups as yolk testosterone levels increased from early- to late-laid eggs. This suggests that ani females can influence nestling competition and chick survival by within-clutch differential T allocation. Unexpectedly, we did not observe an effect of group size on yolk T deposition. Yolk testosterone concentrations may not be a mere reflection of a female's hormonal status as female plasma circulating levels of T did not vary in the same direction as yolk T levels. Results of this study therefore support the idea that females may adaptively manipulate chick behavior through hormonal deposition in eggs.

Keywords: Testosterone; Yolk Androgen; *Crotophaga ani*; Smooth billed ani; Communal breeding; Hormone Deposition; Laying order

Introduction

Studies from several avian species indicate that egg yolks contain significant amounts of maternally derived hormones (Gil, 2003; Groothuis et al., 2005a; Schwabl, 1993). Mothers can affect offspring phenotype and, therefore, influence the development of behavioral and life-history strategies through this transmission of hormones from mother to offspring (Ketterson and Nolan, 1999; West-Eberhard, 2003). Testosterone (T), the best studied hormone deposited in eggs, has been found to vary with laying order and to affect chick begging behavior, growth, and social dominance among nest-mates in many species (Gil, 2003; Groothuis et al., 2005a; Schwabl, 1993, 1996a). For instance, increased T levels in later-laid eggs resulted in increased mass of the hatching muscle (*musculus complexus*), which allows breaking out of the shell and subsequent flexion of the neck during begging, in red-winged blackbirds (*Agelaius phoeniceus*, Lipar and Ketterson, 2000), as well as higher begging levels and growth rates in canaries (*Serinus canaria*, Schwabl, 1996a) and black-headed gulls (*Larus fuscus*, Eising et al., 2001, 2003). Patterns of yolk androgen deposition could provide females with a means to manipulate sibling competition within the clutch, thereby increasing offspring survival and their own fitness (Gil, 2003; Groothuis et al., 2005a). Specifically, females might

adjust androgen deposition depending on the magnitude of hatching asynchrony, a factor that often influences sibling competition and subsequent chick survival (Schwabl et al., 1997; Stoleson and Beissinger, 1995).

The hatching asynchrony adjustment hypothesis states that increases in yolk androgen levels over the laying sequence function to mitigate the disadvantage to later-hatched chicks in species without adaptive brood reduction (Groothuis et al., 2005a; Lipar et al., 1999a; Schwabl, 1993, 1996a). Because hatching asynchrony produces a size and motor-skills disadvantage for the later-hatched chicks, increases in androgen levels with laying order would enable later-hatched chicks to make up their growth deficit. However, in species with adaptive brood reduction (*sensu* Lack, 1968), it may be to the female's advantage to increase the asymmetry between early- versus late-hatched chicks (Mock and Ploger, 1987). In these cases, the hatching asynchrony adjustment hypothesis predicts a decrease in yolk androgen levels over the laying sequence to help older siblings to out-compete younger chicks when food resources are low (Groothuis et al., 2005a; Schwabl et al., 1997). Support for the hatching asynchrony adjustment hypothesis has been found in both species with and without adaptive brood reduction (see Groothuis et al., 2005a for a review). However, because not all species tested to date fit the predictions of the asynchrony adjustment hypothesis (Groothuis et al., 2005a), more studies are needed to determine whether the failures of the hypothesis' predictions can be attributed to particular species (or groups of birds) or unique ecological conditions.

Recent studies suggest that yolk androgen levels within a clutch vary with factors other than laying order. Yolk hormone levels have been found to vary with a female's

social condition, including the levels of aggressive interactions that she experiences around the time of laying, specifically during the yolk deposition stage (Müller et al., 2002; Pilz and Smith, 2004; Schwabl, 1997; Whittingham and Schwabl, 2002). In group-living species in which females lay in a shared communal nest, there may be intense egg-laying and nestling competition. In such species, group size could be another important social factor that affects a female's circulating plasma hormone levels and, ultimately, egg yolk hormone deposition. To our knowledge, such an effect in a communal or cooperatively breeding bird has only been investigated previously in one species. In the guira cuckoo (*Guira guira*), another plural- breeding joint-nester, levels of androstenedione (but not testosterone) increased with both the communal clutch size (i.e., in later-laid eggs) and female group size (Cariello et al., 2006).

We examined yolk testosterone concentrations in eggs of a communally breeding joint-nester: the smooth-billed ani (*Crotophaga ani*). Anis have an unusual mating system in which from one to five females lay their eggs in a single nest (Quinn and Startek-Foote, 2000; GS pers. obs.). Intense egg-laying competition occurs in multi-female groups with eggs laid early having high probabilities of loss because of egg tossing and burial under nesting material beneath later-laid eggs (Quinn and Startek-Foote, 2000; GS pers. obs.). This egg-laying competition intensifies with increased group size, as both the number of eggs laid and lost per female increase with group size (chapter 2). The upper-most layer of eggs (under which earlier-laid eggs may become buried) represents the clutch of eggs that is incubated to hatching. Following this intense competition, group members that are typically unrelated to each other cooperate to incubate eggs and to rear

the young. In single female groups, last hatched chicks only rarely die in the nest (one chick death in 11 single female nests studied) suggesting that adaptive brood reduction does not occur in this species. In multi-female groups, egg-laying lasts longer and hatching asynchrony is more pronounced (chapter 2). The smallest chicks, which are more likely to die, come from eggs laid late. In this typically joint-nesting species, brood reduction would not serve an adaptive function and typically unrelated females should favour their own chicks. Females could potentially do so by depositing more testosterone in eggs, which may result in more intense begging later on in life (Eising et al., 2001, 2003; Schwabl, 1996a).

Here, we tested the predictions of two hypotheses to investigate the effect of both laying order and group size on hormone deposition in anis. First, we tested the predictions of the hatching asynchrony adjustment hypothesis in both single and multi-female groups. In single female nests, the hypothesis predicts that testosterone levels should increase with egg-laying order to mitigate the disadvantage of being a later-laid egg, which almost invariably becomes a later-hatched chick in the brood. In multi-female groups, we also predict an increase of yolk T levels from early- to late-laid eggs because the clutch of eggs that is incubated usually consists of late-laid eggs from different females. We therefore argue that the hatching asynchrony adjustment hypothesis can be extended to communally breeding joint-nesting species which predicts that yolk T levels will increase with laying order in both single and multi-female nests and, thereby allow later-hatched chicks to make up their growth deficit. Second, we tested the predictions of the competitive female hormone investment hypothesis. Competition among unrelated

nestlings in multi-female groups may select for increased androgen deposition in eggs to enhance the competitive abilities of an individual's offspring. This hypothesis predicts that yolk T levels will be higher in multi-female groups when compared to single-female groups. These two hypotheses are not mutually exclusive as the yolk T patterns predicted by the hatching asynchrony hypothesis could occur in both single- and multi-female groups; however, under the predictions of the competitive female hormone investment hypothesis the absolute levels of T in yolks would be predicted to be higher in multi- rather than single-female groups.

Materials and methods

Study sites

We studied smooth-billed anis at the Cabo Rojo and Laguna Cartagena National Wildlife refuges in south-western Puerto Rico (17°59'N, 67°10'W elevation from 2 to 42 m, and 18°01'N, 67°06'W elevation from 55 to 71 m, respectively) during the rainy season (September-January), when most ani breeding occurs, in 2003-4 and 2004-5. The south-western portion of Puerto Rico has a dry tropical climate. Both Cabo Rojo (587 acres) and Laguna Cartagena (794 acres) refuges contain second-growth dry scrubland, open, and disturbed habitats that attract breeding anis (Loflin, 1983; Quinn and Startek-Foote, 2000).

Field procedures

Details about group size determination and nest check regime are provided elsewhere (Chapter 2). In brief, we checked every active nest daily during egg-laying and marked every new egg with non-toxic markers to indicate egg number in the laying sequence. We took hormone samples from eggs that were among the first or last 20 % to be laid in the group's clutch (i.e., early- versus late-laid eggs). These eggs included some that had been tossed or buried, as well as those that remained in nests. We collected yolk samples from freshly laid, un-incubated eggs by inserting a 25 gauge needle through the eggshell at the middle of the long axis of the egg, following the method pioneered by Schwabl (1993). We inserted the needle as far as required to reach the center of the yolk and extracted a small sample (approximately 40 mg) of yolk with a 1 mL disposable syringe. We sealed the insertion hole with new-skin liquid bandage (Medtech, Jackson, WY, USA) and returned the egg to its nest. We transferred yolk samples to 1.5-mL Eppendorf tubes and froze them at -20 °C until samples were shipped overnight on ice to the University of Memphis for assay (see below). We analyzed these eggs for testosterone content in this chapter and assayed the same eggs for estradiol and corticosterone content in chapter 4 and 5 respectively. To verify the consistency of the sampling method, we sampled 15 intact tossed eggs that were freshly laid as described above. We then froze these eggs to separate the yolk from the albumen, and then collected another small sample (approximately 40 mg) of thawed and homogenized whole yolk. We then compared whole egg yolk hormone values to those obtained from

egg puncturing to verify that the sampling method was consistent as sampling from different layers of the yolk is an issue in some species (Lipar et al., 1999b).

Adult Sampling

Adults are routinely captured in our long-term field project for hormonal and parentage analyses. In this study, we wanted to know how plasma T levels in females generally vary from the early to the late egg-laying stages. Since it takes two to three days for the major portion of the yolk to be deposited in an egg (Romanoff and Romanoff, 1949; Schwabl, 1996b), we restricted our dataset to samples from females that were caught either within the first three days or the last three days of the period when eggs were being laid in her group. Because not all females were banded and we could not often observe females while egg-laying, we could not determine whether the captured females were the same females that laid the sampled eggs. Therefore, our adult hormonal analyses are meant to uncover general patterns of female plasma T variation within groups to determine how these plasma levels relate to yolk T levels in early-laid versus late-laid eggs. We analyzed the same samples for circulating plasma estradiol levels in chapter 4. We captured adults using two 60 mm mesh mist nets (18 m length) stacked one upon the other on 7.2m telescoping poles (Meyers and Pardieck, 1993). Adults were also captured using a hardware cloth cylindrical funnel trap that was placed within a group's territory. The trap allowed easy entry but not egress and contained a caged hand-reared lure ani to attract territory defenders (Vehrencamp, 1977). After capture, we collected

300 µL of blood from adults for which hormone analyses were done. Blood was collected by venipuncture of the jugular vein with a 27 gauge needle with a 1 mL syringe. Pressure was subsequently maintained for 20 s with a finger after bleeding to avoid blood loss and to close the wound. Collected blood was subsequently centrifuged for 5 min at 4400 g to separate plasma from the red-blood cell fraction that was used for later microsatellite genotyping (Blanchard and Quinn, 2001; Gregory and Quinn, 2006). We determined the sex of each individual by amplifying an intron in the Chromo-Helicase-DNA binding gene (CHD; Griffiths et al., 1998). Plasma samples were frozen at -20 °C until they were shipped to the University of Memphis for assay. Procedures in this study were approved and conducted under a McMaster University Animal Care Permit (AUP 05-07-40).

Radioimmunoassay

We assayed the concentration of yolk testosterone with a competitive-binding radioimmunoassay (Ball and Wingfield, 1986; Schoech et al., 1996; Wingfield and Farner, 1975; also see Schwabl, 1993 for specific methods that pertain to extraction of steroids from yolk). We weighed a sub-sample (approximately 30 mg) to the nearest 0.01 mg, added 500 µl distilled water and three glass beads to each sample and vortexed them repeatedly to homogenize the yolk. We added approximately 2000 cpm each of [^{3}H]testosterone (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA) to each yolk sample to calculate the recovery percentages after extraction and chromatography. After further mixing, we let the homogenate equilibrate for at least 12 hr at 4°C prior to

extraction. The steroids were extracted from the aqueous phase by adding 3 mL of a petroleum:diethyl ether (30%:70%) solution, that was vortexed, then snap-frozen, and the ether containing the steroids was then poured off. This was done twice for each sample and the resultant 6mL of ether solution was then dried under N_2. We then added 1 mL 90% ethanol to remove excess lipids; samples were stored overnight at -20°C (Schwabl, 1993). We separated precipitated proteins and lipids from the ethanol phase by decanting after centrifugation at 1100 g for 15 min. We dried the samples under N_2, resuspended in 0.5 mL 10% ethyl acetate in isooctane, and ran them through chromatography columns that consisted of a celite:ethylene glycol:propylene glycol (6 g:1.5 mL:1.5 mL) upper phase and a celite:water (3 g:2 mL) lower phase. We collected the T fraction in each column with 4.5 mL of 20% ethyl acetate in isooctane. We compared duplicate values of each sample to a standard curve that ranged in concentration from 500 to 1.95 pg. We measured all yolk T samples in three different assays. The intra-assay coefficients of variation based on six standards for the three assays were 17.7%, 17.9%, and 13.4%, and the inter-assay coefficient of variation was 16.5%. We assayed female plasma samples using a similar technique as for yolk samples (see above), using 70-100 μL of plasma to start the extraction procedure. For plasma samples, we skipped the step where 1 mL 90% ethanol was added to each sample as this step is meant to remove excess lipids found in egg yolks. We measured all plasma T samples in three different assays. The intra-assay coefficients of variation based on six standards for the three assays were 10.6%, 9.6%, and 8.5%, and the inter-assay coefficient of variation was 9.7%.

Data and Statistical Analysis

Plasma and yolk testosterone values were transformed using the reciprocal transformation (1/(X+0.5)) to meet the requirements of parametric tests. For each nest under study, the eggs from single female groups were deposited by different females that were located on different territories. For each nest under study, we averaged T values from different eggs laid during the early (first 20%) period and late (last 20%) period, producing two average T values per nest (early and late). In multi-female nests, each averaged data point (early or late) represents an average from different eggs in a given nest that were laid within 1.4 ± 0.29 d (heretofore, all such data are presented as means ± SEM). Since ani females lay eggs every other day during the laying period (Quinn and Startek-Foote, 2000), eggs in the same category (early or late) were generally laid by different females. The mean lay-date for the early and late egg-laying periods were respectively 60.4 (± 6.5) and 70.0 (± 6.8) days from the beginning of the reproductive season (set as September first). Because of nest failure due to depredation or destruction by high winds we were unable to sample late eggs from four groups; therefore, we only include early egg data for those four nests. Accordingly, we performed statistical tests on 26 groups for the early egg-laying period and 22 groups for the late egg-laying period. To calculate female plasma T concentrations during the early and late egg-laying stages we restricted our sampling to females that were caught either within the first three days or the last three days of the period when eggs were being laid in her group. We averaged female plasma T concentrations for the early stage in 2003 (n = 4) and 2004 (n = 3) and

the late stage in 2003 (n = 9) and 2004 (n = 6). Variation in testosterone levels with laying order (early versus late) and group size were analyzed using an analysis of variance using year and day of year as two factors. Females were only included once in the analyses. We tested year and laying-order effects with a post-hoc test (least significant difference: LSD). T-tests were used to examine the effect of laying order (early versus late) and year on female plasma T levels and to look at possible yolk T concentration differences between tossed/buried and retained eggs. We examined the relationship between T measurements from both sampling methods (puncturing and whole yolk) with a Spearman rank correlation. We conducted power analyses in STATISTICA (version 6.0). All other tests were performed in SPSS 10.0 (SPSS Inc.) as two-tailed tests with an α of 0.05.

Results

Yolk testosterone levels

We monitored and analyzed a total of 88 fresh eggs from 26 different ani groups in 2003 and 2004. From these 88 eggs, a total of 55 eggs had been either tossed or buried. Yolk levels of T were significantly higher in late-laid eggs than in early-laid eggs ($F_{1,38} = 9.63$, $P < 0.01$; Figs. 1 and 2) but T levels did not vary with group size ($F_{3,38} = 1.34$, $P = 0.27$; Fig. 2). We also found that yolk T levels were significantly higher in 2004 than in 2003 ($F_{1,38} = 17.65$, $P < 0.001$; Fig. 1); however, there was no effect of date ($F_{1,38}$

= 0.25, P = 0.62). T levels were greater in late-laid as compared with early-laid eggs in both years of the study, however, the increase in 2004 was not statistically significant (in 2003: $F_{1,22} = 4.46$, P = 0.04; in 2004: $F_{1,8} = 2.27$, P = 0.17; Fig. 1). The power of the test was low because of a small sample size in 2004. A power of 80% for detecting a 25% increase from early to late laying would have required at least 26 eggs per group. We found that there was no significant difference between yolk T levels from eggs that were retained and eggs that were lost to either egg tossing or burial (t-test: $t_{86} = -0.291$, P = 0.77). Excluding the four early data points from the analysis does not change our results.

We found a positive highly significant correlation ($r_s = 0.879$, N =15, $P < 0.001$) between T-levels from puncture samples and whole yolk samples, suggesting either that yolk samples were generally collected from the same yolk layer or that yolk T concentrations are similar across egg layers in anis.

Plasma testosterone levels

Female plasma T levels were significantly higher in 2003 (t-test: $t_{20} = -2.912$, P = 0.009) and tended to decrease in both years of the study between the early and the late egg-laying periods, although this trend was not significant in either year (t-test in 2003: $t_{11} = -0.897$, P = 0.39; t-test in 2004: $t_7 = -0.843$, P = 0.43; Fig. 3). However, because of a small sample size, the power of the test was low. A power of 80% for detecting a 25% decrease from early to late laying would have required at least 10 birds per group in 2003

and 6 birds per group in 2004. Similar results were found when only including females from multi-female groups (n= 18).

Discussion

Our results supported the predictions of the hatching asynchrony adjustment hypothesis. Testosterone levels were higher in late-laid versus early-laid eggs, paralleling findings in previous studies that focused primarily on socially monogamous species. Support for the hatching asynchrony adjustment hypothesis has been found in different species without adaptive brood reduction: canaries (Schwabl, 1993, 1996a), red-winged blackbirds (*Agelaius phoeniceus*, Lipar et al., 1999a), and species with adaptive brood reduction: cattle egrets (*Bubulcus ibis*, Schwabl et al., 1997). However, not all species fit the predictions of the hatching asynchrony adjustment hypothesis. For instance, in the zebra finch, a species without adaptive brood reduction and with a similar degree of hatching asynchrony as in the canary, androgen levels in yolk decline slightly over the laying sequence (Gil et al., 1999). Similarly, there was no relationship between laying sequence and yolk androgen levels in tree swallows (*Tachycineta bicolor*, Whittingham and Schwabl, 2002). Results from our study suggest that female anis adjust their hormone deposition according to laying order, and that steroid secretion into the egg appears to be independent of general systemic secretions (see below). Furthermore, hatching asynchrony adjustment occurs in communal breeders as T levels increased with laying order in both single- and multi-female nests. In the guira cuckoo, a closely related species

to the ani, and the only other communal breeder tested to date, egg yolk levels of androstenedione, a precursor to testosterone, increased with laying order but T levels did not, although levels of both hormones were positively correlated (Cariello et al., 2006). Given the limited current understanding of crotophagid biology, future studies are required to understand these between species differences.

In ani joint-nests, eggs laid early in the sequence are more likely to be lost due to egg tossing and burial independent of communal clutch size (chapter 2). For instance, eggs laid first have approximately an 80% probability of being tossed or buried in communal groups, more than the 10-15% chance of egg loss for last laid eggs. One might, therefore, predict that early-laid eggs in multi-female groups would have lowered hormone deposition because the early-laid eggs are unlikely to hatch. However, as in the guira cuckoo (Cariello et al., 2006), we found no difference between yolk T levels from eggs that were retained and eggs that were lost to either egg tossing or burial. Females therefore do not seem to adjust their androgen deposition in eggs as a function of the probability of an egg being retained or lost.

The finding that yolk T levels in multi-female groups increased in late versus early eggs could be explained in terms of plasma levels in early-laying versus late-laying females. However, we suggest that this is not the case for two reasons. First, if female plasma levels were to explain the observed yolk T patterns than we would expect to see an increase in female plasma T levels over the egg-laying period. Data from this study and from the few other species investigated to date suggest otherwise. In canaries, female plasma T levels peak the day before the first egg is laid and then decrease until reaching a

minimum about four days after the first egg is laid (Schwabl, 1996b). In European starlings (*Sturnus vulgaris*), plasma T levels peak for pre-laying females and then gradually decrease until clutch completion (Williams et al., 2004). In this study, circulating plasma T levels in female anis did not significantly vary with laying order and even tended to decrease during egg-laying (Fig. 3). However, in anis and canaries, yolk T levels increase with laying order (Schwabl, 1993; this study). Female plasma T variations cannot therefore easily explain variations in yolk T levels. Second, yolk T levels were higher in 2004 than in 2003, whereas female plasma circulating T levels exhibited the opposite pattern. The above results suggest that maternal depositions are not a mere by-product of female hormonal state as the increase in yolk T levels with laying order was in contrast with patterns of female plasma circulating T concentrations. The steroid hormone producing thecal and granulosa cells of the ovarian follicle wall, therefore, likely play an important role in steroid secretion into the egg (Groothuis et al., 2005a; Hackl et al., 2003; Williams, 2005). There may be a mechanism whereby steroids are sequestered within yolk. Future research should examine just how this might occur. We suggest that the lipophilic nature of steroid hormones may effectively 'pre-destine' their movement into the lipid-rich yolk rather than aqueous plasma. Alternatively, it may be worth looking more closely at the proteins within yolk as there may be globulins with a steroid-binding affinity.

Various environmental factors are thought to influence T deposition in eggs (Groothuis et al., 2005a). In our study, the main environmental factor that may explain why yolk T levels were higher in 2004 than in 2003 was the amount of rainfall and

associated correlates of prey availability. In 2003, levels of rainfall were higher than in 2004, especially prior to egg-laying (July to September; Lentz et al., in revision). These higher precipitation levels may have affected insect abundance. Food availability prior to egg-laying may be an important factor influencing female egg-laying behavior if females are 'capital' breeders and use stored resources (energy or nutrients) for reproduction (Drent and Daan, 1980; Norris et al., 2004; Stearns, 1989). Few studies have looked at the influence of food availability on yolk T levels. In black-headed gulls, food supplemented females produced eggs with lower yolk T levels while the yolk T levels pattern over laying sequence remained unaffected (Verboven et al., 2003). Results in black-headed gulls suggest that females compensate for lower food abundance (and possibly egg quality) by adding more androgens to eggs (Groothuis and Schwabl, 2002). Since chick survival is probably linked to food availability, increased yolk T levels and the resultant increase in chick begging abilities in poor food years may enhance the likelihood that provisioning group members will provide more food to those nestlings.

In communal joint-nests, nestlings often compete with unrelated nestlings for food and space in the nest while trying to attain a favorable feeding position. Given this rivalry, one might expect yolk T levels to increase with adult group size. Such an increase in yolk T levels could be explained either by an adaptive female investment in response to the heightened levels of competition her offspring will likely encounter at the nest, or by an incidental physiological response whereby mothers in multi-female groups have higher levels of circulating plasma T levels that result from increased agonistic interactions. Interestingly, our results suggest that female yolk T deposition patterns are

independent of group size, suggesting a response that is constrained by either evolutionary or physiological processes, or both. We offer the following three hypotheses to explain why the prediction (yolk T concentration should positively correlate with group size) of the competitive female hormone investment hypothesis was not met.

Hypothesis 1): Females trade-off between the number and quality of eggs laid (Nager et al., 2000). In multi-female groups, egg-laying competition is manifested in an increase in the number of eggs laid per capita, as well as an increase in the egg-laying period (chapter 2). This increased egg-laying investment may limit a female's ability to deposit additional T in eggs in multi-female groups.

Hypothesis 2): The maintenance of high plasma T levels may be detrimental to female reproductive output and survival (Dufty, 1989; Ketterson and Nolan, 1992; Nelson, 2000). The increased yolk T levels observed in eggs of female anis that nest singly may represent the highest adaptive level in yolk T deposition that cannot be increased further without major physiological costs to the laying female or developmental and immunological costs to the growing embryo (Gil, 2003; Groothuis et al., 2005a, 2005b). For instance, T-treatment of breeding females can interfere with reproduction and stop nest building or delay clutch initiation (Clotfelter et al., 2004; Nelson, 2000; Searcy, 1988). Furthermore, faster growth of the embryo induced by maternally derived androgens may cause reduced immune function (Andersson et al., 2004; Groothuis et al., 2005a, 2005b) and ultimately affect long-term survival (Eising et al., 2004).

Hypothesis 3): Tropical birds may use testosterone differently than temperate zone birds during agonistic interactions (Hau et al., 2000). Yolk T values obtained in our

study were low compared to altricial species in the temperate zone but similar to those of another tropical joint-nester, the guira cuckoo. In guira cuckoos, yolk T levels also did not increase with group size (Cariello et al., 2006). Both of these crotophagid birds live in the tropics and tropical birds are frequently characterized by longer breeding seasons (Stutchbury and Morton, 2001). Although not much is known about how plasma T levels vary between females of tropical and temperate zone species, the evidence from the literature on male birds suggests that birds in the tropics have lower plasma T levels than most temperate zone birds (Dittami, 1986, 1987; Hau, 2001; Hau et al., 2000; Levin and Wingfield, 1992; Stutchbury and Morton, 2001). Furthermore, individuals of many tropical species occupy territories year-round and maintain relatively low T levels throughout the year, even during the breeding season when aggressive interactions are more common (Hau et al., 2000; Levin and Wingfield, 1992; Wikelski et al., 1999a, 1999b). Therefore, we may have failed to observe a group size effect on yolk T concentrations because increased agonistic interactions in anis may not translate to higher levels of circulating T levels in females or their eggs (Hau et al., 2000).

This study is the first to our knowledge, to report an increase in testosterone levels over egg-laying in communal nesting birds and, as such provides some support for the hatching asynchrony adjustment hypothesis in a cooperatively breeding species. Numerous studies have focused on the anabolic effect of androgens on offspring development and behavior (see introduction). Ani chicks hatching from later-laid eggs may have a competitive advantage that could enable them to catch up on their growth deficit and increase their chances of survival. Indeed, preliminary results of an ongoing

study suggest that ani chicks with higher levels of circulating plasma testosterone beg at higher intensities than chick with low plasma T levels (chapter 6). Joint-nesting birds provide a natural framework to explore what influences hormone deposition in eggs. For instance, experimental manipulations of the number of females in a group would facilitate study of the influence of egg-laying competition on androgen deposition. In this study, the observed variation in testosterone levels with laying order, and the mismatch between female plasma and yolk T levels can be interpreted as being consistent with the idea that female can control the amount of testosterone deposited in eggs. However, this might alternatively be interpreted as supporting the idea that lipophilic steroid hormones preferentially and passively move into and are sequestered by yolk. Future studies will need to determine whether yolk androgen deposition is active or passive and, if the former, the degree to which females can control movement and, therefore influence offspring phenotype. The fitness consequences of maternal hormonal investment on chick begging abilities during nestling competition are the focus of ongoing studies in our long-term study of anis.

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Figure legends

Figure 1. Mean concentration (± SE) of testosterone (T) in egg yolks as a function of year and laying order. The numbers above the x axis represent the number of nests in each category. T levels were significantly greater in late-laid as compared with early-laid eggs in 2003 ($F_{1,22} = 4.46$, P = 0.04) but not in 2004 ($F_{1,8} = 2.27$, P = 0.17).The early laying order category corresponds to eggs laid during the first 20% of the total clutch of eggs while the late laying order category corresponds to eggs laid during the last 20% of the total clutch of eggs. Laying order categories that differ significantly are denoted by different letters above the bars.

Figure 2. Mean concentration (± SE) of testosterone (T) in egg yolks as a function of laying order and group size (GS = the number of laying females in a group). The numbers above the x axis in the figure represent the number of nests in each category. Yolk levels of T were significantly higher in late-laid eggs than in early-laid eggs ($F_{1,38} = 9.63$, P < 0.01) and T levels did not vary with group size ($F_{3,38} = 1.34$, P = 0.27). The early laying order category corresponds to eggs laid during the first 20% of the total clutch of eggs while the late laying order category corresponds to eggs laid during the last 20% of the total clutch of eggs. Laying order categories that differ significantly are denoted by different letters above the bars.

Figure 3. Mean concentration (± SE) of testosterone (T) in female plasma as a function of year and laying order. The numbers above the x axis represent the number of nests in

each category. Female plasma T levels were significantly higher in 2003 than in 2004 (t_{20} = -2.912, P = 0.009). The early laying order category corresponds to eggs laid during the first 20% of the total clutch of eggs while the late laying order category corresponds to eggs laid during the last 20% of the total clutch of eggs. Laying order categories that differ significantly are denoted by different letters above the bars.

Figure 1.

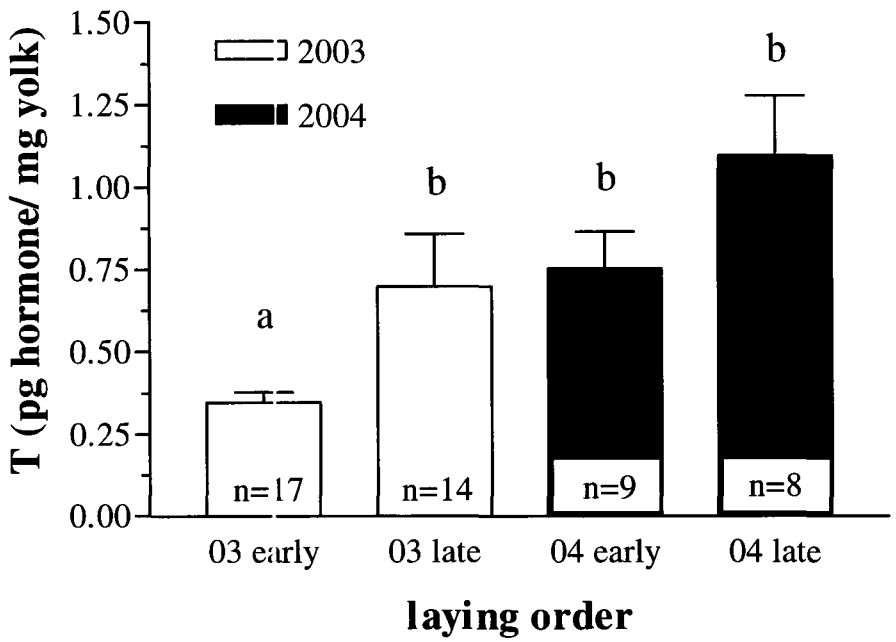

T (pg hormone/ mg yolk)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
2003
2004
a
b
b
b
n=17
n=14
n=9
n=8
03 early
03 late
04 early
04 late
laying order

Figure 2.

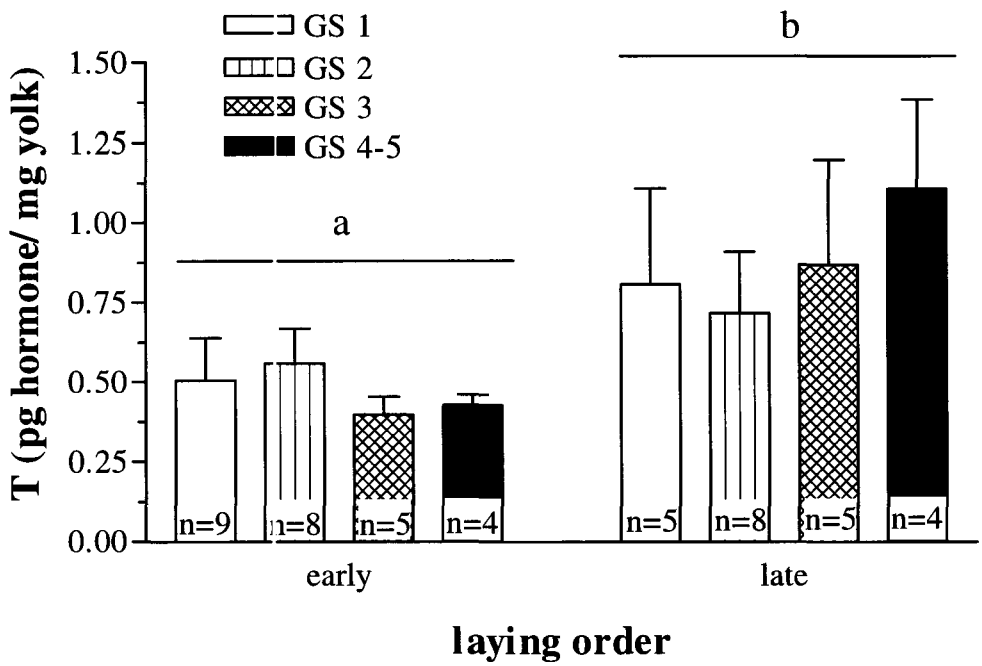
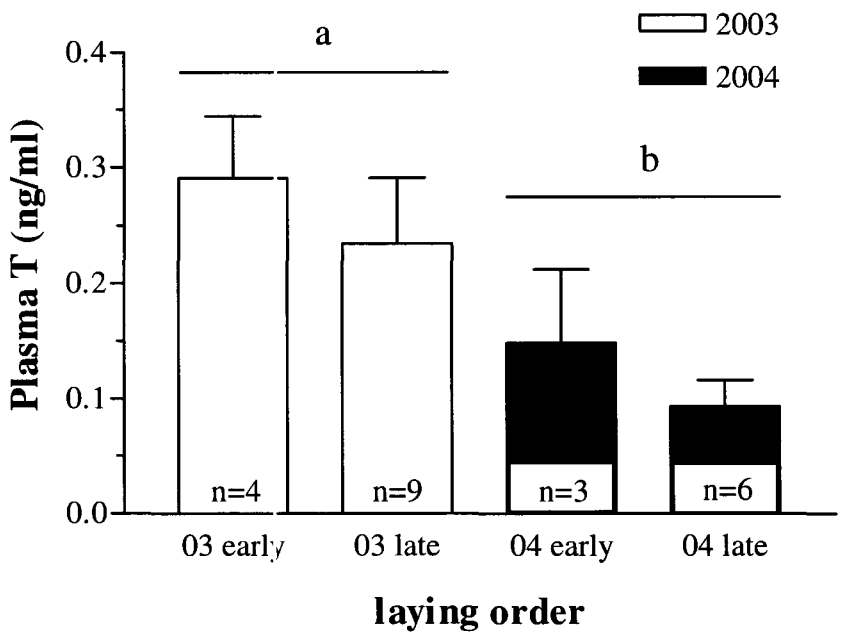

GS 1
GS 2
GS 3
GS 4-5
a
b
T (pg hormone/ mg yolk)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
n=9
n=8
n=5
n=4
n=5
n=8
n=5
n=4
early
late
laying order

Figure 3.


2003
2004
a
b
0.4
0.3
0.2
0.1
0.0
Plasma T (ng/ml)
n=4
n=9
n=3
n=6
03 early
03 late
04 early
04 late
laying order

CHAPTER 4

Maternal estrogens in eggs of a communal breeder: the smooth-billed ani (*Crotophaga ani*).

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Abstract

Avian eggs contain maternally-derived steroid hormones that can have significant effects on offspring phenotype. While numerous studies have focused on the anabolic effects of androgens on offspring development and behavior, little is known of the effects of estrogens. Published research on yolk estrogens suggests that 17β-estradiol (E_2) may have anabolic effects upon offspring development. Furthermore, since estradiol in reproductively active females is involved in the production of yolk precursors and can effect egg mass and size, yolk and plasma levels of E_2 might reflect another manner in which females influence their offsprings' phenotype. We examined female plasma and egg yolk E_2 concentrations in the smooth billed ani (*Crotophaga ani*), a plural-breeding cooperatively breeding joint-nesting species, to determine the relationships between plasma and yolk estradiol levels, laying order, and egg volume. In one year of our two year study, we found that yolk E_2 levels and egg volumes did not vary with laying order. In the other year, both yolk E_2 levels and egg volume increased with laying order while female plasma circulating E_2 levels decreased from the early to late egg-laying stage. Our results suggest that estradiol is another important steroid hormone within yolk that varies with laying order and which may affect offspring phenotype. Additionally, our results suggest that the effect of female plasma E_2 on egg size may be more complex than previously envisioned. Our results further suggest a 'disconnect between plasma and yolk E_2 levels and raise further questions as to whether steroid transfer to the egg is a passive *versus* an active process.

Introduction

It has been more than a decade since Schwabl (1993) first noted the presence of maternally-derived hormones in eggs. Since this discovery, a number of studies have focused on the effects of maternally-derived yolk androgens on offspring development and behavior (for review see Gil 2003; Groothuis et al. 2005). For instance, testosterone (T) has been found to vary with laying order and affect nestling begging behaviour, growth, and social dominance among nest-mates in some species (Schwabl 1993; 1996a; Gil 2003; Groothuis et al. 2005). These studies suggest that patterns of yolk androgen deposition could provide females with a means to manipulate nestling quality within a clutch and, thereby, increase their own fitness (Groothuis et al. 2005). Specifically, females might adjust androgen deposition depending on the magnitude of hatching asynchrony and, thereby influence sibling competition and potentially, survival of later-hatched nestlings (Stoleson & Beissinger 1995; Schwabl et al. 1997). For example, in species with markedly asynchronous hatching, increased androgens in latter laid eggs might mitigate some of the negative effects associate with late hatching (i. e., increase development rates of embryos or nestlings). Support for such adaptive explanations for maternal androgen deposition has been found in some species, including canaries (*Serinus canaria*, Schwabl 1993, 1996a), red-winged blackbirds (*Agelaius phoeniceus,* Lipar et al., 1999a), and cattle egrets (*Bubulcus ibis*, Schwabl et al. 1997). Despite the growing interest in this area of research, with the notable exception of testosterone, other

potentially important steroid hormones that are deposited in egg yolks have largely been neglected.

In his seminal paper, Schwabl (1993) reported that egg yolks contain measurable amounts of sex steroid hormones, including both androgens: androstenedione (A4), testosterone (T), 5α-dihydrotestosterone (DHT), and estrogens: 17β-estradiol (E_2). Because levels of E_2 found in eggs are quite low and, as such, difficult to measure, this steroid hormone has received much less attention than androgens (Williams et al. 2005; Groothuis et al. 2005). To date, to the best of our knowledge only a handful of published studies have focussed on yolk estradiol levels (Adkins-Regan et al. 1995; Schwabl 1993; Williams et al. 2005). Nonetheless, estradiol may be very important in eggs since it can have a number of effects upon development, including sexual differentiation (Adkins-Regan et al. 1995; Balthazart & Adkins-Regan 2002), embryonic development (Balthazart & Adkins-Regan 2002; von Engelhardt et al. 2004), male embryonic mortality (Williams 1999; von Engelhardt et al. 2004), and nestling growth (von Engelhardt et al. 2004). Most studies conducted to date suggest that elevated levels of yolk E_2 levels may be detrimental to the developing embryo (Adkins-Regan et al. 1995; Balthazart & Adkins-Regan 2002; Williams 1999); however, this does not appear to always be the case. In a recent study, when compared with untreated controls, E_2-treated female zebra finches (*Taeniopygia guttata*), laid eggs that 1) were characterized by shorter incubation periods (suggesting increased rates of embryonic development) and 2) produced nestlings that were heavier at day 7 (von Engelhardt et al. 2004). Although yolk E_2 levels were not assayed in the study, the authors argue that the above results were

likely due to the transfer of E_2 to the egg as parental incubation behaviour was not affected by E_2 treatment (von Engelhardt et al. 2004). These results suggest that E_2 in egg yolks has anabolic effects upon offspring development that are similar to those of androgens (Lipar & Ketterson 2000). If so, females could influence offspring phenotype, nestling competition, and nestling survival via differential allocation of both androgens and estrogens to eggs. Furthermore, females could also influence nestling development and survival via differential investment in egg dimensions or mass, quality (e.g., differential make-up of yolk contents, see below), or both.

In consideration of egg quality, it is important to note that during follicle development E_2 plays a key role in regulation of the liver's production of the yolk precursors vitellogenin (VTG) and very low density lipoproteins (VLDL). Because VTG and VLDL are the main sources of yolk proteins and lipids respectively in the egg (Wallace 1985) and because E_2 is also involved in the liver's synthesis and secretion of albumen (Yu et al. 1971), estradiol is thought to play an important role in determining egg dimensions, mass, and quality. In addition to the potential direct effects of yolk E_2 upon offspring development, females could also manipulate offspring phenotype via E_2-mediated differential VTG, VLDL, and albumen contribution to eggs.

Here we examine female plasma and egg yolk estradiol concentrations in a communally breeding joint-nesting species, the smooth-billed ani (*Crotophaga ani*). Anis have an unusual mating system wherein females lay eggs either alone, or in groups of as many as seven females that share a single nest (Quinn & Startek-Foote 2000). Intense egg-laying competition occurs in multi-female groups with eggs laid early having high

probabilities of loss because of egg tossing and burial under nesting material and beneath later-laid eggs (Quinn & Startek-Foote 2000, chapter 2). Egg-laying competition intensifies with increasing group size as both the number of eggs laid and lost per female increase with group size (chapter 2). Following this intense competition, group members, which are usually unrelated to one another, cooperate to incubate eggs and to rear the young. We investigated whether yolk E_2 levels varied with laying order and adult group size; two variables that we considered likely to influence nestling survival and maternal fitness in ani groups. If yolk E_2 has developmental benefits that allow females to 'manipulate' offspring phenotype, then we would expect to find that yolk E_2 levels increase with laying order, group size, or both; similar to what has been previously reported in studies that have considered yolk T levels (Schwabl 1996a; Gil 2003; Groothuis et al. 2005; chapter 3). Additionally, we examined the relationship between egg quality, inferred to be positively correlated with volume, and female plasma E_2 to assess whether females increase egg quality with laying order and whether this is accompanied by increases in plasma levels of E_2 from the early to late egg-laying stages.

Methods

Study sites

We studied smooth-billed anis at the Cabo Rojo (17°59'N, 67°10'W, elevation from 2 to 42 m) and Laguna Cartagena (18°01'N, 67°06'W, elevation from 55 to 71 m) National

Wildlife refuges in south-western Puerto Rico during the rainy season when most ani breeding occurs (September-January, 2003-2004 and 2004-2005, hereafter denoted as 2003 and 2004 seasons, respectively). The south-western portion of Puerto Rico has a dry tropical climate and both Cabo Rojo (587 acres) and Laguna Cartagena (794 acres) refuges contain second-growth dry scrubland, open, and disturbed habitats that attract breeding anis (Loflin 1983, Quinn & Startek-Foote 2000).

Field procedures

Details about group size determination and nest check regime are provided elsewhere (chapter 2). In brief, we checked every active nest daily during egg-laying and marked every new egg with non-toxic markers to indicate egg number in the laying sequence. We sampled eggs that were among the first or last 20 % to be laid in a group's clutch (i.e., early- versus late-laid eggs). We collected yolk samples from freshly laid, un-incubated eggs by inserting a 25 gauge needle through the eggshell at the middle of the long axis of the egg, following the method pioneered by Schwabl (1993). We inserted the needle as far as required to reach the center of the yolk and extracted a small sample (approximately 40 mg) of yolk with a 1 mL disposable syringe. We sealed the insertion hole with New-Skin® liquid bandage (Medtech, Jackson, WY, USA) and returned the egg to its nest. We transferred yolk samples to 1.5-mL eppendorf tubes and froze them until samples were shipped overnight on ice to the University of Memphis (TN, USA) for assay (see below). To verify the uniformity of the sampling method, we also collected

samples from 13 freshly laid intact and marked eggs that had been recently 'tossed' from nests (0-2 day-old eggs). We froze these eggs and, in the laboratory in Memphis, subsequently separated the yolk from the albumen. We then thawed and homogenized the whole yolk, and collected another small sample (approximately 40 mg) for assay. Because Lipar et al. (1999b) found differential levels of hormones across egg layers, we compared hormone values from whole egg yolks with those obtained from egg puncturing (see below). The same eggs where assayed for testosterone and corticosterone content in chapter 3 and 5 respectively.

Adult sampling

We regularly captured adult anis and collected blood samples for hormonal and DNA analyses. Because it takes two to three days for the major portion of the yolk to be deposited in an egg (Romanoff & Romanoff 1949; Schwabl 1996b), we restricted our dataset to blood samples that were collected from females that were caught either within the first three days or the last three days of the period when eggs were being laid in her group's nest. We could not determine whether the captured females were the same females that laid the sampled eggs, so our adult plasma hormone analyses are meant to uncover general patterns of plasma E_2 variation within groups to determine how these plasma levels relate to yolk E_2 levels in early- versus late-laid eggs. The same individuals were assayed for plasma circulating testosterone levels in chapter 3. We captured adults using two 60 mm mesh mist nets (18 m length) stacked one above the other on 7.2m

telescoping poles (Meyers & Pardieck 1993). Adults were also captured using a hardware cloth cylindrical funnel trap that was placed within a group's territory and continuously monitored. The trap allowed easy entry but not egress and contained a caged hand-reared ani to attract territory defenders (Vehrencamp 1977). We collected 300 μL of blood (well under the established 1% of body mass guideline: see McGuill & Rowan 1989) from adults by venipuncture of the jugular vein with a 27 gauge needle and a 1 mL syringe. Samples were kept cool in the field by storing in a cooler. Blood was subsequently centrifuged for 5 min at 4400 g to separate plasma from the red-blood cell fraction that was used for later microsatellite genotyping (Blanchard & Quinn 2001; Gregory & Quinn 2006). We determined the sex of each individual by amplifying an intron in the Chromo-Helicase-DNA binding gene (CHD; Griffiths et al. 1998). Plasma samples were drawn off with a Hamilton® syringe and then frozen and stored in an eppendorf vial until they were shipped to the University of Memphis for hormone assay. All procedures in this study were approved and conducted under a McMaster University Animal Care Permit (AUP 05-07-40).

Radioimmunoassay

All samples from smooth-billed ani eggs were analyzed for the presence of 17β-estradiol (E_2) with a competitive-binding radioimmunoassay (Wingfield & Farner 1975, Ball & Wingfield 1986, Schoech et al. 1996, also see Schwabl 1993 for specific methods that pertain to extraction of steroids from yolk). We weighed a sub-sample (approximately 30

mg) of each yolk sample to the nearest 0.01 mg, added 500 µl distilled water and three glass beads to each sample within an eppendorf vial, and then vortexed the mix repeatedly to homogenize the yolk. We then added approximately 2000 cpm each of [^{3}H]-E_2 (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA) to each yolk sample to calculate the recovery percentages after extraction and chromatography. After further mixing, we let the homogenate equilibrate for at least 12 hr at 4°C prior to extraction. The steroids were extracted from the aqueous phase by adding 3 mL of a petroleum:diethyl ether (30%:70%) solution: the mixture was vortexed, then snap-frozen using dry ice in methanol , and the ether containing the steroids was then decanted. This was done twice for each sample and the resultant 6mL of ether solution was then evaporated in a water bath (30°C) under N_2. We then added 1 mL 90% ethanol to remove excess lipids and stored the samples overnight at -20°C (Schwabl 1993). We separated precipitated proteins and lipids from the ethanol phase by decanting after centrifugation at 1100 g for 15 min. We dried the samples under N_2, resuspended in 0.5 mL 10% ethyl acetate in isooctane, and ran them through chromatography columns that consisted of three parts of a celite:ethylene glycol:propylene glycol (6 g:1.5 mL:1.5 mL) upper phase above one part of a celite:water (3 g:1 mL) lower phase. Increasingly polar volumes of solvents (ethyl acetate:isooctane) that are forced through the columns by pressurized N_2 allow collection of the different steroids based upon their polarity. After the DHT and T fractions were removed, we removed the E_2 fraction from each column with 4.5 mL of 40% ethyl acetate in isooctane, fractions were collected and then dried down under N_2, the sample was then reconstituted in buffer, and stored overnight at 4°C. We compared

duplicate values of each sample to a standard curve that ranged in concentration from 500 to 1.95 pg. Given the large number of samples assayed, yolk samples were assessed in three different assays. The intra-assay coefficients of variation based on six standards measured in each of the three assays were 12.5%, 13.4%, and 7.9%, and the inter-assay coefficient of variation was 13.8%. We assayed female plasma samples (70-100 μL) with a very similar protocol; however, we omitted the 90% ethanol step of the extraction process as this is meant to remove excess lipids from yolk. As was the case for yolk samples, the large number of samples necessitated three assays. The intra-assay coefficients of variation were 16.2%, 13.9%, and 15.6%, and the inter-assay coefficient of variation was 15.1%. Estradiol antibody and standard for both all RIAs were obtained from Biogenesis and Sigma, respectively.

Statistical analyses

We used nonparametric statistics because data were not normally distributed. For each nest under study, we averaged E_2 values from different eggs laid during the early (first 20%) period and late (last 20%) period, producing two average E_2 values per nest (early and late). We calculated egg volumes similarly by averaging volumes from different eggs laid during the early (first 20%) and late (last 20%) period. We used the same eggs for both E_2 and egg volume values. Egg volumes were calculated using Hoyt's (1979) formula: volume (mm^3) = 0.51 LB^2 where B is the maximum width and L the length of an egg measured with calipers (resolution to 0.1 mm). In multi-female nests, each

averaged data point (early or late) represents an average from a variable number of different sampled and marked eggs in a given nest that were laid within 1.4 ± 0.29 d on average. Eggs belonging to the same category (early or late) and nest were therefore, generally laid by different females as individual ani females lay eggs two days apart (Quinn & Startek-Foote 2000). Because of nest failure due to depredation or destruction by high winds, we were unable to sample late eggs from four groups. Therefore, we only include early egg data from those four nests. We monitored and analyzed a total of 86 fresh eggs from 26 different ani groups in 2003 and 2004 during the two years of the study. Of these, a total of 54 eggs had been either tossed or buried. To calculate female plasma E_2 concentrations during the early and late egg-laying stages, we restricted our sampling to females that were caught either within the first three days or the last three days of the period when eggs were being laid in her group. We averaged female plasma E_2 concentrations for the early stage (n = 7) and the late stage (n = 15) and a given female was only included once in the analyses. We used Mann-Whitney U tests and Kruskal-Wallis tests to investigate relationships between estradiol and the variables under study, such as group size and laying order (early versus late). Mann-Whitney U tests were used to examine the effect of laying order (early versus late) and year on female plasma E_2 levels. We examined the relationship between E_2 measurements from both sampling methods (puncturing and whole yolk) with a Spearman rank correlation. Hormone samples below the detection limit of our assays were set at the detection limit as a conservative estimate for statistical analysis. All tests were performed in SPSS 10.0

(SPSS Inc.) as two-tailed tests with an α of 0.05. Means are provided with standard errors (SE).

Results

Yolk estradiol levels

There was a strong year effect on yolk E_2 levels, with higher levels in 2004 than in 2003 (Mann-Whitney U test, N = 48, Z = -4.17, P < 0.001; Fig. 1). Overall, there was no significant effect of laying order on yolk estradiol deposition (Mann-Whitney U test, N = 48, Z = -1.74, P = 0.08). However, yolk levels of E_2 were significantly higher in late-laid eggs than in early-laid eggs in the 2003 season (Mann-Whitney U test, N = 31, Z = -2.14, P = 0.03; Fig. 1) but not in the 2004 breeding season (Mann-Whitney U test, N = 17, Z = -0.43, P = 0.66; Fig. 1). Yolk E_2 levels were not significantly different in single versus multi-female groups in this two-year study (Mann-Whitney U test, N = 48, Z = -0.09, P = 0.93) and when analyzing the years separately (2003 season: Mann-Whitney U test, N = 31, Z = -0.42, P = 0.67; 2004 season: Mann-Whitney U test, N = 17, Z = -1.07, P = 0.28). To investigate whether the between-year difference in yolk E_2 concentration was due to eggs having been sampled at different times within a season, we divided the reproductive season into three periods of 40 days each. We found no evidence that the year-effect on yolk E_2 levels was due to time of collection (Kruskal-Wallis test: N = 48, H_2 = 2.59, P = 0.27). We also considered egg volume and found that egg volumes were

greater in late-laid than in early-laid eggs in 2003 (Mann-Whitney U test, N = 31, Z = -2.3, P = 0.02; Fig. 2), but not in 2004 (Mann-Whitney U test, N = 17, Z = -0.38, P = 0.7; Fig. 2). Because of nest failure, we were unable to sample late eggs from four groups (see above). Excluding the four early data points from the analysis does not change our results. Examination of sampled versus homogenized yolk revealed a significant positive correlation ($r_s = 0.64$, N =13, P = 0.02).

Plasma estradiol levels

Overall, there was no year-effect (Mann-Whitney U test, N = 22, Z = -0.77, P = 0.44) and female plasma E_2 levels were lower in late-laid than in early-laid eggs (Mann-Whitney U test, N = 22, Z = -2.15, P = 0.03). When we limited our analysis to females from multi-female groups (n = 18), we found a similar decrease in plasma E_2 concentration between the early and the late egg laying periods (Mann-Whitney U test, N = 18, Z = -2.55, P = 0.01). Additionally, there were no significant differences in plasma E_2 levels between the two years of the study (Mann-Whitney U test, N = 18, Z = -1.02, P = 0.31). As above, we divided the reproductive season into three equal periods to evaluate whether plasma E_2 levels varied within a reproductive season. Females sampled at different times of the season did not significantly differ in their plasma E_2 levels (Kruskal-Wallis test: N = 22, $H_2 = 0.49$, P = 0.78).

Discussion

During the initial year of our two year study, yolk E_2 levels were higher in late-laid versus early-laid eggs. This is a similar directional trend to what has been reported for androgens in species with a socially monogamous mating system that are not characterized by adaptive brood reduction (Schwabl 1993; 1996a; Lipar et al. 1999a; Groothuis et al. 2005; but see Gil et al. 1999), and this pattern is also similar to what we found in this species (see chapter 3). In species without adaptive brood reduction, hatching asynchrony produces a size and motor-skills disadvantage for later-hatched chicks, and increased yolk androgen levels with laying order have been hypothesized to enable later-hatched chicks to overcome their growth deficit (Gil 2003; Groothuis et al. 2005). If estrogens have similar developmental effects, our observed two-fold increase in yolk E_2 levels between early- and late-laid eggs may serve a similar function. It is interesting to note that patterns of T in ani egg yolks were similar to what we report here for E_2 in both years of our study (chapter 3). However, because the thecal and granulose cells within the follicle respond to the upstream signals of luteinizing (LH) and follicle stimulating hormone (FSH) to produce T and E_2, respectively, and LH and FSH secretion patterns largely mirror one another, perhaps one should expect yolk patterns of these two steroid hormones to parallel one another. This is especially true in the case of estradiol since E_2 is a metabolite of testosterone, and therefore patterns of E_2 may just reflect the patterns seen with T (chapter 3).

As is not uncommon in multi-year studies, our findings are confounded by year effects and, as is often the case may be attributed to inter-year variation in some environmental factor. The main environmental factor that differed between years and; therefore, may explain the observed year differences in our study was the amount of rainfall and the assumed associated increase in resource availability (see Pianka 1988). In 2003, levels of rainfall were higher than in 2004, especially prior to egg-laying (July to September; Lentz et al., in prep). These higher precipitation levels may have affected insect abundance and this, in turn, may have increased egg laying in 2003. Indeed, we found far fewer nests with at least one egg in 2004 (n = 28) than we did in 2003 (n = 40: GS pers. observ.). If female anis are 'capital' breeders and use stored resources (energy or nutrients) for egg production (Drent & Daan, 1980; Norris et al., 2004; Stearns, 1989), food availability in the period prior to the onset of egg-laying would be especially critical. Irrespective of whether a bird is a capital or 'income' breeder (Drent & Daan 1980), access to sufficient resources prior to egg laying is necessary for ovarian and associated tissue growth, as well as recruitment and early development of follicles. Very few studies have looked at the influence of food availability on yolk steroid levels and no studies that we know of have focused on yolk E_2 levels. Results in black-headed gulls (*Larus fuscus*) suggest that females may compensate for lower food abundance by adding more androgens to eggs (Groothuis & Schwabl, 2002; Verboven et al., 2003; but see Sandell et al. 2007). If maternal steroid deposition in eggs is inversely related to food abundance and if our conservative assumption of a positive relationship between rainfall

totals and resource abundance is correct, then our observed patterns of higher yolk steroid hormones, both T and E_2, in 2004, as compared to 2003 is as one might predict.

In our consideration of, plasma E_2 levels of female anis, similar to the findings from the few studies that have, to date examined this issue, we found plasma levels decreased significantly over the laying period (e. g., canaries (Sockman & Schwabl, 1999) and European starlings (*Sturnus vulgaris*; Williams et al., 2004). Because E_2 production is highest in small early stage follicles (Bahr 1983), one might, therefore, expect plasma levels to decrease after laying has begun because fewer follicles are actively secreting steroids given that the follicles that have been recruited for the clutch would have developed beyond this early stage and either have been ovulated or are nearing maturation with ovulation imminent. Our study suggests that maternal deposition of steroid hormones into eggs is not be a mere by-product of female hormonal state, or at least does not co-vary chronologically, as the increase in yolk E_2 levels with laying order are in contrast with the pattern of ani females' plasma circulating E_2 concentrations (see chapter 3). Further support for the idea that yolk steroid levels do not necessarily reflect female plasma levels comes from our finding that yolk E_2 levels were higher in one year of the study (2004 versus 2003), even though female plasma E_2 levels did not vary between years. The relationship between plasma and yolk steroid levels is clearly more complex than previously envisioned. This is further supported by the fact that in anis, testosterone levels in plasma and yolk are also independent of one another (chapter 3). We currently do not know whether females can adjust yolk hormone levels independently of their own plasma levels. We also do not know a mechanism that would make such a

relatively independent transfer of steroids into the yolk possible. While we know of only one other avian species where such a disconnect was found (European starlings: Pilz et al. 2003; Williams et al., 2004), two species of lizards, anoles (*Anolis carolinensis*) and leopard geckos (*Eublepharis macularius*), also are characterized by decoupled levels of yolk and plasma steroid hormones (Lovern & Wade, 2003; Rhen et al. 2006). There is a distinct need for a better understanding of the mechanisms whereby steroid hormones move from thecal and granulosa cells into the yolk, and thereby determine whether this is an active or a passive process. We suggest that the lipophilic nature of steroid hormones may effectively 'pre-destine' their movement into the lipid-rich yolk rather than the aqueous blood stream. Further, as a possible mechanistic explanation for our (and possibly other) findings of higher sex steroid hormone levels in late- versus early-laid eggs, this may merely reflect the fact that late laid follicles have a longer period during which they can 'take up' steroids both from the general circulatory system and local within-ovary movement. Clearly, such a mechanism cannot explain those findings in which steroid hormone levels across a clutch remain static or even decline (Schwabl et al., 1997 ; Gil 2003; Groothuis et al., 2005).

Late-laid eggs also had greater volumes than early-laid eggs in 2003, whereas there were no volume or E_2 differences in 2004. These data do not support the view that circulating female plasma estradiol is the main determinant of egg size or mass (see Introduction), as we found that female plasma E_2 levels decreased during egg-laying in both years of the study whereas egg volumes remained similar or increased with laying order. It should be noted that complete hatching synchrony is quite uncommon and, as a

result, developmental differences across the clutch are virtually the rule. In many passerine species that do not use a brood reduction strategy (i.e., species with relatively synchronous hatching that routinely attempt to fledge all of the young within a brood), egg size tends to remain constant or to increase with laying order (Slagsvold et al., 1984). It has been hypothesized that in species without adaptive brood reduction, such an increase in egg volume serves to compensate for the slightly delayed hatching of the last egg (Slagsvold et al., 1984; Hillstroem, 1999). If, as has been postulated (see above), E_2 is the prime driver of egg size, how does one explain our (and others) finding of an increase in egg volume with laying order while plasma E_2 levels decrease with laying order in the species tested to date (smooth-billed anis and European starlings)? The effect of female plasma E_2 on egg mass or size is almost certainly more complex than previously envisioned. For instance, although exogenous E_2 resulted in elevated plasma VG and VLDL levels in European starlings and zebra finches (Christians & Williams, 1999; Williams & Martyniuk, 2000), a later study from the same research group somewhat paradoxically found that yolk precursor levels in circulation were independent of endogenous plasma E_2 levels in starlings (Williams et al., 2004). Furthermore, treatment of females with E_2 did not result in increased egg mass or size in either starlings or zebra finches (Christians & Williams, 1999; Williams et al., 2005). Clearly, future studies are required to better understand the relationship between female plasma estradiol levels and egg size.

Joint-nesting birds provide a natural framework to explore the influence of within group competition during egg-laying on both circulating hormone levels and on hormone

deposition in eggs. To our knowledge, yolk E_2 concentrations have never been examined in a cooperative breeder. In communal nests, offspring often compete with unrelated nestlings for food and space in the nest while trying to attain a favorable feeding position. Given this, one might expect females to use any means possible to favor their own chicks. If E_2 has beneficial effects upon nestling development (see above), females might be able to give a competitive edge to their chicks via differential allocation of yolk E_2, and this may explain why yolk E_2 levels were higher in late-laid versus early-laid eggs in one year of the study. However, we can only speculate as to mechanisms whereby differential yolk E_2 levels might be beneficial as we have no data on pre- or post-hatching development rates.

Our results suggest that E_2 is another potentially important hormone that varies with laying order and may be used by females to affect egg quality. In addition to the potential effects of yolk E_2 upon offspring development, females could also manipulate offspring phenotype by adjusting egg size or quality via E_2- mediated differential production of yolk precursors. Estradiol has received very little attention to date, possibly because E_2 was seen as detrimental to the developing embryo, levels of E_2 present in the egg are lower than T, or both. We hope that this study will generate more interest and that future studies will lead to a better understanding of the patterns and functions of E_2 deposition in eggs. Von Engelhardt et al. (2004) suggest that yolk E_2 may provide anabolic effects to offspring development similar to that observed from androgen deposition. If anabolic effects are important, then we predict that elevated yolk estradiol levels would lead to the production of offspring with higher growth rates and better

begging abilities. The fitness consequences of maternal hormonal investment on chick begging abilities during nestling competition are the focus of ongoing studies in our long-term study of anis.

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Figure legends

Figure 1. Mean concentration (± SE) of 17-β estradiol (E_2) in egg yolks as a function of year and laying order. E_2 levels were significantly higher in late-laid eggs than in early-laid eggs in 2003 (see text). The early laying order category corresponds to eggs laid during the first 20% of the total clutch while the late laying order category corresponds to eggs laid during the last 20% of the total clutch. Laying order categories that differ significantly are denoted by different letters above the bars. The numbers above the x axis in the figure represent the number of nests in each category.

Figure 2. Mean egg volume (mm^3) (± SE) as a function of year and laying order. Egg volumes were greater in late- than in early-laid eggs in 2003 (see text). The early laying order category corresponds to eggs laid during the first 20% of the total clutch while the late laying order category corresponds to eggs laid during the last 20% of the total clutch. Laying order categories that differ significantly are denoted by different letters above the bars. The numbers above the x axis in the figure represent the number of nests in each category.

Figure 1.

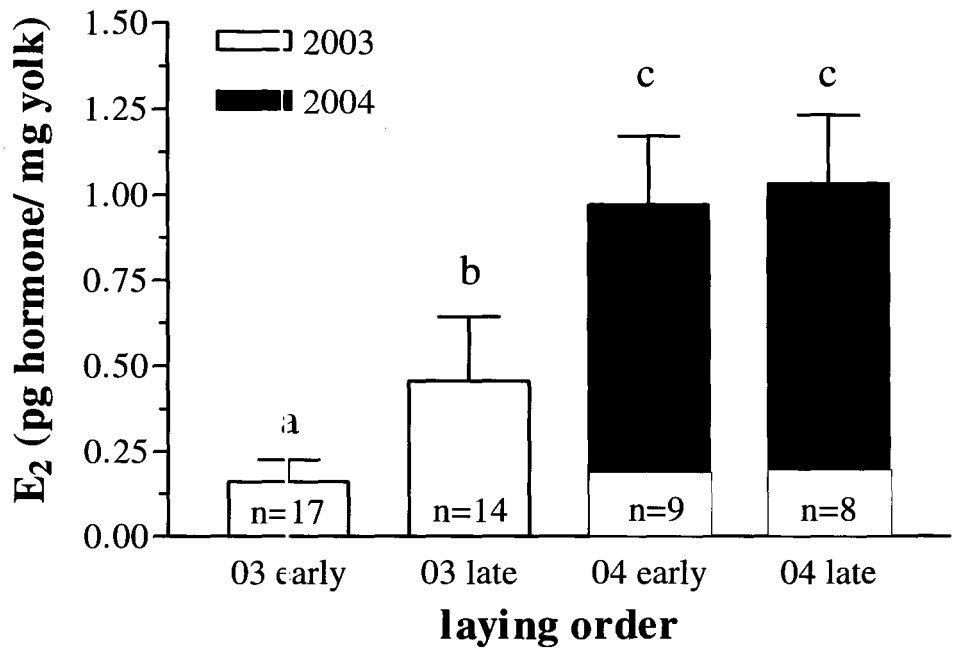
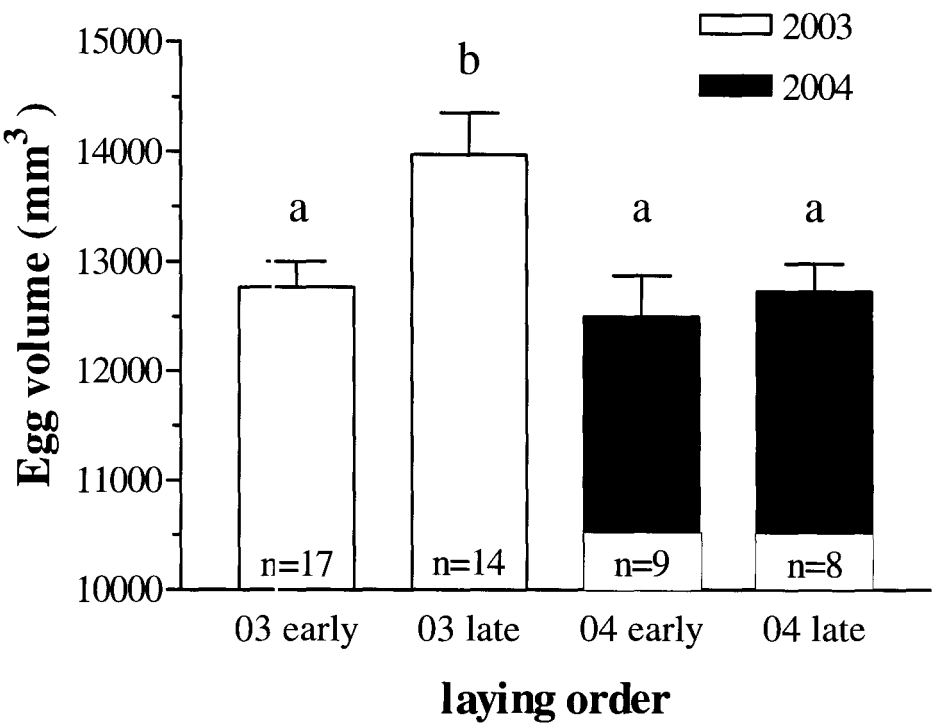

1.50
1.25
1.00
0.75
0.50
0.25
0.00
E_2 (pg hormone/ mg yolk)
2003
2004
a
b
c
c
n=17
n=14
n=9
n=8
03 early
03 late
04 early
04 late
laying order

Figure 2.


2003
2004
15000
14000
13000
12000
11000
10000
Egg volume (mm3)
a
b
a
a
n=17
n=14
n=9
n=8
03 early
03 late
04 early
04 late
laying order

CHAPTER 5

Maternal corticosterone deposition in avian yolk: influence of clutch laying order and group size in a joint-nesting cooperatively breeding species

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Abstract

Assessment of individuals' levels of glucocorticoids (e. g., corticosterone and cortisol) is a widely used method to measure the responsiveness of an individual to a given stressor. These hormones trigger physiological and behavioral responses to stressful events that allow the individual to cope with the stressor. Various environmental perturbations, such as food shortage, adverse weather conditions, predation attempts, and agonistic encounters with conspecifics and individuals of other species often elevate plasma glucocorticoid levels in vertebrates. Because prenatal exposure to maternal glucocorticoids is thought to be detrimental to the growing embryo, maternal stress may have negative carryover effects on offspring fitness. We examined egg yolk corticosterone concentrations in a plural-breeding joint-nesting communally breeding species: the smooth billed ani (*Crotophaga ani*). We investigated whether yolk corticosterone levels varied with clutch laying order and group size, two variables that are likely to influence the level of stress experienced by laying females. Because egg-laying competition leads to physiological and social stress that is intensified with group size and clutch laying order, we predicted that yolk corticosterone levels should increase from the early to the late egg-laying period and from single female to multi-female groups. In this two-year study, we found that yolk corticosterone levels increased from the early to the late egg-laying period in multi-female groups, but not in single-female groups. Results from this study suggest that laying females experience higher levels of stress in multi-female groups and that this maternal stress influences yolk corticosterone concentrations.

This study identifies a novel cost of group-living in cooperatively breeding birds, namely the increase in yolk corticosterone levels and the likely ensuing detrimental effects on offspring development.

Keywords: corticosterone, yolk hormones, cooperative breeding, joint-nesting

Introduction

Avian eggs contain maternally-derived hormones that can have significant effects on embryonic development, post-hatching growth, and offspring survival (Groothuis et al. 2005). Females have been found to vary allocation of hormones to the egg in response to a variety of factors including laying order, paternal quality, and food availability; and further, differential allocation can also affect the sex of the offspring from a given egg (for reviews, see Gil 2003; Groothuis et al. 2005). For instance, testosterone (T) varies with laying order and affects chick begging behavior, growth, and social dominance among nest-mates (Schwabl 1993; 1996; Gil 2003; Groothuis et al. 2005). Overall, these studies suggest that females use yolk hormone deposition to adaptively adjust their reproductive investment in response to the current conditions in a manner that will maximize their fitness (Stoleson & Beissinger 1995; Schwabl et al. 1997; see Gil 2003 & Groothuis et al. 2005 for a review). We emphasize the use of the word *suggest* in the previous sentence. While findings from research in this area are exciting, given contradictory findings across studies and the relatively few published works from which to draw meaningful conclusions, in our opinion caution and further research is needed before one can assume causal links between yolk hormone levels and a variable of interest. Additionally, despite the growing interest in this area of research, there are important steroid hormones (e.g., corticosterone and estradiol) that are known to be deposited in egg yolks have largely been ignored.

In his seminal paper, Schwabl (1993) reported that egg yolks contain measurable amounts of steroid hormones including sex steroid hormones like androgens: androstenedione (A4), testosterone (T), 5-dihydrotestosterone (DHT), and estrogens: 17β-estradiol (E_2), as well as low levels of the adrenal steroid hormone, corticosterone (CORT). Because relatively low levels of CORT are present in eggs, this steroid hormone has received much less attention than androgens (Groothuis et al. 2005), with only a handful of published studies that have focused on yolk corticosterone levels (Hayward & Wingfield 2004; Love et al. 2005; Saino et al. 2005). While corticosterone is an important hormone that can influence both maternal and offspring phenotype, little is definitively known about how it gets into the egg yolk and subsequently affects chick development (Gil 2003; Rettenbacher et al. 2005).

Various environmental perturbations, such as food shortage (Lynn et al. 2003), adverse weather conditions (Wingfield et al. 1983; Astheimer et al. 1995), human disturbance (Wasser 1997; Creel et al. 2002), and predatory encounters (Boonstra et al. 1998; Silverin 1998; Scheuerlein et al. 2001), can elevate plasma glucocorticoid levels in vertebrates. Individuals with high parasitic loads or low body condition also experience high baseline levels of glucocorticoids, such as corticosterone in birds and herptiles (reptiles and amphibians), or cortisol in fish and mammals (note that some animals produce both glucocorticoids and some mammals, e. g., rats, produce corticosterone) (Breuner & Hahn 2003; Kitaysky et al. 1999 a,b; Love et al. 2005). Glucocorticoids trigger what are considered to be adaptive physiological and behavioral responses to stressful events that serve to bring the internal environment of the organism back to

homeostasis (Silverin 1998). These hormones are, therefore, widely used to measure the response of an individual to stress (Broom & Johnson 1993; Von Holst 1998; Sapolsky et al. 2000; Downing & Bryden 2002). High levels of plasma glucocorticoids have been found to suppress reproduction, and affect both foraging and territorial behaviors (Wingfield et al. 1997; 1998; Wingfield & Kitaysky 2002; Salvante & Williams 2003). If the environmental conditions are not harsh to the point where an animal altogether abandons its reproductive effort, but are severe enough to result in elevated CORT levels, for example in a laying female, CORT transferred to yolk may influence offspring phenotype in a number of ways.

Prenatal exposure to maternal glucocorticoids can have negative effects both upon embryonic development (Mashaly 1991; Heiblum et al. 2001; Eriksen et al. 2003), as well as longer-term effects upon other traits, such as offspring body mass (Love et al. 2005; Saino et al. 2005), growth (Hayward & Wingfield 2004; Heiblum et al. 2001; Love et al. 2005), sensitivity to stress (Welberg & Seckl 2001; Hayward & Wingfield 2004), and behavior (Lesage et al. 2004; Rubolini et al. 2005). Corticosterone is produced by the adrenal cortex in response to the blood borne chemical messenger adrenocorticotrophic hormone (corticotrophin, ACTH) that is released from the anterior pituitary in response to the hypothalamic messenger, corticotrophin-releasing factor (CRF) (Holmes & Phillips 1976). And while the adrenal medulla is a known source of sex steroid production, we can find no evidence of gonadal CORT production. Thus, CORT almost certainly enters the egg yolk from the maternal blood circulation. Yolk CORT levels should, to some degree reflect maternal plasma CORT levels as corticosterone, a lipid-soluble hormone,

likely enters the yolk by passive diffusion (Hayward & Wingfield 2004; Groothuis et al. 2005; but see Rettenbacher et al. 2005). But note that the lipid-rich composition of yolk combined with the highly vascularized nature of ovarian follicles and the lipophilic nature of steroid molecules, establishes a scenario where egg yolk could in effect serve as a CORT 'sink'. Because the affinity of CORT for yolk is much greater than it is for plasma, free CORT (i. e., CORT not bound to carrier corticoid binding globulin, CBG), or for that matter CBG-bound CORT, might have a propensity to move into the yolk thus effectively concentrating therein. As a result, yolk levels might represent a longer-term accumulation that doesn't necessarily reflect a laying female's current state. That said, CORT deposition in the egg may: therefore, link offspring development to maternal environmental conditions (Love et al. 2005; Saino et al. 2005). For example, female barn swallows (*Hirundo rustica*) exposed to a predator during egg-laying produced eggs with higher concentrations of CORT than controls (Saino et al. 2005). Further, embryos exposed to higher CORT levels were less likely to fully develop and hatch and, those eggs that did hatch produced young that at fledging were smaller (Saino et al. 2005). Therefore, stressful conditions experienced by mothers may influence egg quality and offspring phenotype, which, in turn, may negatively affect maternal fitness (Sinervo 1998; Rubolini et al. 2005; Saino et al. 2005).

In this study, we examined egg yolk corticosterone concentrations in the smooth billed ani (*Crotophaga ani*), a plural-breeding joint-nesting communally breeding species. Anis are characterized by an unusual breeding system wherein groups of between one and seven socially monogamous pairs share a single nest (Quinn & Startek-

Foote 2000). Intense egg-laying competition occurs in multi-female groups with eggs laid early having high probabilities of loss because of egg tossing and burial beneath later-laid eggs (Quinn & Startek-Foote 2000, chapter 2). This egg-laying competition intensifies as group size increases and both the number of eggs laid and lost per female increase with group size (chapter 2). As a consequence, females in communal groups lay more eggs than females in single groups (6.8 ± eggs versus 5.3 ± eggs, respectively). Following this intense competition, group members, who are usually unrelated to each other, cooperate to incubate eggs and to rear the young (Quinn & Startek-foote 2000). We investigated whether yolk CORT levels varied with group size and clutch laying order (i.e. laying order for all eggs within a specific clutch) and, further, we hypothesize that these two variables likely influence the level of stress experienced by females in ani groups for two different reasons. First, increased competitive interactions with other group members are known to induce a social stress and increase glucorticoid levels in many vertebrate taxa (Abbott et al. 2003; Goymann & Wingfield 2004; Sapolsky et al. 2000; Wingfield et al. 1997). We reason that the increased egg-laying competition experienced by female anis in multi-female groups would induce a similar social stress and we, therefore, predict an increase in yolk CORT levels from single female to multi-female groups. Second, elevated CORT titres coincide with periods of egg production, ovulation, or oviposition in various passerine birds (Hegner & Wingfield, 1986; Kitaysky et al. 1999b; Silverin & Wingfield, 1982; Wingfield and Farner, 1978), and experimental evidence links egg production with elevated plasma CORT levels (Etches et al., 1984; Salvante & Williams 2003; Williams et al., 1985). Salvante and Williams (2003) suggest that the increased

energy expenditure required for egg production is physiologically taxing and is, thereby responsible for the observed elevation in circulating CORT levels during egg-laying. Because reproductive costs and physiological stress likely increase during egg production (Salvante & Williams 2003; Williams 2005), we predict to find an increase in yolk CORT levels from the early to the late egg-laying period. Further, because females in multi-female ani groups both lay more eggs than their single-female counterparts, as well as likely engage in potentially stressful agonistic within-group interactions, we also predict that any clutch laying order effects upon yolk CORT levels should be more pronounced in multi-female groups than in single-female groups. Additionally, given the above referenced expected increase in agonistic encounters over egg laying in multi-female groups that is, by definition absent in single-female groups, we predict yolk levels of CORT to be generally higher in multi- *versus* single-female groups irrespective of laying order.

Methods

Study sites

We studied smooth-billed anis at the Cabo Rojo (17°59'N, 67°10'W elevation from 2 to 42 m) and Laguna Cartagena (18°01'N, 67°06'W elevation from 55 to 71 m) National Wildlife refuges in south-western Puerto Rico during the rainy season when most ani breeding occurs (September-January, 2003-2004 and 2004-2005, hereafter denoted as

2003 and 2004 seasons, respectively). The south-western portion of Puerto Rico has a dry tropical climate and both Cabo Rojo (587 acres) and Laguna Cartagena (794 acres) refuges contain second-growth dry scrubland, open, and disturbed habitats that attract breeding anis (Loflin 1983, Quinn & Startek-Foote 2000).

Field procedures

Details about group size determination and nest check regime are provided elsewhere (chapter 2). In brief, we checked every active nest daily during the egg-laying period and marked every egg with non-toxic markers to indicate egg number in the laying sequence of a given nest. We sampled eggs that were among the first or last 20 % of a group's clutch (i.e., early- versus late-laid eggs). In this study, we were interested in potential differences in CORT levels between early- and laid-laid eggs in the total communal clutch of eggs within a group's nest. Therefore, we use the terminology "clutch laying order" instead of "laying order." We note, however, that although for single-female groups the true "laying order" is known, to facilitate comparisons with multi-females groups, we express and compare eggs from single-group females in the same manner (i. e., compare the first and last 20% of eggs laid). We collected yolk samples from freshly laid, un-incubated eggs by inserting a 25 gauge needle through the eggshell at the middle of the long axis of the egg, following the method pioneered by Schwabl (1993). We inserted the needle as far as required to reach the center of the yolk and extracted a small sample (approximately 40 mg) of yolk with a 1 mL disposable syringe. We sealed the

insertion hole with New-Skin® liquid bandage (Medtech, Jackson, WY, USA) and returned the egg to its nest. We transferred yolk samples to 1.5-mL Eppendorf tubes that were kept cool on ice until return to the lab where they were frozen and stored until shipment overnight on ice to the University of Memphis for assay (see below). We assayed the same eggs for testosterone and estradiol content in chapter 4 and 5 respectively. To verify the uniformity of the sampling method, we also collected samples from 14 freshly laid intact eggs that had been recently 'tossed' from nests (0-2 day-old eggs). We froze these eggs, and then, separated the yolk from the albumen. We subsequently thawed and homogenized the whole yolk, and collected another small sample (approximately 40 mg) for assay. Because both Lipar et al. (1999) and Rettenbacher et al. (2005) found differential levels of hormones across egg layers, we compared hormone values from homogenized whole egg yolks with those obtained from the yolk center by egg biopsy (see below).

Radioimmunoassay

All yolk samples were analyzed for the presence of CORT with a competitive-binding radioimmunoassay (Wingfield & Farner 1975, Ball & Wingfield 1986, Schoech et al. 1996, also see Schwabl 1993 for specific methods that pertain to extraction of steroids from yolk). We weighed a sub-sample (approximately 30 mg) to the nearest 0.01 mg, added 500 µl distilled water and three glass beads to each sample and vortexed them repeatedly to homogenize the yolk-water mixture. We added approximately 2000 cpm of

[^{3}H]corticosterone (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA) to each yolk sample to calculate the recovery percentages after extraction and chromatography. After further mixing, we let the homogenate equilibrate for at least 12 hr at 4°C prior to extraction. The steroids were extracted from the aqueous phase by adding 3 mL of a petroleum:diethyl ether (30%:70%) solution, that was vortexed, then snap-frozen, and the ether containing the steroids was then decanted. This was done twice for each sample and the resultant 6mL of ether solution was then evaporated in a water bath (30°C) under N_2. We then added and vortexed 1 mL 90% ethanol to the tubes to remove excess lipids and stored the samples overnight at -20°C (Schwabl 1993). We separated precipitated proteins and lipids from the ethanol phase by decanting after centrifugation at 1100 g for 15 min. We dried the samples under N_2, resuspended in 0.5 mL 10% ethyl acetate in isooctane, and ran them through hand-packed chromatography columns that consisted of a celite:water (3 g:1 mL) lower phase and a celite:ethylene glycol:propylene glycol (6 g:1.5 mL:1.5 mL) upper phase . Increasingly polar volumes of solvent mixtures (ethyl acetate:isooctane) that are forced through the columns by pressurized N_2 allow collection of the different steroids based upon their polarity. After the DHT, T, and E_2 fractions were removed, we collected the CORT fraction from each column with 4 mL of solution containing equal parts ofethyl acetate and isooctane. Each elute containing the CORT was dried under N_2, the sample was then reconstituted in buffer, and stored overnight at 4°C. We compared duplicate values of each sample to a standard curve that ranged in concentration from 2000 to 7.81 pg. Given the large number, yolk samples were spread across three different assays. The intra-assay

coefficients of variation based on six standards for the three assays were 5.7%, 9.6%, and 10.3%, and the inter-assay coefficient of variation was 8.7%. Corticosterone antibody and standard for both assay procedures were obtained from Biogenesis and Sigma, respectively.

Data and statistical analysis

We used nonparametric statistics as the data were not distributed normally. For each nest under study, we averaged CORT values from different eggs laid during the early (first 20%) period and late (last 20%) period, producing two average CORT values per nest (early and late). In multi-female nests, each data point (early or late) represents an average from different sampled and marked eggs that were laid within 1.4 d (± 0.29) on average. Eggs belonging to the same category (early or late) and nest were therefore generally laid by different females as ani females lay eggs every two days during the laying period (Quinn & Startek-Foote 2000). Because of nest failure due to depredation or destruction by high winds, we were unable to sample late-laid eggs from four groups. Therefore, we only include early egg data for those four nests. We monitored and analyzed a total of 87 fresh eggs from 26 different ani groups in 2003 and 2004 during the two years of the study. Of these, a total of 55 eggs had been either tossed or buried. We used Mann-Whitney U tests and Kruskal-Wallis tests to investigate relationships between corticosterone and group size and laying order (early versus late). We examined the relationship between CORT levels from both sampling methods (puncturing and

whole yolk) with a Spearman rank correlation. All tests were performed in SPSS 10.0 (SPSS Inc.) as two-tailed tests with an α of 0.05. Means are provided with standard errors (SE).

Results

There was no direct significant effect of clutch laying order (early versus late) on yolk levels of corticosterone (Mann-Whitney U test, N = 48, Z = -0.3, P = 0.76; Fig. 1). However, yolk CORT levels significantly increased with group size (Mann-Whitney U test, N = 48, Z = -2.36, P = 0.018; Fig. 2), and this effect was driven by an increase in yolk CORT levels in late-laid eggs (Mann-Whitney U test, N = 22, Z = -2.19, P = 0.025; Fig. 2). Yolk CORT levels did not differ between years (Mann-Whitney U test, N = 48, Z = -0.46, P = 0.64; Fig. 1). To investigate whether yolk CORT levels had a seasonal component that might underlie the observed early- *versus* late-laid difference, we divided the reproductive season into three periods of 40 days each. There was no such seasonality effect within years (Kruskal-Wallis test: N = 48, $H_2 = 4.77$, P = 0.09). Note that exclusion of the four early-laid data points for which we do not have samples from late-laid eggs (due to nest failure, see above) from the analyses does not qualitatively change our results. In addition, CORT levels from egg puncture samples were significantly correlated with levels from the homogenized whole yolk samples ($r_s = 0.86$, N =14, P < 0.001).

Discussion

We found different patterns of allocation of yolk corticosterone in single- and multi-female groups, indicating that increased group size probably acted as a source of stress. It is likely that the increased competition experienced by female anis in multi-female groups induced a social and physiological stress that ultimately resulted in increased yolk corticosterone levels. Unfortunately, the difficulty of trapping anis necessitates use of a trapping method that precludes measurement of circulating plasma levels of CORT (see chapter 3). As a result, we are unable to compare yolk and plasma CORT levels as we have been able in other studies (chapter 2 & 3). Regardless, it is reasonable to assume that CORT that is deposited in yolk is derived from adrenally-produced circulating hormone as we know of no evidence for ovarian CORT production.

We postulate that stress in multi-female ani groups is caused by two different competitive factors. First, the levels of direct competitive interactions (i. e., those that are exclusive from those linked to laying access [see below]), are almost certainly and literally by definition going to be higher in multi-female ani groups than for females that nest singly. As has been found in other group-dwelling species, increased within group competitive interactions induce a social stress that results in increased glucocorticoid levels (Abbott et al. 2003; Goymann & Wingfield 2004; Sapolsky et al. 2000; Wingfield et al. 1997). Second, in multi-female ani groups, there is an intense egg-laying competition between females for access to the layer of eggs that will subsequently be incubated: many eggs are buried beneath later-laid eggs and, as a result are not

successfully incubated (e. g., 56% of all eggs laid in 40 communal nests were lost to either egg tossing or burial; chapter 2). In multi-female groups, the egg-laying period lasts longer and individual females lay more eggs than than do single-female 'groups' (chapter 2). As has been suggested for other species (Salvante & Williams 2003), the increased energy expenditure for egg production may be physiologically taxing, thereby elevating circulating, and in anis, yolk CORT levels. Our findings that yolk CORT levels increased from single-to multi-female groups only in late-laid eggs supports the above two proposed mechanisms: both group size and clutch laying order are important variables that influence yolk CORT depositions in eggs.

Given that to date, relatively few studies have measured CORT levels in yolk and examined their effect upon offspring phenotype, one can not yet state unequivocally that yolk CORT has negative developmental effects. While Japanese quail chicks hatched from eggs laid by CORT-implanted mothers tended to be lighter at hatching, grew more slowly, and showed a higher sensitivity to stress as adults than controls, however, as is explained below, these traits may be beneficial under some circumstances (Hayward & Wingfield 2004). However, the majority of studies support the view that exposure to elevated CORT levels either pre- or post-hatch, are detrimental to offspring development. For instance, Rubolini et al. (2005) found that eggs injected with CORT in yellow-legged gull (*Larus michahellis*) had increased time to hatching, decreased hatchling mass, decreased rate and loudness of late embryonic vocalizations, decreased intensity of chick begging display, and depressed T-cell mediated immune response. This begs the questions; how and why do females transfer corticosterone to the egg? It seems likely that

the transfer of CORT from the maternal circulation to the egg is passive and that CORT produced by the adrenals enters circulation and subsequently moves into the yolk via diffusion (Groothuis et al. 2005; but see Rettenbacher et al. 2005). This view is supported by research in which CORT-implanted female Japanese quail (*Coturnix coturnix japonica*; Hayward & Wingfield 2004) and European starlings (*Sturnus vulgaris*; Love et al. 2005) laid eggs with higher yolk CORT levels than those of controls. Similarly, in natural experiments, females that were exposed to stressful conditions subsequently laid eggs with higher concentrations of CORT that females that did not experience such stress (predation related stress: Saino et al. 2005; social stress: Downing & Bryden 2002). Our results are consistent with a passive transfer of CORT from the maternal circulation to the egg with highest yolk CORT levels in the late-laid eggs of multi-group females.

While the above, to a degree, address the issue of "how" CORT is transferred, the question "why" CORT is transferred to the yolk remains largely unaddressed. This is an especially vexing issue as given its largely negative developmental effects; one might expect that a protective mechanism would have evolved. However, it may be unrealistic to expect a protective mechanism to be in place to respond to transient rapid changes in CORT levels due to exposure to a short-term stressor. This 'argument' does not necessarily apply for cases of chronic or longer term secretion of CORT by the female. It seems that in the communal breeding ani, in which exposure to social stress via laying competition is predictable and occurs regularly and that females that either were capable of suppression of the stress response or possessed some type of protection mechanism (see below) would not subject their developing offspring to negative effects of CORT and

would; therefore; be fitter than females without such characteristics. There are several examples of birds with a suppressed stress response during the breeding season, though Wingfield et al. (1997) postulate that doing so minimizes the expression of hypothesized behavioral effects (e. g., nest abandonment) by the breeders (see Astheimer et al. 1992; and see too Wingfield et al. 1997; Breuner et al. 2003). Clearly, an alternative hypothesis for breeding season dampening of the adrenalcorticoid response to stress is that it serves to minimize yolk CORT levels and accompanying negative effects of CORT upon developing offspring. While dampening CORT production and secretion at its source is one way to minimize the effects of CORT, *immobilizing* CORT in circulation and, thereby rendering it incapable of diffusing into the lipid-rich yolk environment seems a logical alternative protection method. Such a protection method has been hypothesized, though not in this specific regard, by Breuner and Orchinik (2002) who propose corticosteroid binding globulin (CBG) that binds CORT with high affinity as "a tissue buffer against potentially deleterious effects of elevated CORT." While this hypothesized function of CBG has been debated in the context of whether the CBG-CORT complex renders CORT unavailable to tissue versus its serving as a mechanism to deliver CORT to specific tissues (see Schoech et al. 2007), unless ovarian follicles possess specific receptors for the CBG-CORT complex this aquaphilic molecule could serve to render CORT unavailable to yolk.

Alternatively, it has been hypothesized that transference of glucocorticoids from the maternal circulation to the egg may, under certain circumstances, be beneficial. Some recent studies suggest that yolk CORT deposition may be adaptive over the long-term

under suboptimal environmental conditions (Hayward & Wingfield 2004; Saino et al. 2005). More specifically, it is postulated that stress and elevated CORT levels during early development may lead to hyper-responsiveness to stressful stimuli as adults, suggesting HPA axis 'programming' for a stressful environment (Pravosudov & Kitaysky 2006, Seckl & Meaney 2006, Zhang et al. 2006). Further, that maternal exposure to stressors results in increased deposition of CORT into yolk and that this hyper-responsiveness enables the animal to better cope with stressors encountered in a harsh environment. It is clear that the exposure to maternal corticosterone links maternal condition with offspring development (Hayward & Wingfield 2004; Saino et al. 2005). What is less clear; however, is how often offspring benefit from elevated yolk glucocorticoid levels.

We know that glucocorticoid levels may be elevated in females that experience low food availability (Lynn et al. 2003). Further, in yellow-legged gulls, injection of CORT into freshly laid eggs resulted in reduced rates and loudness of late embryonic vocalizations, as well as the intensity of chick begging (Rubolini et al., 2005). It is reasoned that in the event of a food shortage, inducing a slower rate of growth in her offspring may be adaptive for both mother (reduced parental care) and offspring (reduced energetic requirements) and, thereby maximize fitness under the constraints of the local environment (Hayward & Wingfield 2004). Additionally, CORT-induced slower growth and higher levels of 'fearfulness' (see Cockrem 2008) or sensitivity to stress may be beneficial in an environment with high predator densities as exposure in fearful animals is lessened (Hayward & Wingfield 2004; Saino et al. 2005). In those situations, long-term

benefits may be traded against short-term costs such as reduced growth. In this study, in multi-female ani groups, offspring are often not related to each other, chicks vigorously compete for incoming food items, and chick mortality in multi-female ani nests is high (chapter 2.). In a communal joint-nesting system where nest mates belonging to different mothers compete for survival, it is difficult to invoke an adaptive underpinning to CORT in yolks given its multiple negative developmental effects. Seemingly, yolk CORT deposition is a result of passive diffusion that reflects the level of stress experienced by ani females and is a maladaptive byproduct of a physiological response that under most circumstance has adaptive functions for the female.

Although the influence of group size and social stress on circulating CORT levels has been established in previous work (Abbott et al. 2003; Goymann & Wingfield 2004; Sapolsky et al. 2000; Wingfield et al. 1997), very few studies have investigated how various social and environmental variables affect CORT deposition in eggs (Saino et al. 2005). We know of no other study that has examined the influence of group size and laying order on yolk CORT deposition. Such studies are, however, important if we want to understand fully how maternal effects influence offspring phenotype. Results from this study suggest a new potential cost of group-living in cooperatively breeding birds, namely the increase in yolk corticosterone levels that might result in detrimental effects on offspring development and behavior. The potential effects of glucocorticoids on chick behavior are the subject of on-going work.

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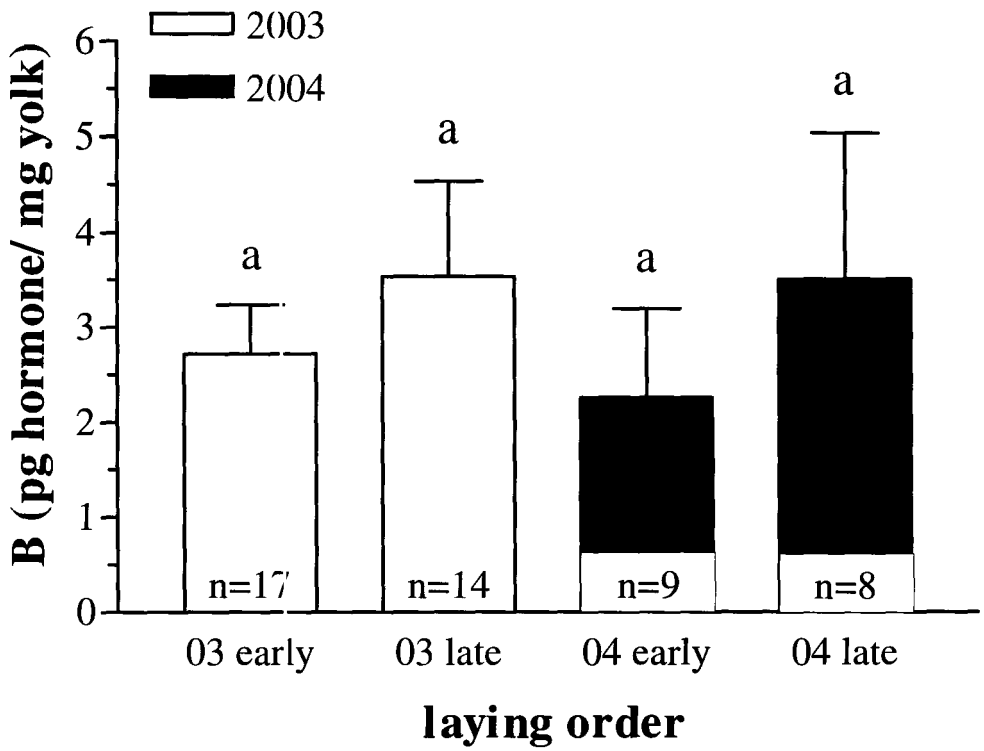
Figure legends

Figure 1. Mean concentration (± SE) of corticosterone (CORT) in egg yolks as a function of year and clutch laying order. There was no significant effect of clutch laying order (early versus late) or year on CORT yolk levels. The early laying order category corresponds to eggs laid during the first 20% of the clutch while the late category corresponds to eggs laid during the last 20% of the clutch. There were no significant differences between the different categories (as denoted by the same letter above the bars). The numbers above the x axis in the figure represent the number of nests in each category.

Figure 2. Mean concentration (± SE) of corticosterone (CORT) in egg yolks as a function of clutch laying order and group size (i.e., GS; the number of laying females in a group) for both years of the study. Yolk CORT levels increased significantly with group size, an effect due to increased yolk CORT levels in late-laid eggs. The early laying order category corresponds to eggs laid during the first 20% of the clutch while the late category corresponds to eggs laid during the last 20% of the clutch. Laying order categories that differ significantly are denoted by different letters above the bars. The numbers above the x axis in the figure represent the number of nests in each category.

Figure 1.


2003
2004
B (pg hormone/ mg yolk)
0
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2
3
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5
6
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n=17
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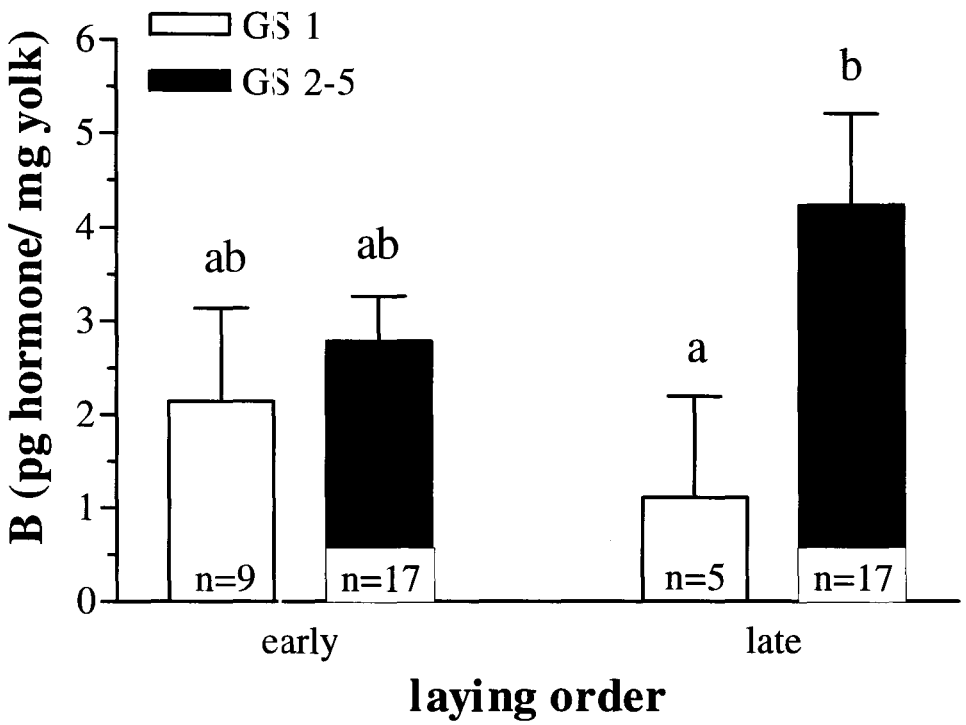
n=9
n=8
03 early
03 late
04 early
04 late
laying order

Figure 2.


GS 1
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early
late
laying order

CHAPTER 6

Effect of relatedness and nestling testosterone on begging behaviour in the communally breeding smooth-billed ani.

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Abstract

The avian egg contains maternal hormones that affect behavior, growth, morphology, and offspring survival. These maternal effects studies suggest that steroid hormones are involved in the regulation of begging behaviour. We examined the potential influence of testosterone (T) on three measures of begging behaviour (maximum height of begging stretch, wing flapping and call rates) in a plural-breeding joint-nesting cooperatively breeding species. We measured circulating plasma T concentrations and begging responses in smooth-billed ani (*Crotophaga ani*) chicks temporarily removed from nests. We found marked variations in T levels between different years and between different broods for chicks at the same age. In one year of this two year study, T levels were higher in nestlings with better begging abilities. In the other year, nestlings hatched from eggs laid late in the laying sequence had better begging abilities. Finally, nestlings more closely related to each other (i.e. from small ani groups) had higher circulating T levels. Taken together these results suggest that: 1) testosterone is an important controlling mechanism of begging behaviour, 2) female T depositions influence begging behavior, and 3) maternal T depositions in eggs have long lasting effects on embryonic and nestling development. Furthermore, results suggest that begging abilities may not always be under control of T levels and that different physiological mechanisms may be at play in different years.

Introduction

Begging displays of nestling birds are often seen as selfish attempts to influence parental food allocation. Models for the evolution and maintenance of begging behaviour predict that this parent-offspring conflict may be resolved by scramble competition between nestlings, or by costly honest signalling of offspring need to parents (Godfray, 1995; Mock and Parker, 1997; Johnstone and Godfray 2002). Despite much research in this area, our understanding of the mechanisms that control begging behaviour during nestling competition remains limited. Such information may facilitate understanding the selective pressures shaping begging behaviour. Recent studies of maternal effects provide new insight, suggesting that steroid hormones are involved in the regulation of begging behaviour.

Studies from several avian species indicate that mothers can affect offspring begging through hormone deposition into eggs (Gil, 2003; Groothuis et al., 2005a; Schwabl, 1993). For instance, increased testosterone (T) levels in later-laid eggs within a clutch resulted in increased mass of the hatching muscle (*musculus complexus*), which allows breaking out of the shell and subsequent flexion of the neck during begging, in red-winged blackbirds (*Agelaius phoeniceus*, Lipar and Ketterson, 2000), as well as higher begging levels and growth rates in canary (*Serinus canaria*, Schwabl, 1996) and black-headed gull chicks (*Larus fuscus*, Eising et al., 2001, 2003). Results from the above studies suggest that maternal testosterone (T) deposition controls T production or some other regulatory mechanism of begging in nestlings. Patterns of yolk androgen deposition

could therefore provide females with a means to manipulate nestling competition within the brood, thereby increasing offspring survival and their own fitness (Gil, 2003; Groothuis et al., 2005a).

It remains unclear how pre-natal maternal exposure to androgens positively affects begging behavior within broods. Maternally derived T is unlikely to influence chick begging behavior directly, as T degrades in a matter of hours (Goymann et al. 2002). Given that endogenous T production in altricial nestlings begins during the embryonic stage (Adkins-Regan et al. 1990; Ottinger & Abdelnabi 1997), a more likely explanation is that maternally derived T influences chick begging indirectly by affecting nestling physiology, namely endogenous T production. To date many studies have looked at the effect of maternal T on offspring phenotype, but very little work has been conducted on the effect of maternal T on endogenous chick T production, or on the effect of circulating plasma chick T levels on begging behavior. Results differ in the few studies that have investigated the potential role of nestling circulating plasma T as a control mechanism of nestling begging behavior. In white storks (*Ciconia ciconia)*, the first-hatched chicks, which had higher plasma T levels, responded faster to feeding events and received more food (Sasvári et al. 1999). In pied flycatchers (*Ficedula hypoleuca*) and thin-billed prions (*Pachyptila belcherie*), nestlings with the most intense begging had the highest plasma T levels (Goodship & Buchanan 2006; Quillfeldt et al. 2006). However, in black-headed gulls, testosterone-implanted chicks had suppressed begging compared to controls (Groothuis & Ros 2005).

In this study, we examined circulating plasma testosterone concentrations in smooth-billed ani (*Crotophaga ani*) chicks. Anis have an unusual mating system wherein females lay eggs either alone, or in groups of as many as seven females that share a single nest (Quinn & Startek-Foote 2000). Intense egg-laying competition occurs in multi-female groups with eggs laid early having high probabilities of loss because of egg tossing and burial under nesting material (Quinn & Startek-Foote 2000, chapter 2). Egg-laying competition intensifies with increasing group size as both the number of eggs laid and lost per female increase with group size (chapter 2). The upper-most layer of eggs (under which earlier-laid eggs may have been buried) represents the combined-clutch of eggs that is incubated to hatching. Following this intense competition, group members that are typically unrelated to each other cooperate to incubate eggs and to rear the young. In single female groups, last-hatched chicks only rarely die in the nest (chapter 2) suggesting that adaptive brood reduction does not occur in this species. In multi-female groups, egg-laying lasts longer and hatching asynchrony is more pronounced (chapter 2). The smallest chicks, which are more likely to die, typically come from eggs laid late (chapter 2). In this typically joint-nesting species, brood reduction would not serve an adaptive function and typically unrelated females should favour their own offspring.

Here, we describe an experiment under controlled single-chick conditions using individuals removed temporarily from ani nests to determine the influence of circulating plasma T levels on begging behaviour. If testosterone is an important controlling mechanism of begging behaviour, we predict that nestlings with higher T levels should have better begging abilities than nestlings with low T levels (prediction 1). In anis, yolk

testosterone levels increase significantly with laying order, both in single and multi-female groups (chapter 3). Therefore, if maternal T depositions in eggs have long lasting effects on embryonic and nestling development, we also predict that ani chicks hatched from late-laid eggs should have better begging abilities than chicks hatched from earlier eggs (prediction 2). Furthermore, in this joint-nesting species, the degree of relatedness among nestlings should vary considerably in nests of different adult group sizes. Here, we investigate for the first time the importance of nestling relatedness levels on nestling begging behaviour and T levels. In multi-female nests, group members, typically unrelated to each other, have to share reproduction. This should lead to low levels of relatedness among nestlings within the brood. Given the increased egg-laying competition experienced in multi-female groups compared to single female groups situations, we predict that nestling begging abilities should be highest in nests where average relatedness values are low (prediction 3).

Materials and methods

Study sites and study subjects

We conducted smooth-billed ani chick begging experiments at the Cabo Rojo and Laguna Cartagena National Wildlife refuges in south-western Puerto Rico (17°59'N, 67°10'W elevation from 2 to 42 m, and 18°01'N, 67°06'W elevation from 55 to 71 m, respectively) during the rainy season (September-January), when most ani breeding

occurs, in 2003-4 (henceforth called 2003) and 2004-5 (henceforth called 2004). The south-western portion of Puerto Rico has a dry tropical climate. Both Cabo Rojo (587 acres) and Laguna Cartagena (794 acres) refuges contain second-growth dry scrubland, open, and disturbed habitats that attract breeding anis (Loflin, 1983; Quinn and Startek-Foote, 2000). During this two-year study, we found a total of 65 active nests with at least one egg laid (n = 37 in 2003, n = 28 in 2004) and 40 active nests with at least one chick hatched (n = 21 in 2003, n = 18 in 2004). Over the two study years, we tested chicks that belonged to 24 nesting ani groups (n = 14 in 2003, n = 10 in 2004). We selected these 24 nests because we had accurate information about brood size, hatching date (hatching = day 0), hatching time (± 5 h), and nestling age (± 5 h) for each chick being tested. We tested 74 chicks during this two-year study (n = 40 in 2003, n = 34 in 2004).

Field procedures

For the 24 focal groups, adult group size was determined before, during, and after the nesting effort, recording both total number of birds and number of banded and unbanded adults whenever visible during each visit to the group's territory (typically every other day). Group compositions and group sizes were different from year to year for any groups on any particular location. Groups were therefore considered independent (see chapter 2 for details). In south-western Puerto Rico, anis use predominantly two species of thorned trees, mesquite (*Prosopis pallida*) and rólon (*Pithecellobium dulce*) for nesting, and do not reuse old nests (Quinn & Startek-Foote 2000). Nests were found by

following adult group members or by checking trees for recent nest structures. Each nest under study was checked daily until egg-laying stopped and eggs felt warm (i.e. incubation started). Nest checks were resumed two days prior the anticipated first hatching date. During the egg hatching period (lasting 2-4 days in general), nests were checked two to three times daily to get accurate hatching date and time for test subjects. Accurate hatching times were also obtained by watching videotapes from a time-lapse camera (Sandpiper Technologies) placed over some of the study nests (n = 8). For identification purposes, we marked new chicks by clipping a toenail on different toes for each individual.

Experimental protocol

We performed our experiments on chicks at 4 days of age when begging is intense but before they become ambulatory. Ani chicks grow rapidly and can climb out of nest if disturbed by day 5 or 6 (Quinn & Startek-Foote 2000). For each nest under study, we selected chicks for which we had accurate age information (age estimation ± 5 h) that were 4 days of age (± 5 h) during routine nest visits. We temporarily (less than 5 h) removed each chick from its nest and transferred it to a field lab location for the experiment. We weighed each chick immediately after removing it from its nest. We placed each nestling alone in a heated artificial nest (Tupperware container lined with black cloth) and covered over with similar black cloth. To monitor temperature in the nest, we placed a temperature probe next to the chick. We monitored temperature (always

observed at 37°C (± 1.5°C) throughout the experiment. The heat source was provided by a lamp placed behind the nest. In front of the nest, we placed a Sony handycam camera (CCD-TRV 138) 0.4 m away from the nestling.

Each experiment started within 20 to 40 min after a given chick was removed from its nest. For the following three hours, we recorded begging trials performed every 15 min (total of 12 begging trials per experiment). For each begging trial, we removed the black cloth cover and stimulated the nestling to beg by making four "chip" noises in succession while waving a 1 mL syringe 5-6 cm above the head of the nestling. The syringe was continuously waved for about 12 s and then the syringe was lowered to feed the nestling. After the chick was fed, we placed the black cloth cover over the nest. Nestlings were fed with a synthetic liquid casein/starch-based diet developed for insectivorous nestlings (Lepczyk et al. 1998). Chicks were fed 0.45 cc of this liquid diet for each trial. We selected this amount of food to approximate natural food deliveries at wild ani nests (based on videotaped observations; GS unpublished data). With this amount of food, we were able to maintain subjects at a constant level of begging (see results). For each begging trial we recorded: (i) the number of nestling calls (i.e. uninterrupted period of buzzing), (ii) the number of wing flaps, (iii) the begging stretch determined by estimating the distance from the highest begging position of the head relative to the lowest (resting) position in units estimated using the size of the nestling's head , and (iv) the trial duration (in s from the start of the begging stimulus until feeding event). We then calculated a calling rate and a wing flapping rate by dividing the number of nestling calls and the number of nestling wing flaps by the trial duration. All chicks

were blood sampled just after the last (12^{th}) feeding event to assess circulating plasma T concentration. Chicks were then weighted again (accuracy 0.1 g), fed to satiation, and returned to their original nest. Each chick was only tested once. All experimental trials were performed by the same observer (GS). The observer assigned an individual arbitrary code (e.g. D5) for each experimental chick to ensure blind scoring of the videos. Videotapes were scored by two naive observers.

Blood sampling and genotyping

Blood (150 µl) was collected by jugular venipuncture with a 30 gauge needle with a 1 mL syringe. Pressure to the venipunctured hole was maintained for 20 s with a finger after sampling reduces hematomas. Blood was subsequently centrifuged for 5 min at 4400 g to separate plasma from the red-blood cell fraction that was used for later microsatellite genotyping (Blanchard and Quinn, 2001; Gregory and Quinn, 2006). We determined the sex of each nestling by polymerase chain reaction (PCR) amplifying an intron in the Chromo-Helicase-DNA binding gene (CHD; Griffiths et al., 1998). We were unable to determine sex in one instance because CHD gene amplification failed. To calculate within group relatedness levels among chicks, we genotyped all nestlings belonging to the study groups (i.e. we sampled all nestmates of chicks included in trials; n = 142) at four different microsatellite markers (ANI500C5, ANI500C14, ANI450B2, ANI9546). Conditions for the amplification and electrophoresis of the four *C. ani* have previously been described ((Blanchard and Quinn, 2001). Alleles frequencies were

calculated using the Cervus software (Cervus; Marshall *et al.* 1998) and a large number of both adult and nestling anis (n = 640). Relatedness values were calculated using the Kinship software (Kinship; Goodnight & Queller 1999). We were unable to determine relatedness in one nest (2 nestlings) because DNA amplification failed for one of the nestling. Plasma samples were frozen until they were shipped to the University of Memphis for assay (see below). Procedures in this study were approved and conducted under a McMaster University Animal Care Permit (AUP 05-07-40).

Radioimmunoassay

We assayed the concentration of plasma testosterone with a competitive-binding radioimmunoassay (Ball and Wingfield, 1986; Schoech et al., 1996; Wingfield and Farner, 1975). Detailed information of our assaying protocol is provided elsewhere (chapter 3). Briefly, we added approximately 2000 cpm each of [3H] testosterone (New England Nuclear Corp., Boston, MA) to each sample to calculate the recovery percentages after extraction and chromatography. After mixing, we let the homogenate equilibrate for at least 12 hr at 4°C prior to extraction. We used 70-100 µL of plasma to start the extraction procedure. The steroids were extracted from the aqueous phase by adding 3 mL of a petroleum:diethyl ether (30%:70%) solution, that was vortexed, then snap-frozen, and the ether containing the steroids was then poured off. This was done twice for each sample and the resultant 6mL of ether solution was then dried under N_2. We separated precipitated proteins and lipids from the ethanol phase by decanting after

centrifugation at 1100 g for 15 min. We dried the samples under N_2, resuspended in 0.5 mL 10% ethyl acetate in isooctane. We ran the samples through chromatography columns that consisted of a celite:ethylene glycol:propylene glycol (6 g:1.5 mL:1.5 mL) upper phase and a celite:water (3 g:2 mL) lower phase. We collected the T fraction in each column with 4.5 mL of 20% ethyl acetate in isooctane. We compared duplicate values of each sample to a standard curve that ranged in concentration from 500 to 1.95 pg. We measured all plasma T samples in three different assays. The intra-assay coefficients of variation based on six standards for the three assays were 6.4%, 10.6%, and 11.1%, and the inter-assay coefficient of variation was 9.3%.

Data and Statistical analysis

Plasma testosterone values were transformed using the square root transformation ($\sqrt{(X+1)} + \sqrt{(X)}$) to meet the requirements of parametric tests. We performed a principal component analysis (PCA) on our three measures of begging (nestling begging stretch, wing flapping rate, and call rate) to evaluate whether these variables were correlated to one another, and if so, to combine them in a single measure of begging (PCA axis 1). We were able to determine nestling origin (i.e. the egg from which the offspring hatched) in 50 instances. For those nestlings, we calculated an egg order index for each egg within the communal clutch to determine the effect of laying order on begging abilities. To do so, we divided the position of a given egg in the clutch by the total number of eggs laid (chapter 2). For instance, if a chick hatched from the second egg laid in a clutch of 10

eggs, this nestling would have an egg index score of 0.2. We calculated average relatedness levels within nests by averaging relatedness levels among all nestlings belonging to a given group. We constructed three different general linear mixed models (GLMMs) to evaluate the influence of different variables on nestling testosterone and begging behavior. In the first model, we aimed at testing whether nestling testosterone levels were independent of other explanatory variables of begging behavior. We evaluated whether variations in time of day, date, year, nest brood size, nestling sex, nestling weight, and average relatedness levels within nests could explain variations in nestling testosterone. In the second model, we evaluated whether variations in time of day, date, year, nest brood size, nestling sex, nestling weight, average relatedness levels within nests, and nestling testosterone could explain variations in begging intensities (i.e. begging score from PCA). In the third model, we added the egg order index to the list of variables that could potentially affect begging intensities. We also included in each analysis a nest of origin variable (nest ID) to account for the non-independence of nestlings from the same group. For each analysis, we removed in a stepwise fashion biologically relevant interactions and main factors that were not significant. To investigate inter-family variations in testosterone, mean T levels (± SE) were calculated for 15 families in which three or more nestlings were tested. All tests were performed in SPSS 10.0 (SPSS Inc.) as two-tailed tests with α set at 0.05. Averages are provided with standard errors (SE).

Results

Begging variables and overall begging score

The concordance between the scoring of the two observers was high for the three measures of begging behavior (nestling begging stretch: ($r_s = 0.93$, N =30, $P < 0.001$; wing flapping rate: ($r_s = 0.97$, N =30, $P < 0.001$); call rate: ($r_s = 0.95$, N =30, $P < 0.001$).

We observed within-subject variance in levels of calling rates, wing flapping rates, and begging stretches during the experiment as chicks took a few trials to get used to the conditions and as chicks came into the experiment at different hunger levels. After an hour of feeding trials, chick begging stabilized and reached a plateau for each of the three begging variables. We calculated an average chick calling rate, wing flapping rate, and begging stretch based on the last 7 trials of the experiment as the individual slopes for the three begging variables did not significantly differ from zero for those trials.

The first principal component (PCA1 defined as begging score) explained most of the total variance in calling rate, wing flapping rate, and begging stretch 87.4 % and was the only axis retained in the analysis (Eigenvalue of 2.6 for PCA1 and 0.2 for PCA2).

T and family effect

There was a strong year effect in this study as nestling T levels were significantly higher in 2003 than in 2004 (Table 1; Fig. 1.). There was also a significant inter-family

effect as T levels varied between different ani nests (nest ID variable) (Table 1; Fig. 1.). There was a significant interaction between average within group relatedness values and nest ID (Table 1), indicating that the extent to which T was affected by average within group relatedness values depended on nest ID. Overall, T levels increased as average within group relatedness values increased (r_p= 0.30, N= 55, P = 0.01; Fig. 2.). There was a significant interaction between brood size and year (Table 1) indicating that year influenced the relationship between brood size and T.

T and begging behavior

There was a significant interaction between nestling testosterone and year (Table 2), indicating that the extent to which begging was affected by T depended on the year effect. Begging score significantly increased with increasing values of T in 2003 (GLMM in 2003: $F_{1,36}$ = 55.03; P < 0.001; Fig. 3) but not in 2004 (GLMM in 2004: $F_{1,30}$ = 0.10; P = 0.75; Fig. 3). In a separate analysis, egg order significantly varied with begging score (Table 2). Begging score significantly increased with egg order in 2004 (GLMM in 2004: $F_{1,21}$ = 5.83; P = 0.02; Fig. 4) but not in 2003 (GLMM in 2003: $F_{1,19}$ = 0.09; P = 0.76; Fig. 4). In this analysis, the effect of nest ID became marginally significant as sample size decreased (n = 50 here instead of n = 74 in the previous analysis).

Discussion

In this study, we investigated for the first time the influence of nestling T levels on begging abilities in a cooperatively breeding species. Results from this study support our first prediction in one year of this two-year investigation. In 2003, testosterone levels were higher in nestlings with better begging abilities (prediction 1; Fig. 3), paralleling findings in previous studies that focused primarily on socially monogamous species. Support for the effect of nestling T on begging behavior has been found in different species: white storks (Sasvári et al. 1999), pied flycatchers (Goodship & Buchanan 2006), and thin-billed prion (Quillfeldt et al. 2006). However, not all species fit this potential direct positive effect of testosterone on begging behavior (reversed relationship in black-headed gulls; Groothuis & Ros 2005). Our results suggest that, under certain ecological conditions, testosterone is an important controlling mechanism of begging behaviour. Furthermore, the current results suggest a causative effect of T on begging behavior. However, the majority of studies conducted to date, including this one, remain correlative. Future studies should therefore directly manipulate circulating T levels in nestlings to establish a causal link between T levels and begging behavior.

In addition to important year effects, results of this study suggest that there are marked differences in T levels from one brood to the next for chicks at the same age (Fig. 1). Such differences may explain between brood effects: why begging levels can be very different from one nest to the next (Price & Ydenberg 1995). Within brood variations (the standard errors within each column: Fig. 1) along with egg-laying order effects found in

this study could potentially explain the differences in begging between nest mates (Lotem 1998), along with brood hierarchical effects (Schwabl 1993, Groothuis et al. 2005a), and differences in growth rates within clutches (Mock and Parker, 1997; Nisbet et al. 1998).

In 2004, begging abilities covaried with the egg order index: nestlings hatched from eggs laid late in the laying sequence had better begging abilities, independent of brood size. Since in anis, yolk T levels increase with laying order in single and multi-female groups, these results suggest that: 1) under certain ecological conditions and in certain years, female T depositions influence begging behavior, and 2) maternal T depositions in eggs have long lasting effects on embryonic and nestling development (prediction 2). Studies that link maternal condition and investment in offspring are scarce (Groothuis et al., 2005a). Evidence to date suggests that maternal yolk T depositions may affect begging behavior by increasing metabolism and growth rate (Smith & Montgomerie 1991; Schwabl 1996), enhancing perception of cues which stimulate begging behavior (Andrew 1975), or by increasing mass of muscles associated with begging (Lipar & Ketterson 2000). Interestingly, despite the significant difference in nestling T level in 2003 and 2004, levels of begging remained similar in both years (Fig.1 & 3). This result suggests that different physiological mechanisms may be at play in different years, depending on the environmental conditions. Therefore, begging abilities may not always be under control of T levels. Such year-to-year variations were found in other studies (Goodship & Buchanan 2006). Alternatively, there may not be a causal relationship between T and begging behavior, but T production may be a proxy for indices of condition. Furthermore, late-hatching young live in an environment where they

have to compete with older nestlings. This may ramp up their begging intensity of necessity, especially in years of low food availability (see below). This may help explain the differences observed between 2003 and 2004. Future manipulative work is needed to link maternal depositions, to nestling testosterone, and begging behavior.

Various environmental factors are thought to influence T deposition in eggs or circulating T levels in birds (Groothuis et al., 2005a, 2005b; Eising et al., 2004; Hau et al., 2000; Stutchbury & Morton, 2001). In our study, the main environmental factor that may explain why nestling T levels were higher in 2003 and why begging abilities increased with T levels in 2003 but not in 2004 is the amount of rainfall and associated correlates of prey availability. In 2003, levels of rainfall were higher than in 2004, especially prior to egg-laying (July to September; Lentz et al., in revision). These higher precipitation levels may have affected insect abundance, which in turn may differentially affect nestling growth rates and T levels (Smith & Montgomerie 1991).

To our knowledge, this study is the first to investigate the effect of relatedness on begging and nestling T levels. Overall, we found no effect of the average relatedness level on begging score (prediction 3 not supported). However, relatedness levels were correlated with nestling T levels and nestlings that were more closely related to each other had higher circulating T levels. Nests in which average nest mate levels of relatedness are high tend to be small ani groups in which females lay fewer eggs than in larger ani groups (correlation between adult group size and average levels of relatedness within nests: $r_p = -0.58$; $p < 0.001$). Females may trade-off between the number and quality of eggs laid (Nager et al., 2000). In multi-female groups, egg-laying competition

is manifested in an increase in the number of eggs laid per capita, as well as an increase in the egg-laying period (chapter 2). This increased egg-laying investment may limit a female's ability to deposit additional resources (hormones, yolk, albumen, antioxidants) in eggs in multi-female groups. Therefore, the finding that closely related nestlings have elevated T levels may be specific to joint nesting species where egg-laying competition occurs.

Numerous studies have focused on the anabolic effects of androgens on offspring development and behavior (see introduction). Joint-nesting birds provide a natural framework to explore what influences hormone depositions in eggs have on hormonal levels in offspring and ultimately on begging behavior. In this study, we found that nestling T levels varied with brood size. Therefore, nestling T levels may be influenced by: 1) maternal effects and, 2) social factors (i.e. number of competing nestlings). Experimental cross-fostering manipulations of nestlings would improve our understanding of these two important factors that influence begging behavior. Future studies are also needed to determine the relative importance of maternally and endogenously produced T levels to begging behavior. Such studies would also shed light on how and to what extent females can influence offspring phenotype via hormonal depositions.

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Table 1. Variables in the general linear mixed model (GLMM) which explain individual variation in nestling testosterone of 4-day old smooth-billed ani chicks during 3 h feeding experiments. The variables included were time of day, date, year, nest brood size, nestling sex, nestling weight, average relatedness levels within nests, and nest number (n = 74). The analysis was conducted over the two-year period (2003 and 2004).

source	d.f.	*F*	*p*
nestling testosterone			
year	1	10.84	0.002
nest ID	1	3.85	0.054
nest brood size	1	5.72	0.020
relatedness	1	11.01	0.001
year X nest brood size	1	5.16	0.026
nest ID X nest brood size	1	5.15	0.027
nest ID X relatedness	1	8.68	0.004

Table 2. Variables in the general linear mixed models (GLMM) which explain individual variation in begging score of 4-day old smooth-billed ani chicks during 3 h feeding experiments. The variables included in (a) were time of day, date, year, nest brood size, nestling sex, nestling weight, average relatedness levels within nests, nest number, and nestling testosterone (n=74). The analysis was conducted over the two-year period (2003 and 2004). In (b), the egg order index was added to the analysis which reduced sample size from n = 74 to n = 50.

source	d.f.	*F*	*p*
(a) *begging score*			
nestling testosterone	1	2.82	0.098
year	1	13.16	0.001
date	1	7.85	0.007
nest ID	1	7.38	0.008
year X nestling testosterone	1	4.85	0.031
(b) *begging score*			
nestling testosterone	1	3.08	0.086
year	1	12.42	0.001
date	1	5.40	0.025
nest ID	1	3.96	0.053
egg order index	1	4.93	0.032
year X nestling testosterone	1	5.20	0.028

Figure legends

Figure 1. Mean (±SE) testosterone (T) levels of 15 broods (A-O) of smooth-billed anis chicks (N=59) sampled at 4 d old in 2003 (A-H) and 2004 (I-O).

Figure 2. The relationship between the average within group relatedness and circulating testosterone (T) levels in 4 d old smooth-billed ani nestlings in 2003 (filled circles) and 2004 (open circles).

Figure 3. The relationship between begging intensity (begging score) and circulating testosterone (T) levels in 4 d old smooth-billed ani nestlings in 2003 (filled circles) and 2004 (open circles).

Figure 4. The relationship between begging intensity (begging score) and egg order in 4 d old smooth-billed ani nestlings in 2003 (filled circles) and 2004 (open circles).

Figure 1.

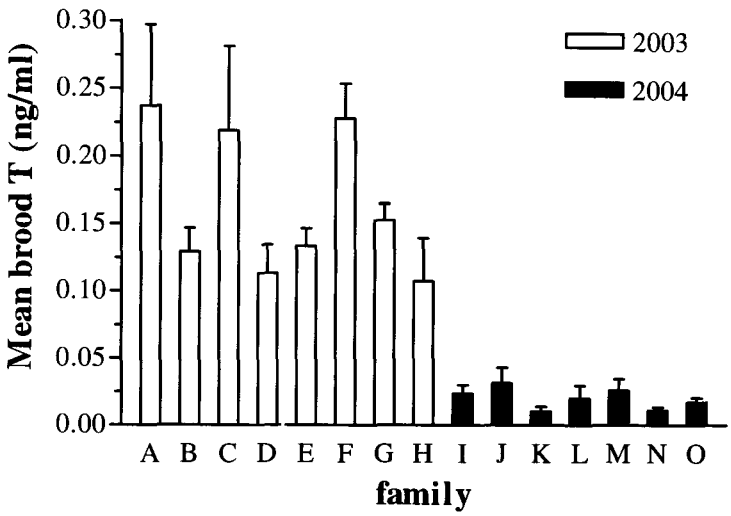

0.30
0.25
0.20
0.15
0.10
0.05
0.00
Mean brood T (ng/ml)
2003
2004
A B C D E F G H I J K L M N O
family

Figure 2.

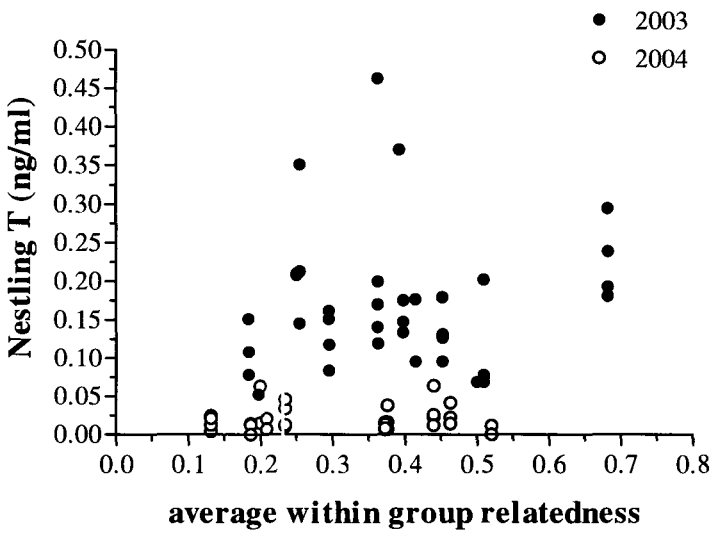

2003
2004
Nestling T (ng/ml)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
average within group relatedness

Figure 3.

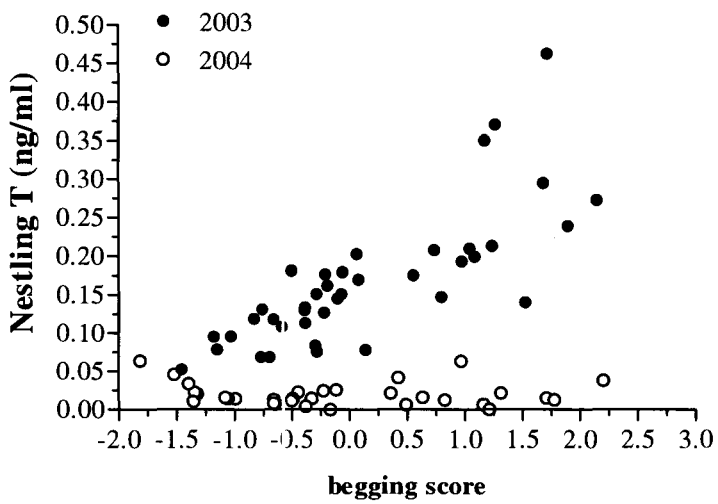
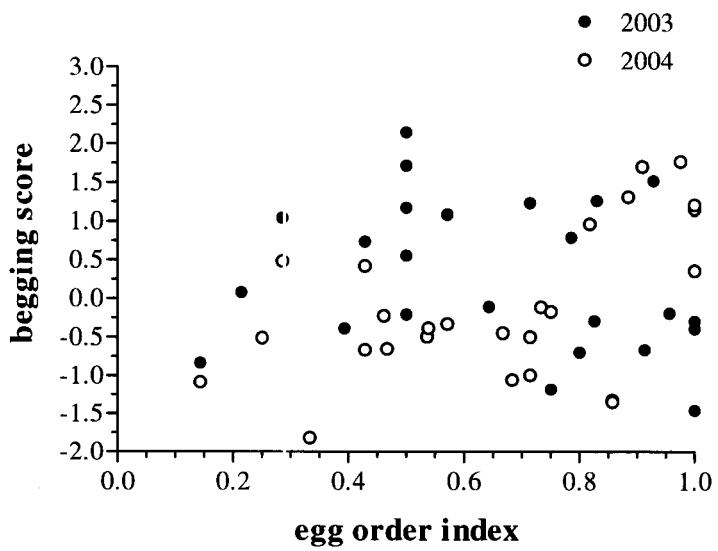

2003
2004
Nestling T (ng/ml)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0 2.5 3.0
begging score

Figure 4.


2003
2004
begging score
egg order index
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
0.0
0.2
0.4
0.6
0.8
1.0

CHAPTER 7

Synthesis and Recommendations for Future Research

7.1 Maternal Effects in Joint-Nesting Birds

It has been generally acknowledged that mothers in oviparous species not only provide information about environmental conditions to their offspring, but also strongly influence the environmental conditions their offspring will experience. The main objective of this thesis was to examine the maternal effects that take place in a joint-nesting species and to evaluate whether these maternal effects were adaptive from the mother's perspective. Different lines of evidence suggest that mothers adaptively manipulate offspring phenotype. First, females responded to increased competitive pressures in larger ani groups by adjusting their egg-laying behaviour (chapter 2) in a manner that is consistent with an adaptive strategy (i.e. trying to skew the content of the incubated clutch of eggs in their favour). Furthermore, in both single- and multi-female groups, yolk testosterone and estradiol deposited by females in eggs increased from early- to late-laid eggs (chapter 3 and 4). This suggests that ani females can influence nestling competition and chick survival by within-clutch differential steroid hormone allocation. Increases in yolk steroid levels over the laying sequence may function to mitigate the disadvantage of being a later-hatched chick in species that lack adaptive brood reduction. In one year of the study, egg volumes increased with laying order (chapter 4). It has been hypothesized that in species without adaptive brood reduction, such an increase in egg volume serves to compensate for the slightly delayed hatching of the last eggs (Slagsvold et al. 1984; Hillstroem 1999). Finally, in the first year of a two-year study, circulating plasma testosterone levels were higher in nestlings with better begging abilities. Additionally, nestlings hatched from eggs laid late in the laying

sequence had better begging abilities in the second year of the study. Taken together these results suggest that female testosterone depositions in eggs have long lasting effects on offspring development and behavior, and this may enhance both maternal and offspring fitness. Furthermore, as anticipated (section 1.9), both group size and laying order influenced maternal strategies. I will look at each of these in turn.

7.2 Influence of Group Size on Maternal Effects

Most clutch size and maternal effect studies have focused on species that breed singly as socially monogamous pairs. Studies of communally breeding joint-nesting species are essential if we want to better understand the influence of group size in shaping the development of behavioral and life-history strategies. Results from this thesis clearly indicate that in a communally breeding joint-nesting species there is intense egg-laying competition and egg loss (chapter 2). These competitive behaviours are very important in explaining results found in this study. One important conclusion is that maternal egg-laying behaviour and physiological status are strongly influenced by group size. Tossing and burial were absent from single female groups but these two behaviours became more and more prevalent as group size increased. Egg loss was common in multi-female groups and can be seen as a clear indicator of the intense egg-laying competition that exists in multi-female groups (chapter 2). Females responded to this increased competition in multi-female groups by laying more eggs, apparently as a strategy to negate the detrimental effects of egg tossing and burial (Vehrencamp 1977; Macedo 1992; Macedo et al. 2004).

Patterns of allocation of yolk corticosterone were different in single- and multi-female groups (chapter 5). It is likely that the increased competition experienced by female anis in multi-female groups induced a physiological and/or social stress that ultimately resulted in increased yolk corticosterone levels (Abbott et al. 2003; Goymann and Wingfield 2004; Sapolsky et al. 2000; Wingfield et al. 1997). For instance, the increased energy expenditure for egg production experienced by females in multi-female groups may be physiologically taxing, thereby elevating circulating and yolk corticosterone levels (Salvante and Williams 2003). It therefore seems that group living strongly influences egg-laying strategies and that the selective pressures facing pair-nesting socially monogamous species and communal joint-nesters can be very different.

7.3 Influence of Laying Order on Maternal Effects

As highlighted above, group size effects may influence the development of behavioural strategies that are specific to communal joint-nesters. However, results from this thesis suggest that pair-nesting socially monogamous species and communal joint-nesters can be remarkably similar when it comes to the differential allocation of sex steroid hormones deposited into eggs. Results reported in chapters 3 and 4 supported the predictions of the hatching asynchrony adjustment hypothesis. Testosterone and estradiol levels were higher in late-laid versus early-laid eggs, paralleling findings in previous studies that focused primarily on socially monogamous species. Support for the hatching asynchrony adjustment hypothesis has been found in different socially monogamous species: canaries (Schwabl 1993, 1996a), red-winged blackbirds (Lipar et al. 1999), and

cattle egrets (Schwabl et al. 1997). Here, hatching asynchrony adjustment occurs in communal breeders as testosterone and estradiol levels increased with laying order in both single- and multi-female nests. In other words, females behaved similarly with regards to sex steroid hormonal deposition (independent of group size). Therefore, group size, a variable that influence both egg-laying behaviour and corticosterone deposition (see section above) seems to have very little effect on sex steroid hormone deposition in eggs. In communal joint-nests, nestlings often compete with unrelated nestlings for food and space in the nest while trying to attain a favorable feeding position. Given this rivalry, one might expect yolk androgen levels to increase with adult group size. Such an increase in yolk testosterone levels could be explained either by an adaptive female investment in response to the heightened levels of competition her offspring will likely encounter at the nest, or by an incidental physiological response whereby mothers in multi-female groups have higher levels of circulating plasma testosterone levels that result from increased agonistic interactions. Interestingly, our results suggest that female yolk testosterone deposition patterns are independent of group size, suggesting a response that is constrained by either evolutionary or physiological processes, or both. This result contrasts with the fact that corticosterone levels increased with laying order in multi-females groups, and suggests that mechanisms of transfer of sex steroid hormones and glucocorticoids may be different (see below).

7.4 Hormone Deposition in Eggs: Passive Diffusion or Active Transfer?

Results from this thesis suggest that two different mechanisms are at play when it comes to the transfer of steroids into eggs. First, our results are consistent with a passive transfer of glucocorticoids from the maternal circulation to the egg with highest yolk corticosterone levels in the late-laid eggs of multi-group females (chapter 5). These results are similar to natural experiments where females that were exposed to stressful conditions subsequently laid eggs with higher concentrations of corticosterone than females that did not experience such stress (Downing and Bryden 2002; Saino et al. 2005). Therefore it is likely that corticosterone produced by the adrenals enters the systemic circulation and subsequently moves into the yolk via diffusion (Hayward and Wingfield 2004; Groothuis et al. 2005; Love et al. 2005).

Second, our results are consistent with the idea that females can control the amount of sex steroid hormones (i.e. testosterone and estradiol) deposited in eggs. We observed variation in testosterone and estradiol levels with laying order, and a mismatch between female plasma and yolk sex steroid levels (chapter 3 and 4). This active transfer hypothesis is supported by the handful of species (canaries, European starlings, anoles lizards, and leopard geckos) in which both plasma and yolk sex steroid levels have been examined (albeit in different studies). For instance, in canaries, female plasma testosterone levels peak the day before the first egg is laid and then decrease until reaching a minimum about four days after the first egg is laid (Schwabl 1996b). Similarly, estradiol plasma levels decrease significantly over the laying period in canaries (Sockman and Schwabl 1999). However, in canaries, yolk sex steroid levels increase with

laying order (Schwabl 1993). Therefore, results form the canary studies and this thesis suggest that maternal deposition of sex steroid hormones into eggs is not be a mere by-product of female hormonal state.

7.5 Hormone Deposition in Eggs: Similarities in Testosterone and Estradiol Profiles

Results from the canary and starling studies (see above) along with the results from this thesis (chapters 3 and 4) reveal similarities in the transfer of both testosterone and estradiol into the egg, although estradiol is present in eggs in lower levels. Since the upstream production of estradiol and testosterone is regulated by the same hormones (luteinizing (LH) and follicle stimulating (FSH) hormone) one may expect the patterns of variation for testosterone and estradiol to largely mirror one another. If so, we could wonder if it is worth spending time and resources to study both hormones. I argue that it is for two reasons. First, we currently do not know if estradiol has similar anabolic effects than testosterone. Such an understanding is essential if we want to better understand how maternal effects shape offspring phenotype. Second, it has been postulated that estradiol is a prime driver of egg size (chapter 4; Williams et al. 2005). If so, the study of the transmission of estradiol into eggs is very important because it could potentially increase our understanding of the mechanisms that control egg size, another important maternal effect that affect offspring phenotype and survival.

7.6 Influence of Environmental Variations on Maternal Effects

In chapters 3, 4, 5, and 6, various hypotheses were tested in 2003 and 2004. I found that many of the variables under study varied from one year to the next (see summary table below). For instance, I found increases in egg volumes and in yolk testosterone and estradiol levels with laying order in one year of the study and not the other. Similarly, endogenous testosterone levels covaried strongly with begging intensities in one year but not the other. The main environmental factor that may explain variations between years was the amount of rainfall and associated correlates of prey availability. Various environmental factors are thought to influence steroid deposition in eggs (Groothuis et al. 2005). In our study, the main environmental factor that may explain the observed year differences was the amount of rainfall and associated correlates of prey availability. In 2003, levels of rainfall were higher than in 2004, especially prior to egg-laying (July to September; Lentz pers com). These higher precipitation levels may have affected insect abundance. Food availability prior to egg-laying may be an important factor influencing female egg-laying behavior if females use stored resources (energy or nutrients) for reproduction (Drent and Daan 1980; Norris et al. 2004; Stearns 1989). Furthermore, access to sufficient resources prior to egg laying is necessary for ovarian and associated tissue growth, as well as recruitment and early development of follicles. Variations in food availability may also have affected insect abundance, which in turn may differentially affect nestling growth rates and testosterone levels (Smith and Montgomerie 1991). Such year-to-year variations were also found in another study that examined the influence of nestling testosterone on begging intensities (Goodship and

Buchanan 2006). One important conclusion from this multi-year study is that certain important variables such as begging abilities may not always be under control of testosterone levels and that different physiological mechanisms may be at play in different years within the same species.

When we look at the overall results in this thesis, it is important to try to understand the results in the context of year effects (see summary table below). Few studies have looked at the influence of food availability on yolk T levels. In black-headed gulls, food supplemented females produced eggs with lower yolk T levels while the yolk T levels pattern over laying sequence remained unaffected (Verboven et al., 2003). Results in black-headed gulls suggest that females compensate for lower food abundance by adding more androgens to eggs (Groothuis and Schwabl, 2002). This may explain whay we have found higher yolk sex steroid hormones in 2004 compared to 2003. When we look at circulating levels of testosterone in both females and nestlings, we observed higher levels of testosterone in 2003 compared to 2004. . If food abundance was higher in 2003, both females and nestling may be in better condition and this may potential affect testosterone levels positively (Smith & Montgomerie 1991). In 2003, begging could then be influenced by high levels of circulating testosterone in nestlings. If females were indeed in better condition in 2003, this may also explain why we observed increased egg volumes in 2003 compared to 2004.

Summary table of years effects on various variables measured in this thesis.

Year	2003	2004
Yolk testosterone	Low	High
Yollk estradiol	Low	High
Yolk corticosterone	Similar in 2003 and 2004	Similar in 2003 and 2004
Egg volume	High	Low
Female Testosterone	High	Low
Nestling Testosterone	High	Low
Chick Begging and testosterone levels	Begging similar in 2003 and 2004. Begging and testosterone correlate positively	Begging similar in 2003 and 2004.No correlation between testosterone and begging

7.7 Long-Term Consequences of Maternal Effects

Numerous studies have focused on the anabolic effects of androgens on offspring development and behavior. Evidence to date suggests that maternal yolk testosterone depositions may affect begging behavior by increasing metabolism and growth rate (Smith and Montgomerie 1991; Schwabl 1996a), enhancing perception of cues which stimulate begging behavior (Andrew 1975), or by increasing mass of muscles associated with begging (Lipar and Ketterson 2000). In anis, nestlings hatched from eggs laid late in the laying sequence had better begging abilities, independent of brood size in 2004

(chapter 6). Since in anis, yolk testosterone levels increase with laying order in single and multi-female groups (chapter 3), these results suggest that: 1) under certain ecological conditions and in certain years, female testosterone depositions influence begging behavior, and 2) maternal testosterone depositions in eggs have long lasting effects on embryonic and nestling development.

7.8 Costs of Group-Living in Anis

There are many costs associated with group-living in anis. Some of these costs include shared parentage and the resulting decrease in per capita reproductive success, or increased chick mortality in multi-female ani nests (chapter 2; Quinn and Startek-Foote 2000.). Larger communal groups also take more time to synchronize egg-laying since more females need to lay eggs at roughly the same time for this synchronization to occur. Group membership in larger communal groups may therefore come with additional costs when compared to smaller communal groups. A similar result was found in greater rheas were nests with over 30 eggs had longer laying periods and had lower hatchability (Fernández and Reboreda 1998). This cost contributed to lower reproductive success per capita with increasing group size in anis and other crotophagids (Koford et al. 1990; Macedo 1992; C. Lentz, J. Quinn, G. Schmaltz, unpublished data). Furthermore, egg destruction is an extreme manifestation of reproductive competition and affected over half of all eggs laid communally. Such egg production waste is predicted by "tug-of-war" skew models (Johnstone 2000; Reeve and Shen 2006). Group living therefore appears to come with short-term egg production costs to females (Monaghan and Nager 1997;

Vézina and Williams 2002; Williams 2005). If females trade-off between production of eggs and efficiency at incubating eggs or rearing chicks, both mothers and offspring may incur additional survival costs (Monaghan and Nager 1997; Monaghan et al. 1998). Interestingly, males perform most of the energetically costly nocturnal incubation in anis. This male nocturnal incubation may be an important feature in the evolution and maintenance of joint-nesting systems by freeing females from nocturnal incubation duties, thus allowing them to spend more energy during egg-laying and less during incubation (Vehrencamp 2000).

Results from this thesis reveal additional costs that may have been overlooked previously. Because prenatal exposure to maternal glucocorticoids is thought to be detrimental to the growing embryo, maternal stress (experienced in large groups with high levels of egg-laying competition) may have negative carryover effects on offspring fitness. Although the influence of group size and social stress on circulating corticosterone levels has been established in previous work (Abbott et al. 2003; Goymann and Wingfield 2004; Sapolsky et al. 2000; Wingfield et al. 1997), very few studies have investigated how various social and environmental variables affect corticosterone deposition in eggs (Saino et al. 2005). Results suggest a new potential cost of group-living in cooperatively breeding birds, namely the increase in yolk corticosterone levels that might result in detrimental effects on offspring development and behavior. In conclusion, group-living in joint-nesting species is unlikely to lead to high short-term per capita reproductive benefits (Vehrencamp 1977; Koenig 1981; Macedo 1992). Furthermore, in smooth-billed anis and guira cuckoos, dispersal options do not seem to

be constrained by a lack of suitable breeding territories (Macedo and Bianchi 1997; C. Lentz, J. Quinn, G. Schmaltz, unpublished data; but see Koford et al. 1986). Given this lack of habitat saturation and the reproductive costs highlighted in this thesis, it seems that lifetime fitness benefits are required to explain the maintenance of this social system. Long-term field studies are therefore needed to clarify individual lifetime benefits associated with communal breeding in joint-nesting species. I will provide below additional ideas and needs that will need to be addressed in the future.

7.9 Directions for Future Research

Anabolic effects of estradiol – It is generally acknowledged that testosterone has anabolic effects upon offspring development. However, we still know very little on the effect of estrogens onto offspring development. If estrogens have similar developmental effects than testosterone, then future studies should measure these estradiol levels in eggs to better understand the environmental conditions in which the embryo develops. Furthermore, such studies will also help us better understand the importance of estrogens in determining egg volume. The effect of estrogens on egg mass or size is almost certainly more complex than previously envisioned. For instance, although exogenous estrogens resulted in elevated plasma protein levels in European starlings and zebra finches (Christians and Williams 1999; Williams and Martyniuk 2000), a later study from the same research group somewhat paradoxically found that yolk precursor levels in circulation were independent of endogenous plasma estrogen levels in starlings (Williams et al. 2004).

Influence of environmental variables – Studies that focus on the influence of nestling testosterone on begging behaviour are very recent. One important conclusion from this thesis is that testosterone levels do not always correlate with begging abilities within the same species. This finding suggests that future studies should include at least two years of data (preferably under different controlled conditions) in order to assess the influence of environmental variations (e.g. food availability) to begging behaviour.

Maternal hormonal influence on begging behaviour – Nestling testosterone levels vary with brood size in anis. Nestling testosterone levels can potentially be influenced by two different factors: maternal effects and nestmate rivalry (i.e. direct aggressive interactions or scramble competition for incoming food items). Experimental cross-fostering manipulations of nestlings would improve our understanding on how these two factors influence testosterone levels and begging intensities. Studies that link maternal condition and investment in offspring are scarce (Groothuis et al. 2005). Future manipulative studies are needed to link maternal depositions, to nestling testosterone, and begging behavior. Such studies would establish a causal link between testosterone levels and begging behavior, and would also shed light on how and to what extent females can influence offspring phenotype via hormonal depositions.

Passive or active process for sex steroid hormones – Our results are consistent with the idea that females can control the amount of sex steroid hormones deposited in eggs.

However, this might alternatively be interpreted as supporting the idea that lipophilic steroid hormones preferentially and passively move into and are sequestered by yolk. Future studies should investigate whether the lipophilic nature of steroid hormones may effectively 'pre-destine' their movement into the lipid-rich yolk rather than aqueous plasma. However, even if such a mechanism were to exist, we still cannot explain those findings in which steroid hormone levels remain static or even decline across a clutch (Schwabl et al. 1997 ; Gil 2003; Groothuis et al. 2005). Clearly, the relationship between plasma and yolk sex steroid levels is more complex than previously envisioned.

Stress and corticosterone in females – It is reasonable to assume that corticosterone that is deposited in yolk is derived from adrenally-produced circulating hormone as we know of no evidence for ovarian corticosterone production. However, direct manipulative studies with corticosterone implants could shed some light as to whether the uptake of corticosterone in the egg is a passive process. Future egg removal studies could also help us better understand whether social or physiological costs are responsible for the increased yolk corticosterone levels in eggs. One would predict that removing more eggs would encourage females to lay more eggs (increase in physiological costs) while not affecting direct aggressive interactions between individuals (holding social costs constant).

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