

Testosterone, social status and parental care in a cooperatively breeding bird



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ABSTRACT

The steroid hormone testosterone not only plays an important role in gamete production, but also influences social and aggressive behavior. Testosterone varies seasonally, peaking when competition for mates is high and declining during parental care. Surprisingly, little is known about how testosterone mediates social conflict and parental care behavior in highly social species like cooperative breeders, where group members compete for breeding opportunities and provide parental or alloparental care. We examined how testosterone differs across breeding roles in the tropical cooperatively breeding superb starling, *Lamprotornis superbus*. We determined whether testosterone was elevated in larger groups, and whether testosterone was negatively related to total levels of parental and alloparental care. We found that male breeders had higher testosterone than male helpers and female breeders and helpers during incubation. However, breeding males exhibited a significant decline in testosterone from incubation to chick rearing, and all individuals had similar levels during the chick rearing stage. Additionally, helpers—but not breeders—in large social groups had higher testosterone than those in small groups. Finally, testosterone was not correlated with nestling provisioning rates during chick rearing, suggesting that natural variation in the low levels of testosterone observed during periods of high parental care does not affect nestling provisioning. Together, these results offer insight into how testosterone is related to breeding roles, intra-group conflict, and parental care in a highly social species.

1. Introduction

Testosterone is a steroid hormone that is not only critical for male spermatogenesis, but also plays a role in the development of secondary sexual characteristics—such as song and plumage among others (Lindsay et al., 2011; Van Roo, 2004; Wickings and Dixson, 1992; Wingfield et al., 1990)—suggesting that it can indirectly influence fitness (McGlothlin et al., 2010). Testosterone may also influence reproductive success due to its role in regulating aggressive behavior in both males and females during the breeding season (Hau et al., 2000; Muller and Wrangham, 2004; Sandell, 2007). Aggressive interactions often vary seasonally, typically peaking prior to mating and decreasing with the onset of parental care behavior (Wingfield et al., 1990). Testosterone follows similar seasonal patterns, reaching lowest levels during non-breeding periods and increasing during breeding for sperm production and reproduction (Hau, 2007). When intra-specific competition for mates is high, testosterone can increase above breeding levels before decreasing rapidly once mating has occurred and parental care begins. Elevated breeding testosterone is not maintained for prolonged

periods because it can be costly, leading to immune suppression and even increased mortality (Casto et al., 2000; Hau, 2001; Nolan et al., 1992; Olsson et al., 2000; Wingfield et al., 1990). Similarly, testosterone can inhibit parental care behavior such that individuals that maintain high levels of testosterone throughout offspring rearing often reduce their parental care investment, which could subsequently lead to reduced reproductive success (Horton et al., 2010; Lynn et al., 2009; Nunes et al., 2000; Rosvall, 2013; Silverin, 1980). Therefore, testosterone secretion is thought to be tightly regulated during breeding, increasing when aggressive behavior is important for reproduction but decreasing when the costs of maintaining high levels outweigh these benefits. Despite these general patterns, the role of testosterone in shaping aggression and parental care varies greatly between species, as some species may be behaviorally insensitive to the hormone (Lynn et al., 2005)—elevated testosterone is not always associated with increased aggression (Wiley and Goldizen, 2003), and the trade-off between high testosterone and reduced parental care is not observed in all species (DeVries and Jawor, 2013; Ketterson et al., 2005).

In cooperatively breeding species where both breeders (i.e. parents)

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and helpers (i.e. alloparents) care for young, the role of testosterone in shaping behavior remains unclear. Cooperatively breeding species are often characterized by complex social hierarchies in which one or more dominant individuals monopolize reproduction (Nelson-Flower and Ridley, 2015; Young et al., 2006). For example, in cooperatively breeding pied kingfishers, *Ceryle rudis* (Reyer et al., 1986), bell miners, *Manorina melanophrys* (Poiani and Fletcher, 1994), superb fairy wrens, *Malurus cyaneus* (Peters et al., 2001), and Florida scrub jays, *Aphelocoma c. coerulescens* (Schoech et al., 1991), dominant males have elevated testosterone relative to helper subordinate males, suggesting that testosterone is related to social status in males of these species (DuVal and Goymann, 2011; Wingfield et al., 1990). In contrast, testosterone does not differ between breeder and helper males in red-cockaded woodpeckers, *Picoides borealis* (Khan et al., 2001; Malueg et al., 2009), Harris's hawks, *Parabuteo unicinctus* (Mays et al., 1991), or Australian magpies, *Gymnorhina tibicen* (Schmidt et al., 1991). Thus, it remains unclear why testosterone levels differ between male breeding roles or with social status in some species of cooperatively breeding birds but not others.

Even less is understood about how testosterone relates to social status and influences behavior in females of cooperatively breeding species, particularly in birds. As in males, female testosterone levels in many avian species peak during the breeding season but remain low during the non-breeding season (Ketterson et al., 2005). Testosterone is secreted during pre-ovulation and is important for female reproduction as a precursor for estradiol synthesis (Johnson, 2000; Staub and De Beer, 1997). In eusocial mole-rats, *Cryptomys hottentotus natalensis* and *Fukomys damarensis*, and cooperatively breeding African cichlids, *Neolamprologus pulcher*, breeding females have been shown to have higher testosterone than helper females (Lutermann et al., 2013; Aubin-Horth et al., 2007, respectively). Testosterone has also been suggested to play a role in shaping female dominance hierarchies in other species that live in social groups, including chacma baboons, *Papio hamadryas ursinus* (Beehner et al., 2005) and spotless starlings, *Sturnus unicolor* (Veiga et al., 2004). Females in cooperatively breeding species—particularly plural cooperative breeders where multiple males and females breed per group—are as likely as males to experience elevated social conflict for access to breeding positions, mates, or nesting sites (Clutton-Brock and Huchard, 2013; Rubenstein and Lovette, 2009). Yet, very little is known about testosterone in females of cooperatively breeding bird species. Thus, testosterone may help shape both male and female breeding roles in cooperatively breeding species, though little data exist from avian species.

Although breeders in cooperatively breeding systems are often socially dominant to helpers (Clutton-Brock et al., 2004; Nelson-Flower and Ridley, 2015; Rubenstein, 2007d) and may have elevated testosterone during periods of high social conflict (Sandell, 2007; Vleck and Brown, 1999; Wingfield et al., 1990), they still invest in parental care, often to a greater degree than helpers (Carranza et al., 2008; Rubenstein, 2016; Valencia et al., 2006). Therefore, cooperatively breeding systems are useful for examining how testosterone and parental care covary because offspring care represents a mixture of both parental and alloparental care and varies widely among individuals; care may differ with breeding roles (Mumme et al., 1990; Rubenstein, 2006), among the sexes (Brouwer et al., 2014), with relatedness to the offspring (Browning et al., 2012), with individual body condition (Van de Crommenacker et al., 2011), or with environmental conditions (Wiley and Ridley, 2016). Yet, surprisingly little is known about how testosterone affects natural variation in offspring care in cooperatively breeding species. More generally, testosterone has been shown to decrease in breeders during chick rearing in both polygamous (Silverin, 1980; Wingfield, 1984) and monogamous species (Ketterson et al., 1992; Van Roo, 2004). In cooperative species, breeders may be able to reduce parental care investment compared to non-cooperative species, since alloparents may be able to compensate for reduced parental care from breeders. However, since among-group conflict in both males and

females over resources or territories, as well as within-group conflict over mates, breeding sites, or breeding roles is likely to be high in cooperative breeders (Hau et al., 2008; Peters et al., 2001; Smith et al., 2005), testosterone may be modulated differently in cooperative species during chick rearing because of the presence of alloparents. Thus, cooperatively breeding species may offer unique insight into testosterone's role in shaping male and female behaviors.

Cooperatively breeding species of birds (Jetz and Rubenstein, 2011) and mammals (Lukas and Clutton-Brock, 2017) are overwhelmingly found in tropical environments with low and variable rainfall, though studies of both tropical and cooperatively breeding species and testosterone are still rare. Seasonal changes in testosterone in tropical species are thought to be dependent upon the length of the breeding season and the degree of territoriality, as well as environmental conditions, which can differ between tropical and temperate species (Goymann et al., 2004). Here we examine how testosterone varies across breeding roles and with group size, as well as how it relates to parental care behavior, in the plural cooperatively breeding superb starling, *Lamprolaima superbus*. In this sexually monomorphic species, since multiple breeding pairs reproduce within each social group, both breeding males and females face competition for mates (Apakupakul and Rubenstein, 2015), and both sexes use song in a social context (Pilowsky and Rubenstein, 2013). First, we determined whether testosterone differed between sexes and among breeding roles in two breeding stages: prior to offspring care (i.e. incubation), when social conflict remains high due to asynchronous breeding, extra-pair mating, and mate switching; and during chick rearing, when social conflict decreases in favor of parental care, which is crucial for successfully raising young. We predicted that testosterone (1) would be higher in breeders of each sex relative to helpers because breeders are dominant to helpers in superb starlings and helpers rarely breed despite being sexually mature (Rubenstein, 2007b, 2007d), and (2) would decline in both sexes from incubation to chick rearing, when individuals shift to performing parental and alloparental care. Next, we examined how group size and the proportion of individuals breeding in a group influenced testosterone, as larger groups or the availability of breeding positions may be proxies for increased social conflict (Shen et al., 2014). We predicted that individuals in larger groups, but those with fewer breeding positions, would have higher testosterone as there may be more conflict over breeding roles when there are fewer available. Finally, we explored whether testosterone is associated with parental or alloparental care, predicting that individuals with elevated testosterone during chick rearing would provide reduced nestling care as measured by nestling provisioning rates (Hau, 2007; Wingfield et al., 1990).

2. Materials and methods

2.1. Study system

We have studied a population of free-living superb starlings at the Mpala Research Center in Laikipia, central Kenya (0°17'N, 37°52'E) since 2001. All individuals from nine social groups have been uniquely marked with a numbered metal band and a combination of four colored bands (Rubenstein, 2007a). Superb starlings breed twice per year during the short (approx. Nov–Dec) and long rainy seasons (approx. Mar–Jun). These periods have an increased availability of insects, the primary source of food delivered to nestlings (Rubenstein, 2007d; Rubenstein, 2016). Superb starlings live in plural cooperatively breeding groups of up to 50 birds where individuals care for young, either as breeders (i.e. the social parents at a nest) or helpers (i.e. subordinate individuals that guard and/or provision nestlings) (Rubenstein, 2007d). Helpers can be of either sex, and include individuals that are both related and unrelated to the breeding pairs. In addition to breeders and helpers, every breeding season some group members can forgo caring for young by taking on a non-breeding/non-helping role. Within each social group, multiple breeding pairs

(typically 3 or 4) will reproduce in a breeding season, and between 1 and 14 helpers will contribute to providing offspring care at each nest (Rubenstein, 2016). From previous observations of aggressive interactions at feeding platforms, we know that breeding roles differ in social dominance: breeders are dominant to all other roles, helpers are subordinate to breeders and non-breeders/non-helpers, and males tend to be dominant to females (Rubenstein, 2007d).

Even when incubation begins at one nest, subordinate helpers may still have the opportunity to breed if that nest fails (92% of nests fail before fledging over the course of this study) or via extra-pair paternity or mate switching within the social group. Subordinate individuals are indeed sexually mature and able to reproduce if given the opportunity because (i) male helpers are known to occasionally gain reproductive success via extra-pair fertilizations (though only 10% of chicks are the result of extra-pair fertilizations) (Rubenstein, 2007b) and (ii) mate switching (or divorce) within or among breeding seasons is common and subordinate helpers that do not gain a breeding position at the onset of a breeding season could later have to opportunity to breed (Rubenstein, 2007b; Pollack and Rubenstein, 2015; Rubenstein, 2016). Breeding roles are flexible, as individuals can acquire a breeding position as the season progresses, or change their role from one breeding season to the next. Changes in breeding roles are in part shaped by breeding rainfall where a larger proportion of group members typically breed in rainier breeding seasons (Rubenstein, 2007d; Rubenstein, 2016).

2.2. Behavioral observations

Breeding roles were identified by focal nest observations during incubation, while offspring care behavior was quantified by performing focal nest observations during chick rearing (mean \pm SE = 4.4 \pm 0.4 h per nest, between 8:00 and 16:00). Repeated observations at each nest were attempted but not always possible due to high nest predation. We identified the social mother as the individual incubating the nest and with a brood patch, while the social father was the male guarding the incubating mother (Rubenstein, 2007c). We confirmed parentage genetically when this was possible (i.e. a nest hatched successfully) as described elsewhere (Rubenstein, 2005, 2007b). All other individuals identified at a nest during incubation and/or chick rearing were classified as helpers. We identified individuals that arrived within 20 m of a nest using a spotting scope and monitored whether an individual entered or exited the nest, and whether they delivered food into the nest. Provisioning rates were calculated for each individual breeder or helper as the number of trips where food was delivered into the nest per hour.

2.3. Sample collection

Superb starlings were captured at active nests using food-baited ground traps or mist-nets, and blood samples were collected from the brachial vein within 3 min of capture. Blood samples were centrifuged in the field and plasma was stored on ice for up to 4 h, and subsequently frozen at -30 °C. Plasma samples were then transported to Columbia University in New York on dry ice at the end of each field season (up to 5 months after sample collection), and stored at -80 °C until analysis. Sampling was restricted to the long rainy season, but the specific timing of sampling varied from year to year due to variability in the timing and length of the breeding season; samples were collected from April to June in 2013 (N = 32), March to April in 2014 (N = 17), April to July in 2015 (N = 31), and January to June in 2016 (N = 40). Birds were sampled during both incubation (N = 84) and chick rearing (N = 37), where 18 of these individuals were re-sampled during both breeding stages (Table 1). Although dominance hierarchies appear to be established prior to the onset of reproduction during the dry pre-breeding period in superb starlings (Rubenstein, 2007d), incubation represents a life-history stage where breeding roles are likely to still be flexible

Table 1

The distribution of sample sizes across breeding stages, sex and breeding role.

Stage	Sex	Role
Incubation N = 84	Male N = 28	Breeder N = 9 Helper N = 19
	Female N = 56	Breeder N = 39 Helper N = 17
Chick rearing N = 37	Male N = 17	Breeder N = 5 Helper N = 12
	Female N = 20	Breeder N = 10 Helper N = 10

because breeding is not always synchronous, mate switching can occur, and the number of breeding pairs per group varies from season to season according to rainfall (Rubenstein, 2007d). Therefore, even during early breeding where some birds have begun to incubate, there likely remains conflict over whether other individuals within a group are also able to undertake a breeding attempt or not, or whether males will gain extra-pair fertilizations (Green et al., 1995; Peters et al., 2002; Rubenstein, 2007b). Conversely, once nestlings hatch at a nest, both parental and alloparental care become crucial for fledging young successfully (Rubenstein, 2007c). At this time, investment in offspring care should be favored over social conflict in superb starlings. Whenever possible, individuals were recaptured during both the incubation and chick rearing stages. However, high rates of nest predation during incubation meant that it was not always possible to re-sample the same individuals during chick rearing. Since superb starlings are sexually monomorphic, we determined sex using polymerase chain reaction (PCR) (Griffiths et al., 1998) that has been validated previously for this species (Rubenstein, 2007c). In total, we sampled 75 females and 46 males (which included 64 breeders and 57 helpers; Table 1). This research was conducted with approval from Columbia University's IACUC and Kenyan Authorities (see Acknowledgements).

2.4. Group size

Group size was calculated as the long-term average number of individuals alive in each social group from 2002 to 2015 (sensu Pollack and Rubenstein, 2015). We also determined the proportion of breeding positions available as a proxy for the intensity of competition for breeding opportunities, as social groups with fewer breeding positions may face more intense competition. The availability of breeding positions within each social group changes seasonally based on rainfall and resource availability, and we calculated the relative proportion of individuals that bred in a group by dividing the number of breeding pairs observed in a group within a breeding season by our measure of group size (Cant and English, 2006).

2.5. Testosterone assay

Plasma testosterone was quantified using a commercially available enzyme-linked immunosorbent assay, ELISA (product number 582701; Cayman Chemical, Ann Arbor, MI, USA). The manufacturer reports this assay to have a detection range from 3.9–500 pg/ml with an estimated sensitivity of 6 pg/ml. We followed the manufacturer's instructions with minor modifications; we used a diethyl ether extraction protocol after an initial kit validation indicated cross reactivity with interfering substances in the plasma. Briefly, 4 ml of diethyl ether were added to a known volume of plasma. Samples were vortexed and snap frozen using a dry ice and ethanol bath, at which point the ether layer was transferred to a clean test tube. The ether extract was dried in a water bath (40 °C) under a gentle stream of nitrogen. Dried samples were reconstituted in 500 μ l of assay buffer and stored overnight at 4 °C. The amount of plasma available for extraction ranged across samples from 25 to 100 μ l, such that the dilutions used ranged from 1:5 to 1:20. As

outlined by the manufacturer's instructions, we tested for interfering substances by diluting a pool of plasma to three different dilutions (1:5, 1:8, 1:17). We found that different dilutions of the same sample yielded similar hormone concentrations (average = 3.1% difference), suggesting that no interference occurred after the extraction protocol (Appendix A; Fig. S1). Samples were assayed in duplicate and absorbance was read at a wavelength of 405 nm. Testosterone concentrations were expressed in ng/ml of testosterone in plasma (correcting for the appropriate dilution factor). Extraction recovery (mean \pm S.D. = 78.9 \pm 6.9%) was estimated via cold spike in five samples. Samples were assayed across 4 plates, and the position of each sample was randomly distributed across plates. The mean inter-assay coefficient of variation was 3.22% (calculated for each standard across all plates and averaged), while the intra-assay coefficient of variation was 2.45% (calculated across duplicates and averaged for all samples).

2.6. Statistical analyses

Since some superb starlings were re-sampled between incubation and chick rearing, we used general linear mixed models (LMM) to account for the non-independence of samples (Bolker et al., 2008). Testosterone levels were natural log-transformed to achieve normality. We ran several models to best address our different hypotheses about the relationships between testosterone and breeding roles, group size, or parental investment. In all LMMs, testosterone was used as the response variable and we included a nested random effect of individual ID within a social group and year to control for repeated captures. We constructed models in the R package *nlme* v3.2.0 (R Core Team, 2015). We first built a LMM to test whether testosterone differed with breeding role and sex, or changed across the breeding season: we included sex, breeding role (breeder or helper), age, sampling stage (incubation or chick rearing), and the interactions between sampling stage and sex, role, or both as predictor variables. We calculated effect sizes for pair-wise comparisons of testosterone across sampling stage, role and sex using *Cohen's D*, the standardized difference between the mean of two groups (Cohen, 1988). Next, we performed a LMM to examine whether testosterone increased with group size and the proportion of breeding positions (i.e. proxies for social conflict) as predictor variables, along with age, sampling stage, breeding role, sex and the interactions between role, sex, and either group size or the proportion of breeders. Lastly, we built a LMM to examine whether chick rearing testosterone titers were negatively correlated to parental care investment. In this model, we included age, sex, breeding role, and the provisioning rate along with the interactions between nestling provisioning rate and sex, role or both. For fixed effects in all models, we present coefficient estimates and their standard error, degrees of freedom, the *F*-value, and associated *P*-value ($\alpha = 0.05$), as well as likelihood ratios as a measure of relative effect size. Briefly, we calculated likelihood ratios by comparing full LMMs to a series of reduced models where each fixed effect was removed in isolation (Pinheiro and Bates, 2000); larger ratios indicate a stronger effect size.

3. Results

3.1. Breeding role, sex and breeding stage

Testosterone differed among breeding roles (mean \pm SE for breeders: 0.30 \pm 0.03 ng/ml, helpers: 0.18 \pm 0.02 ng/ml, *Cohen's D* = 0.48) and sexes (mean \pm SE for males: 0.35 \pm 0.04 ng/ml, females: 0.18 \pm 0.01 ng/ml, *Cohen's D* = 0.67). Male breeders had higher testosterone than male helpers (mean \pm SE for male breeders: 0.66 \pm 0.06 ng/ml, male helpers: 0.21 \pm 0.03 ng/ml, *Cohen's D* = 1.33), as well as female helpers, and female breeders (Table 2, Fig. 1). In contrast, female breeder testosterone was not higher than female helper testosterone (mean \pm SE for female breeders: 0.20 \pm 0.02 ng/ml, female helpers: 0.13 \pm 0.02 ng/ml, *Cohen's*

Table 2

Results from a LMM testing the influence of breeding role, sex, and sampling stage on testosterone, including likelihood ratios (χ^2). Significant *P* values are indicated in bold.

Predictor variable	Estimate \pm SE	DF	<i>F</i> value	<i>P</i> value	<i>L</i> ratio
Intercept	-2.09 \pm 0.31	72	628.8	< 0.0001	
Sampling stage	0.04 \pm 0.26	18	1.22	0.28	6.26
Role	-0.02 \pm 0.36	18	9.99	0.005	20.44
Sex	1.01 \pm 0.42	72	23.06	< 0.0001	35.52
Age	0.03 \pm 0.03	18	0.7	0.41	0.95
Sampling stage * role	-0.36 \pm 0.4	18	0.02	0.88	0.85
Sampling stage * ex	0.47 \pm 0.49	18	5.81	0.027	5.23
Role * sex	-1.17 \pm 0.53	18	8.62	0.009	9.23
Sampling stage * role * sex	0.36 \pm 0.63	18	0.33	0.57	5.27

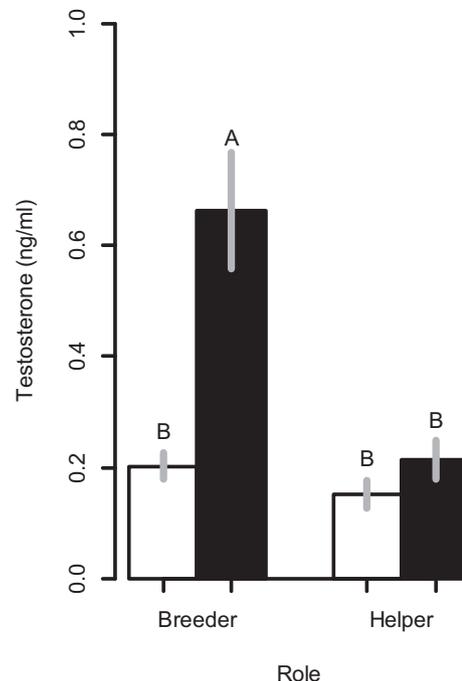


Fig. 1. Testosterone differed among superb starlings of different roles and sexes, where breeding males had significantly higher testosterone than helper males and breeding or helping females (white = females, black = males). Both breeding stages are pooled together for this figure. Grey lines are standard errors. Letters indicate significantly different means based upon a Tukey post-hoc test.

D = 0.33), and there was no difference in testosterone between male and female helpers (Fig. 1).

Testosterone also varied with sampling stage (mean \pm SE for incubation: 0.26 \pm 0.02 ng/ml, chick rearing: 0.21 \pm 0.03 ng/ml, *Cohen's D* = 0.23), but in a sex- and role-specific manner (Table 2). Male breeders had higher testosterone during incubation than female breeders, female helpers, and male helpers (Table 2). However, there were no differences in testosterone during chick rearing among any of the roles or sexes. Finally, testosterone decreased from incubation to chick rearing only for male breeders (Fig. 2).

3.2. Group size and breeding positions

Testosterone increased with group size in helpers, but not in breeders (Table 3; Fig. 3A). Moreover, there was no effect of sex on the relationship between testosterone and group size in helpers, suggesting that both male and female helpers have elevated testosterone in larger groups. However, we did not find a relationship between testosterone and the proportion of breeding positions available within a group for either breeders or helpers, or in males or females (Fig. 3B).

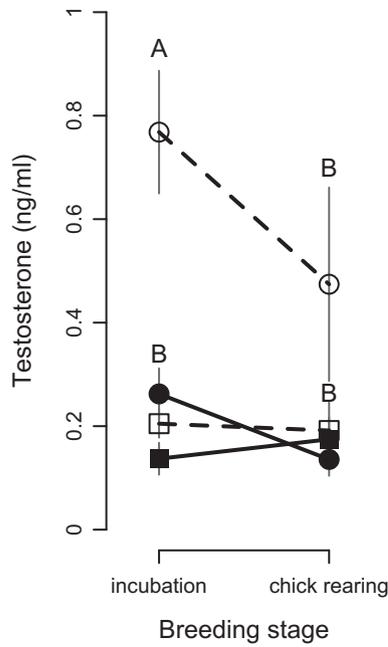


Fig. 2. Testosterone changed between the incubation and chick rearing stages, but only in breeding males. Shapes represent mean testosterone and grey lines represent standard errors. Open shapes represent breeders, while closed shapes represent helpers; circles represent males, and squares represent females. Letters indicate significantly different means based upon a Tukey post-hoc test.

Table 3

Results from a LMM testing the influence of breeding role, sex, group size, and the proportion of breeding roles (Prop. Breed) on testosterone, including likelihood ratios (χ^2). Significant *P* values are indicated in bold.

Predictor variable	Estimate ± SE	DF	F value	P value	L ratio
Intercept	-2.19 ± 0.66	70	592.42	< 0.0001	
Group size	-0.00 ± 0.02	17	0.48	0.56	7.21
Prop. breed	-0.00 ± 0.02	17	0.48	0.5	1.17
Role	-1.38 ± 1.2	17	10.9	0.004	24.08
Sex	2.11 ± 1.15	70	21.61	< 0.0001	33.87
Sampling stage	-0.10 ± 0.16	17	0.62	0.44	0.69
Age	0.06 ± 0.03	17	0.94	0.35	3.34
Group size * role	0.04 ± 0.03	17	4.49	0.049	5.97
Prop. breed * role	-0.02 ± 0.04	17	0.17	0.68	0.89
Group size * sex	-0.02 ± 0.03	70	0.70	0.41	0.68
Prop. breed * sex	0.02 ± 0.05	70	0.001	0.98	0.11
Role * sex	-1.8 ± 1.69	17	9.24	0.007	11.35
Group size * role * sex	0.02 ± 0.04	17	0.28	0.6	6.01
Prop. breed * role * sex	-0.02 ± 0.07	17	0.07	0.79	0.89

3.3. Parental behavior

There was no relationship between testosterone and parental or alloparental care behavior during chick rearing (Table 4), as an individual's investment in nestling provisioning rate did not correlate with its testosterone level (Fig. 4). Breeders and helpers, as well as males and females, exhibited similar patterns.

4. Discussion

To address the role of testosterone in shaping social and parental behavior in cooperatively breeding superb starlings, we examined whether testosterone differed across breeding roles and between the sexes, as well as whether testosterone was correlated to social group size or nestling provisioning rates. Breeding males had high testosterone during incubation but not during chick rearing. Female breeders, female helpers, and male helpers' testosterone was lower than male breeder's testosterone and did not change from incubation to chick

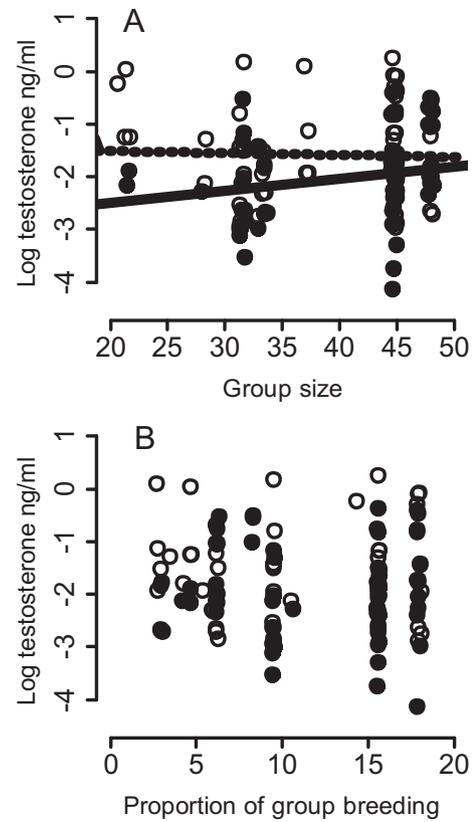


Fig. 3. Testosterone pooled from both breeding stages increased with (A) group size in helpers (closed symbols, solid line) but not breeders (open symbols, dotted line). Conversely, (B) testosterone was unrelated to the proportion of group members with a breeding position for both breeders and helpers.

Table 4

Results from a LMM testing the influence of breeding roles, sex, and provisioning rate on testosterone, including likelihood ratios (χ^2).

Predictor variable	Estimate ± SE	DF	F value	P value	L ratio
Intercept	-1.16 ± 0.54	14	144.91	< 0.0001	
Provisioning rate	-0.50 ± 0.29	14	0.27	0.61	4.62
Role	-0.98 ± 0.43	2	3.90	0.19	6.30
Sex	0.03 ± 0.41	14	0.02	0.89	0.04
Age	-0.01 ± 0.07	2	0.04	0.87	0.003
Provisioning rate * role	0.90 ± 0.66	14	1.82	0.20	2.54
Provisioning rate * sex	-0.08 ± 0.57	14	0.02	0.89	0.04

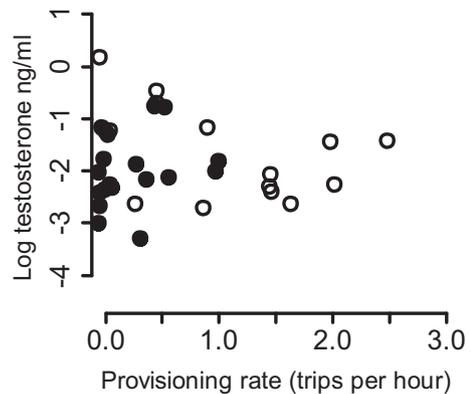


Fig. 4. Testosterone in both sexes was unrelated to nestling provisioning rate in parental (open circles) and alloparental (closed circles) care behaviors.

rearing. Testosterone also increased with group size in helpers, but not in breeders. Finally, although testosterone was low in all individuals during chick rearing, natural variation in testosterone during this life-history stage was unrelated to investment in chick provisioning.

Our results suggest that testosterone differs across life-history stages and between breeding roles. We found that only male breeders had elevated testosterone during incubation, which is similar to previous findings in a variety of other birds, including European starlings, *Sturnus vulgaris* (Calisi et al., 2011; Pinxten et al., 2007). In these temperate-breeding starlings, testosterone remained elevated throughout incubation due to either high breeding densities, sustained male-male aggressive interactions, or prolonged mating effort. Therefore, in asynchronously breeding superb starlings, sustaining high testosterone may be beneficial to males as it could enable them to maintain sperm production and participate in extra-pair mating (Rubenstein, 2007b). Competition for extra-pair fertilizations occurs both within and between social groups in superb starlings (Rubenstein, 2007a, 2007b), such that within- and among-group intra-sexual competition may remain elevated throughout incubation in males. Similarly, superb starlings are multi-brooded during the long rainy season due to high nest predation and often extended rains (Rubenstein, 2006), suggesting that breeding males may maintain elevated testosterone levels throughout incubation in the event of egg predation, when re-nesting is likely to occur. Breeding males also decreased in testosterone from incubation to chick rearing, but this was not observed in breeding females or helpers of either sex. Similar sex-specific patterns have been observed in other species ranging from arctic to tropical study systems, as testosterone typically decreases in males at times when parental care becomes important for an individual's fitness (Goymann and Wingfield, 2004; Hunt et al., 1999; Lynn et al., 2009; Wingfield et al., 1990). In superb starlings, offspring care is still essential for chick survival—even though fathers provision nestlings less than mothers or helpers (Rubenstein, 2006), the cumulative care performed by all parental and alloparents combined is an important driver of fledging success (Guindre-Parker and Rubenstein, unpublished data). Therefore, if testosterone inhibits offspring care and yet care during chick rearing is essential, testosterone should decrease during this breeding stage as was observed in breeding male superb starlings. Alternatively, elevated levels of testosterone may not bring a competitive advantage during offspring care, particularly at the end of the breeding season where extra-pair mating and mate switching become less likely. More research will be needed to reconcile why testosterone differs with breeding status or role in some species but not others.

In contrast to males, we found that breeding females did not have elevated testosterone relative to helpers during either breeding stage. Instead, breeding females had testosterone levels that were similar to male and female helpers throughout incubation and chick rearing. We predicted that dominant females would have higher testosterone compared to subordinate females because both male and female breeders face competition for mates in superb starlings (Apakupakul and Rubenstein, 2015; Rubenstein, 2007c; Rubenstein and Lovette, 2009), and aggression in both sexes is thought to be shaped, in part, by testosterone (Ketterson et al., 2005; Langmore, 1998; Muck and Goymann, 2011; Voigt and Leitner, 2013). However, our results suggest that testosterone may not reflect breeding roles in female superb starlings, despite doing so for males. A possible explanation for why testosterone in female breeders remained low during incubation is that heightened testosterone can delay reproduction in females (Clotfelter et al., 2004). Similarly, since breeding females are the primary individuals performing incubation, elevated testosterone prior to hatching could inhibit female incubation behavior and reduce hatching success (Cantarero et al., 2015; Rosvall, 2013; Rubenstein, 2007b; Schoech et al., 1991; Schwabl, 1996). Although we did not sample individuals prior to egg laying, increased female testosterone prior to laying has been shown to elevate testosterone in egg yolk (Schwabl, 1993; Schwabl, 1996), which could lead to reduced survival and growth of

offspring (Andersson et al., 2004; Rubolini et al., 2006; Sockman and Schwabl, 2000). As a result, females may have decreased testosterone during incubation relative to breeding males due to egg laying and sex-specific differences in incubation care behavior. Indeed, superb starling chicks appear sensitive to environmentally-induced maternal affects (Rubenstein et al., 2016), a topic that warrants further study in this and other cooperative breeders. Finally, female reproductive and aggressive behaviors may be influenced by hormones other than testosterone (reviewed in Duque-Wilckens and Trainor, 2017), including estradiol (Pärn et al., 2007), progesterone (Goymann et al., 2008), and prolactin (reviewed in Angelier and Chastel, 2009). Future studies should compare testosterone and other hormones during the pre-breeding season to determine whether breeding females have elevated testosterone compared to female helpers, and whether pre-breeding testosterone is similar between the sexes and across life-history stages.

Our study is also consistent with the idea that testosterone may be related to social conflict in cooperative species, since helpers in large groups, where conflict is thought to be higher (Shen et al., 2014), had elevated testosterone relative to those in small groups. Although we did not directly measure the relationship between testosterone and social conflict, testosterone is predicted to increase during times of heightened social conflict (Rose et al., 1975; Wingfield et al., 1990; reviewed in Hirschenhauser and Oliverira, 2006). In contrast, testosterone in breeders did not vary with group size, though breeders had higher testosterone than helpers. A possible explanation for this result is that breeder testosterone levels might already be at elevated breeding levels, such that variation in group size cannot further increase testosterone levels; however, several studies using gonadotropin-releasing hormone (GnRH) injections have demonstrated that testosterone is not usually maximally elevated even during breeding (Apfelbeck and Goymann, 2011; Jawor et al., 2006; Moore et al., 2002). Our results are similar to previous studies in cliff swallows, *Petrochelidon pyrrhonota*, where testosterone in males and females was higher in large groups due to increased competition for mates (Smith et al., 2005). Consequently, if superb starling breeders are dominant to helpers and suppress them from breeding (Rubenstein, 2007d), elevated testosterone in helpers could be a result of competition for breeding opportunities or due to receiving aggression from breeders. In some species, such as the cooperatively breeding cichlid fish, *Neolamprologus pulcher*, breeders may threaten helpers with aggression and expulsion from the group to enforce helping behavior (Bender et al., 2006). Helpers in this species that are more submissive have reduced testosterone compared to helpers that are less submissive (Bender et al., 2006), suggesting that testosterone may mediate behavioral tactics to cope with social conflict. Furthermore, it may be easier for dominant members of smaller groups to monitor helper behavior (Fischer et al., 2014), which may also contribute to helpers in smaller groups being submissive to reduce breeder aggression.

Both breeders and helpers in cooperatively breeding species may experience a variety of forms of social conflict besides competition for mates, including conflict over feeding effort, resources, territory defense, mate guarding, and competition for social rank (Kazama et al., 2011; Reyer, 1986; Smith et al., 2005; Wingfield et al., 1991). Since breeders and helpers likely experience social conflict differently, this may be reflected by physiological differences as conflict increases with group size. However, group size is not necessarily linearly or positively correlated to social conflict for all species, and a number of other social and ecological factors can influence social conflict in addition to group size (Shen et al., 2014). Future studies examining intra-group conflicts during pre-breeding and breeding life-history stages in superb starlings should focus on identifying the sources of social conflict across breeding roles and group sizes.

Finally, there was no relationship between testosterone and nestling provisioning rates across breeding roles or sexes. During chick rearing, parents and alloparents with higher testosterone did not have reduced nestling provisioning rates. A likely explanation for this finding is that

testosterone was naturally low for all individuals during chick rearing, and only when testosterone increases above breeding baseline levels do we expect to see inhibited parental care behavior (Wingfield et al., 1990). An alternative explanation for why we do not see a relationship between testosterone and nestling provisioning rates could be due to the importance of parental care for superb starlings, where parental and alloparental care are strong drivers of reproductive success (Rubenstein, 2006; Guindre-Parker and Rubenstein, in prep). In plural cooperatively breeding species, where total offspring care may be crucial to successfully raise young, essential parental and alloparental care may lead to the evolution of behavioral insensitivity to testosterone (Lynn et al., 2005; Lynn, 2008) due to strong selection to perform care despite variation in testosterone levels (Van Duyse et al., 2000). Testosterone levels are unrelated to nestling provisioning rates in a number of temperate avian species, including northern cardinals, *Cardinalis cardinalis*, dark-eyed juncos, *Junco hyemalis*, and black redstarts, *Phoenicurus ochruros*, (DeVries and Jawor, 2013; McGlothlin et al., 2007; Villavicencio et al., 2014). The “essential paternal care” hypothesis proposes that in species where male parental care is critical for offspring survival, males may not reduce parental care even when testosterone is elevated (Lynn et al., 2005). While female insensitivity to testosterone has been less studied, females dark-eyed juncos are behaviorally insensitive to testosterone during incubation (Clotfelter et al., 2004; Ketterson et al., 2005). Although natural variation in testosterone alone did not appear to constrain nestling provisioning rates in superb starlings, determining whether superb starlings are behaviorally insensitive to testosterone would require manipulation of testosterone with hormone implants or altering the offspring care workload of parents and alloparents by manipulating brood size.

5. Conclusion

Testosterone is thought to be important in both male and female reproduction and fitness. By examining testosterone in superb starlings during two breeding stages, and by comparing levels in male and female breeders and helpers, we found that testosterone differed with breeding role in male but not female superb starlings. As in other socially monogamous birds, male breeder superb starlings are dominant to helpers, and testosterone may contribute to dominance hierarchies in this species. The increased levels of testosterone that we observed in helpers in larger groups may be the result of increased social conflict. Although both sexes in cooperatively breeding species may experience similar levels of conflict over reproductive opportunities, testosterone levels were not reflective of breeding roles in females, suggesting that dominance hierarchies may be shaped via different physiological mechanisms in males and females of cooperatively breeding species. Finally, testosterone in superb starlings was low for all individuals during chick rearing, suggesting that either (i) offspring care is essential and individuals are behaviorally insensitive to natural variation in testosterone, or (ii) testosterone is regulated tightly and lowered during offspring care so as not to inhibit care behavior. Thus, our study offers new insight into the role that testosterone plays in the establishment of dominance rank, and social conflict more broadly, in cooperatively breeding species.

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Appendix A. Supplementary data

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