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Testosterone Might Not Be Necessary To Support Female Aggression In Incubating Northern Cardinals

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Testosterone might not be necessary to support female aggression in incubating northern cardinals

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Testosterone's (T) influence on male aggression has been well established in many vertebrate species, but the impact of T on female aggressive behaviour is poorly understood. Among birds, a link between T and female aggression is plausible, as females of many species exhibit a seasonal peak in T concentrations at the onset of breeding when social instability is greatest and they may have circulating T through much of the breeding season. However, investigations examining the relationship between T and female aggression are few and have yielded conflicting results, with experimentally or endogenously elevated T supporting aggressive behaviour in females of some species but not others, and T elevating with aggression at some points of the reproductive cycle but not others. We examined the relationship between endogenous levels of T and female aggression in the northern cardinal, *Cardinalis cardinalis*, a resident temperate species in which pairs exhibit prolonged territoriality and females have measurable levels of T year-round, including all stages of reproduction (incubation, nestling feeding, etc.). Using simulated nest intrusions, we assessed aggressive responses of incubating females to intrasexual 'intruders' at the nest and quantified T levels after each aggressive encounter. Displays of aggression towards 'intruders' varied among females; yet, individuals showing greater levels of aggression did not demonstrate higher levels of T. These results imply that T might not support maternal aggression in this species.

corticosterone

female

incubation

maternal aggression

nest defence

northern cardinal

testosterone

Quantifying relationships between steroid hormones and vertebrate aggression has received much investigative attention. Collectively, such studies suggest that the relationship between sex steroids and aggressive behaviour is complex, as the connection between a hormone and a behaviour can vary among reproductive contexts, seasons, sexes and species (Adkins-Regan, 2005; Goymann, Landys, & Wingfield, 2007). Among birds, testosterone's (T) influence on male aggression has been researched extensively, with many studies indicating that elevated levels of T support male aggressive behaviour (e.g. Beletsky, Orians, & Wingfield, 1990; Geslin, Chastel, & Eybert, 2004; Hau, Wikelski, Soma & Wingfield, 2000; McGlothlin, Jawor, & Ketterson, 2007; Wingfield, 1984, 1994). However, not all species show covariation between T and aggression (Apfelbeck & Goymann, 2011; Apfelbeck, Stegherr, & Goymann, 2011; DeVries, Winters, & Jawor, 2012; Hunt, Hahn, & Wingfield, 1997; Lynn & Wingfield, 2008; Moore, Walker, & Wingfield, 2004) and T elevation with aggression may be context specific (Rosvall, Peterson, Reichard, & Ketterson, 2014). This has prompted recent and interesting suggestions that the association between T and aggression in males be re-evaluated (Goymann et al., 2007).

If the relationship between T and aggression in males is complex, the relationship between T and female aggression remains largely inconclusive. Even though female birds of many species behave aggressively (e.g. Cain, Rich, Aisworth, & Ketterson, 2011; Rosvall, 2008, 2011; Sandell, 1998; Yasukawa & Searcy, 1982), and they can have circulating levels of T throughout the breeding season (e.g. Gill, Alfson, & Hau, 2007; Jawor, 2007; Rosvall, 2013), they have received much less investigative attention

(Ketterson, Nolan, & Sandell, 2005) with relatively little emphasis on hormone expression during aggression.

The few studies that have examined relationships between T and female aggression in birds have focused on aggressive behaviour prior to nesting and have yielded conflicting results. Females of several species (e.g. red-winged black birds, *Agelaius phoeniceus*: Cristol & Johnson, 1994; cliff swallows, *Petrochelidon pyrrhonota*: Smith, Raouf, Bomberger Brown, Wingfield, & Brown, 2005) demonstrated a seasonal peak in T levels during periods of intense social instability (e.g. territorial establishment, mate attraction), implying that greater concentrations of T might support aggressive behaviour. Several studies have attempted to determine whether elevations of T accompany displays of female aggression as proposed by the 'challenge hypothesis' (Wingfield, Hegner, Dufty, & Ball, 1990), yet results are mixed from studies attempting to quantify such a relationship. For example, elevated levels of T were not present following aggressive behaviour in female European stonechats, *Saxicola torquata* (Canoine & Gwinner, 2005; Gwinner, Rödl, & Schwabl, 1994), song sparrows, *Melospiza melodia* (Elekonich & Wingfield, 2000), spotted antbirds, *Hylophylax n. naevioides* (Hou, Stoddard, & Soma, 2004), dark-eyed juncos, *Junco hyemalis* (Jawor, Young, & Ketterson, 2006), and African black coucals, *Centropus grillii* (Goymann, Wittenzellner, Schwabl, & Makomba, 2008), but T concentrations were higher in buff-breasted wrens, *Thryothorus leucotis* (Gill et al., 2007), following exposure to a female decoy, and in dunnocks, *Prunella modularis* (Langmore, Cockrem, & Candy 2002), after experimental removal of males encouraged competition among females.

The aforementioned studies are unified in that they assessed relationships between elevated T and female territorial aggression. Almost nothing is known about connections between levels of T (neither nonelevated nor elevated) and other forms of aggressive behaviour performed by female birds, such as maternal aggression. Performance of this type of aggression is essential for insurance of a female's reproductive success as she defends eggs/offspring from potential destruction and/or brood parasitism (Nelson, 2006). Females of many avian species have detectable T levels during incubation/nestling feeding (Ketterson et al., 2005), which in some cases are not significantly lower than peak concentrations of T characteristic of the prebreeding period (e.g. Jawor, 2007; Jawor et al., 2007). Considering that circulating T is present during periods when females of many species are aggressively defending nests, it is plausible that a relationship between T and avian maternal aggression could exist. Maternal aggression has been well studied in mammals (particularly rodents), and modulation of this behaviour has been linked to a number of hormones including oxytocin, vasopressin, progesterone and neurotransmitters such as serotonin (Angoa-Pérez et al., 2014; Bosch & Newman, 2012; de Sousa et al., 2010; Heiming et al., 2013; Kelly & Goodson, 2014; Sabihi, Dong, Durosko, & Leuner, 2014), but many of these hormones are also used in lactation, a process that birds do not undergo, and their links with aggression may be secondary to their main use. Here we compare circulating levels of T in female northern cardinals, *Cardinalis cardinalis* (hereafter 'cardinals'), that were defending their nest from a simulated conspecific intruder to better understand how T and avian maternal aggression covary.

Several behavioural and physiological characteristics of female northern cardinals render them ideal candidates to examine relationships between T and maternal

aggression. Cardinals are year-round residents ranging from Central America to southern Canada (Halkin & Linville, 1999). This species is socially monogamous and multibrooded, displays prolonged territoriality, which in some areas is year-round (Halkin & Linville, 1999; Jawor, Gray, Beall, & Breitwisch, 2004; Gentry, n.d.), and has a lengthy breeding season (6+ months; Halkin & Linville, 1999). From a behavioural perspective, female cardinals are unique among other female temperate zone passerines in that they actively participate in territorial defence through the display of male-like aggression (e.g. conflict, song) towards intra- and intersexual conspecifics for most of the year (Halkin & Linville, 1999; Jawor et al., 2004). Cardinals are also open-cup nesters that suffer a high depredation rate (Filliater, Breitwisch, & Nealen, 1994; this study), intraspecific brood parasitism is known to occur (Linville, 1997), and cardinals actively defend their nests (Jawor et al., 2004). Consequently, displays of maternal aggression at the nest are potentially frequent as female cardinals protect their reproductive investment (Jawor et al., 2004; M. S. DeVries, personal observations; J. M. Jawor, personal observations).

Female cardinals also demonstrate unique patterns of T production. For example, female cardinals have measurable concentrations of T present year-round (Jawor, 2007) that are higher than many values previously reported for females of other avian species (reviewed in Ketterson et al., 2005). Furthermore, the annual T profile of female cardinals is nearly identical to that of males (Jawor, 2007). Prior work with the population assessed here also suggests that female cardinals can significantly increase T concentrations in response to standardized gonadotropin-releasing hormone (GnRH) injections during the nonbreeding through prebreeding periods (see DeVries, Holbrook,

Winters, & Jawor, 2011; Jawor, Hooker, & Mohn, 2014, for further details) but not during offspring feeding (DeVries & Jawor, 2013). Given that female cardinals are highly aggressive at the nest and have considerable concentrations of circulating T year-round, we hypothesize that a relationship exists between T and maternal aggression in this species. In this study we assessed whether circulating T levels of incubating female cardinals vary by behavioural context (aggressive versus nonaggressive) and whether baseline T concentrations covary with the degree of aggressive behaviour displayed in response to a simulated intrasexual, conspecific 'intruder' at the nest.

METHODS

General Field Methods

This research was conducted at the Lake Thoreau Environmental Research and Education Center property owned by the University of Southern Mississippi in Hattiesburg, Mississippi. In this population of cardinals, females begin building nests in late March to early April and the breeding season often continues through mid- to late September. This study was performed during April–June within the 2008–2011 breeding seasons. Nests were located through systematic searching from mid-March through early June. To determine whether circulating T covaries with behaviour during aggressive interactions, female cardinals were either captured (using mist nets) at the nest following aggressive response to a simulated nest intruder ($N = 29$) or captured at the nest without exposure to a potential 'intruder' ($N = 17$). Most females were randomly subjected to only one treatment (aggressive context, $N = 24$; nonaggressive context, $N = 12$); however, because of the relatively finite size of this population, some individuals were captured in

both aggressive and nonaggressive contexts ($N = 5$; accounted for in statistical analyses; see below). To prevent subjecting an individual to the potential stress of repeated capture at the nest within a short period (which might have negative impacts on incubation behaviour), females that were exposed to both treatments were only exposed to one randomly selected treatment within a single breeding season (e.g. a female captured during an aggressive encounter in 2010 was captured in a nonaggressive context in 2011). No individuals were repeatedly sampled within a single treatment. All simulated intrusions were performed and all birds were captured and processed (blood collected, banded) between 0600 and 1200 hours. Upon capture, time of removal from net was noted and birds were processed following procedures previously described for cardinals (DeVries et al., 2011, 2012). Processing included blood collection (brachial vein puncture) for T and corticosterone (CORT) analyses, banding individuals (U.S. Fish and Wildlife (USFWS) band, plus a unique combination of plastic colour bands) and recording morphometric measures as part of ongoing research (handling time <10 min). Birds were released from the point of capture at the conclusion of processing. Blood samples were centrifuged and plasma was extracted and frozen at –20 °C until hormonal analyses. All procedures performed in this study were in accordance with ASAB/ABS Guidelines for the treatment of animals in behavioural research and teaching and European Union Directive 2010/63/EU for animal experimentation. Furthermore, this work was conducted under USFWS banding permit number 23479, Mississippi Department of Wildlife, Fisheries, and Parks scientific collecting permit number 0201101 and was approved by the Institutional Animal Care and Use Committee of the University of Southern Mississippi (protocol number 11092214). Salvage of adult cardinals for

taxidermic decoy preparation was approved under USFWS special purposes permit number MB135338-0.

Simulated Nest Intrusions

We conducted simulated nest intrusions from April to June in 2008 ($N = 3$), 2009 ($N = 9$), 2010 ($N = 14$) and 2011 ($N = 3$) to capture female cardinals following aggressive behaviour at the nest. After each nest was located, 1 h incubation watches were performed on two separate days to determine each female's preferred flight paths to and from the nest. Pilot work with this species has found that simulated nest intrusions performed prior to the third day of incubation could induce nest abandonment (Jawor, 2000); therefore, simulated nest intrusions were only performed on days 3–12 of incubation (no nest abandonment occurred after we performed simulated nest intrusions in this study). All simulated nest intrusions were conducted as follows. While a focal female was away from her nest, we placed a female cardinal taxidermy mount within 1 m of the nest cup and erected closed mist nets across preferred flight paths. Camouflaged researchers retreated 10–15 m away from the nest to observe the female's reaction to the 'intruder'. Upon the return of the focal female to the nest area, the trial began and her behavioural response to the decoy was assigned an aggressive proximity score modified from a similar study (Kontiainen et al., 2009): 1 = aggressive posturing (e.g. crest flattening, head lowering, wing waving) from a distance >5 m; 2 = aggressive posturing from distances of 2–5 m; 3 = aggressive posturing within 2 m; 4 = 'mild' attack on decoy (e.g. swooping, pecking at decoy's feet, face); 5 = 'major' attack on decoy (e.g. multiple hits on decoy in quick succession, knocking decoy off of perch). The decoy was allowed

to remain in position for 5 min or until it was physically struck by the focal female. After either 5 min following the return of the focal female to the nest area, or a physical attack on the 'intruder', the decoy was removed and we opened mist nets to capture females upon returning to the nest post-intrusion. Following the female's response to the decoy, nets remained open for 1 h. Once captured, we removed females from the net and bled them within 3 min of capture in the net to assess circulating T levels. Aggressive score (1–5), time during simulated nest intrusion for closest approach to the decoy (mean: 56 s, range 1–300 s), time of capture (time recorded seconds until capture following the completion of the simulated nest intrusion: mean: 1270 s, range 100–3120 s) and day of incubation (day 3–12) were recorded and each individual was quickly processed. If females were not captured within 1 h of the behavioural trial, the capture attempt was stopped. Because male and female cardinals share monomorphic song and vocalizations (Halkin & Linville, 1999) and because male pair members were present during 62% of trials, additional measures such as song or chipping frequency performed exclusively by females could not be reliably determined and thus are not included in our analyses. Capture success using the aforementioned procedure was 72.5% (29 captures/40 attempts).

<H2>*Nonaggressive Sampling*

To quantify levels of circulating T in incubating females not engaged in aggressive behaviour (i.e. controls), we captured females ($N = 17$) at the nest without exposure to a simulated nest intrusion during the 2010 ($N = 8$) and 2011 ($N = 9$) breeding seasons. Similar to techniques used for simulated nest intrusions, we determined the

preferred flight paths of 'control' females to and from the nest during 1 h incubation

watches conducted on two separate days prior to a capture attempt. During a capture

attempt, nets were placed in a female's preferred flight paths while she was away from

the nest. When captured upon returning to the nest, we removed individuals from the net

and bled them (via brachial vein puncture) within 3 min of capture to assess circulating

levels of T, then we banded them, noted the day of incubation and released them at the

point of capture. Nets remained open for 1 h or until successful capture.

Testosterone Analyses

We analysed testosterone using an enzyme immunoassay (EIA; Enzo Life

Sciences, Inc., Farmingdale, NY, U.S.A., number 901-065, antibody sensitivity =

5.67 pg/ml plasma) following methods outlined in DeVries et al. (2011, 2012) and Jawor

(2007). This assay has fairly low cross-reactivity with other androgens (testosterone

100%; 19-hydroxytestosterone 14.6%; androstendione 7.2%; dehydroepiandrosterone

0.72%; and oestradiol 0.40%). Tritiated testosterone (2000 cpm, H^3-T; PerkinElmer) was

added to each sample (30 µl of plasma) to allow for the calculation of recoveries

following extraction (3×) with diethyl ether (mean recoveries = 89%). Extracts were

resuspended and diluted to 350 µl with ethanol and assay buffer. Concentrations of T

were calculated using a four-parameter logistic curve-fitting program (Microplate

Manager; Bio-Rad Laboratories, Hercules, CA, U.S.A.) and corrected for incomplete

recoveries. Plasma samples from each individual were analysed on the same plate and

locations of all samples were randomized within plates. Standards of known T

concentration were also placed within each plate for calculation of intra- and interassay

variation. Intra-assay variation for T analyses ranged from 5.30 to 9.00%; interassay variation was 3.7%.

Corticosterone Analyses

Considering that the presence of a simulated 'intruder' at the nest might elicit a stress response in incubating females, we also assessed levels of corticosterone (CORT) in all individuals captured in both behavioural contexts. Similar to our T analyses, we used an EIA (Arbor Assays, LLC, Ann Arbor, MI, U.S.A., number K014-H5, antibody sensitivity 16.9 pg/ml) to determine levels of circulating CORT (as in DeVries & Jawor, 2014). This assay has limited cross-reactivity with other glucocorticoids (corticosterone 100%; desoxycorticosterone 12.3%; aldosterone 0.62%; cortisol 0.38%). Approximately 2000 cpm of H^3-CORT (PerkinElmer) was added to each sample (10 µl of plasma) to allow for the calculation of recoveries following triple extraction with diethyl ether (mean recoveries = 88%). Extracts were resuspended with 400 µl of assay buffer. Concentrations of CORT were calculated using a four-parameter logistic curve-fitting program (Microplate Manager) and corrected for incomplete recoveries. Plasma samples from each individual were analysed on the same plate and the location of all samples was randomized. Samples from a homogenized plasma pool (northern bobwhite, *Colinus virginianus*) served as standards and were placed in four random locations within each plate for calculation of intra- and interassay variation. Intra-assay variation for CORT analyses ranged from 4.41 to 9.19%; interassay variation was 4.78%.

Statistical Analyses

Data were analysed with SPSS 16.0 (SPSS, Chicago, IL, U.S.A.). To account for interassay variation, we calculated correction factors from multiple standards of known hormone concentration placed randomly throughout each assay and applied a correction factor to all T and CORT samples. All corrected T and CORT values were then ln-transformed for statistical analyses. We used a linear mixed model (LMM) to examine the effects of numerous variables on T and CORT concentrations. Fixed factors within the model included behaviour context (simulated nest intrusion versus control), year and day of incubation, while mass and hormone levels were entered as covariates. Individual identity was the random repeated effect to account for repeated sampling across treatments. We also used general linear models (GLMs) to examine the effects of aggression score (1–5), time of closest approach to decoy and time until capture on levels of T and CORT of females captured during simulated nest intrusions. Because blood sampling of all individuals was completed within 3 min, we excluded handling time from statistical analyses.

RESULTS

Behavioural Context Comparison and Hormone Levels

All females assessed during simulated nest intrusions displayed some level of aggressive behaviour during the trial: level 1 ($N = 0$), level 2 ($N = 2$), level 3 ($N = 10$), level 4 ($N = 5$), level 5 ($N = 12$). Overall, the highest level of response (physically striking the intruder) was the most prevalent. Males were present in 62% of the trials; yet male presence had no effect on focal female T levels (independent samples t test: $t_{27} = 1.6$, $P = 0.12$), CORT concentrations ($t_{24} = 0.42$, $P = 0.67$) or level of aggression shown

($t_{27} = 1.36$, $P = 0.18$). Males were not observed during nonaggressive, control captures.

Results indicate that T concentrations of incubating female cardinals captured following aggressive nest defence or during nonaggressive contexts did not significantly differ (Table 1). In addition, there was no significant effect of year, day of incubation, concentrations of CORT or mass on T concentrations in either behavioural context (Table 1, Fig. 1). Concentrations of CORT also did not significantly differ between behavioural contexts ($F_{1, 20} = 0.54$, $P = 0.47$). Furthermore, there was no significant impact of year, day of incubation, concentrations of T or mass on CORT concentrations in simulated nest intrusion and control females (all F values < 1.78, all P values > 0.14). Table 2 summarizes means and ranges of T and CORT concentrations of females captured within each behavioural context.

<H2>*Additional Variables within Simulated Nest Intrusions*

When examining females captured within aggressive contexts, results suggest that year, day of incubation, mass, concentrations of CORT, aggression score, time of closest approach and time until capture were not significantly associated with T concentrations of females captured following simulated nest intrusions (all F values < 3.01, all P values > 0.16; Fig. 2). The aforementioned variables also were not significantly associated with circulating levels of CORT of incubating females assessed following aggressive encounters (all F values < 0.49, all P values > 0.40; Fig. 3). Relationships between aggression scores and year assessed, day of incubation, mass, time of closest approach to the decoy and time until capture were also nonsignificant (all F values < 1.75, all P values > 0.24). Because T levels can increase with time following aggressive interactions,

we compared T and CORT in individuals captured before and after 15 min (post-simulated nest intrusion) as this is an accepted time point for when T concentrations might increase based on hypothalamic–pituitary–gonadal axis functioning (DeVries et al., 2011, 2012; Jawor et al., 2007). We found no effect of time on T concentrations (females captured pre-15 min, $N = 11$; females captured post-15 min, $N = 18$; $t_{27} = 1.5$, $P = 0.14$) or on CORT concentrations (pre-15 min, $N = 11$; post-15 min, $N = 15$; $t_{24} = 0.17$, $P = 0.86$).

DISCUSSION

Testosterone and Maternal Behaviour

Even though females of several avian species exhibit peak concentrations of T at the onset of breeding (e.g. Cristol & Johnson, 1994; Smith et al., 2005), relatively few studies have reported elevations of T accompanying displays of female aggression during the prebreeding period (but see Gill et al., 2007; Langmore et al., 2002). In addition, correlations between nonelevated (baseline) T and aggression have not been demonstrated (Jawor et al., 2006). This current study indicates a similar relationship for female cardinals in that T did not significantly differ for females captured following aggressive behaviour during incubation when compared to nonaggressive birds captured during incubation. Nor did T covary with the level of aggression shown during defence of the nest; yet, the hormone was readily circulating at concentrations not unlike those previously reported for the prebreeding period (mean T concentrations: prebreeding = 0.87 ng/ml, DeVries et al., 2011; incubation = 0.89 ng/ml, this study). Female cardinals in this population can increase circulating T in response to GnRH injections immediately

prior to breeding (DeVries et al., 2011), but they cannot significantly respond to such injections when feeding offspring (DeVries & Jawor, 2013), suggesting that females of this species might lose the ability to transiently increase T concentrations during offspring care. Experimentally elevated T has been reported to delay the onset of breeding (Clotfelter et al., 2004), decrease immune function (Zysling et al., 2006), reduce maternal care (Viega & Polo, 2008), reduce hatching success (Rosvall, 2013) and alter the primary sex ratio of broods (Veiga, Viñuela, Cordero, Aparicio, & Polo, 2004) in females of some avian species. In addition, high concentrations of egg yolk androgens can have detrimental effects on embryo hatchability, growth, immunity and survival (Mazuk, Bonneaud, Chastel, & Sorci, 2003; Navara, Hill, & Mendonça, 2005; Sockman & Schwabl, 2000). It is plausible that selection has not favoured significant elevations in circulating T in female birds regardless of stimuli during some parts of the breeding season because high concentrations of T, and the behaviours influenced by T, might be detrimental to female fitness (Rosvall, 2011, 2013; but see Navara, Siefferman, Hill, & Mendonça, 2006; Whittingham & Schwabl, 2002, for examples in which aggression leads to higher egg T but does not change adult behaviour).

A relationship between circulating T and maternal aggression could exist for female cardinals, but 'baseline' (or nonelevated) concentrations of the androgen may sufficiently support aggressive behaviour during incubation. In this study, T concentrations were not statistically different between aggressive and nonaggressive contexts; however, T concentrations of simulated nest intrusion birds were slightly higher than those of controls. While we did not detect a statistically significant effect of time after experiencing a simulated nest intrusion on T concentrations, there could still be an

effect of minor T increases on behaviour. Steroid hormones are powerful even at low

concentrations (Adkins-Regan, 2005) and subtle increases in concentrations could be of

biological importance even if not statistically significant. In many studies of male

aggressive behaviour significant increases in T are not observed until 10–15 min after an

aggressive stimuli is perceived (Goymann et al., 2007; Wingfield et al., 1990). While T

concentrations in female cardinals experiencing a simulated nest intrusion were not

significantly different before or after 15 min, nor did general T concentrations at the time

of capture covary with time following exposure to a simulated nest intrusion, we cannot

rule out completely that increases in T (even minor ones) play a role here. This could

only be resolved if individual females were captured before and after simulated nest

intrusions. Males of some tropical species that show prolonged territoriality have been

proposed to be more sensitive to low concentrations of circulating T (Hau et al., 2000).

Greater numbers of androgen receptors in the brain might allow such species to use T

more efficiently (Adkins-Regan, 2005) and avoid potential costs of chronically elevated

circulating androgens (e.g. energetic costs, decreased immunity, higher mortality;

Wingfield, Lynn, & Soma, 2001). Very little is known about the efficiency with which T

is used by female birds, but considering that high concentrations of T could interfere with

multiple aspects of female reproduction (Clotfelter et al., 2004; Rosvall, 2013), greater

sensitivity to lower concentrations of the hormone through regulation of androgen

receptor density in the brain could be a likely adaptive mechanism supporting female

aggression. Research in male birds has shown that in some circumstances circulating

concentrations of steroid hormones do not change; instead, it is the brain that changes,

allowing for a greater or lesser behavioural response (Canoine, Fusani, Schlinger, & Hau,

2007; Rosvall et al., 2012; Soma, Hartman, Wingfield, & Brenowitz, 1999; Soma et al., 2000; Soma, Sullivan, & Wingfield, 1999). Given that female cardinals have measurable concentrations of circulating T year-round (Jawor, 2007) but do not intensely elevate T following aggressive encounters or GnRH stimulation within the breeding season (DeVries & Jawor, 2013; this study), it is possible that females of this species could have increased sensitivity to lower concentrations of T due to greater numbers of androgen receptors in the brain and that receptor expression could change through the breeding season. Future studies assessing receptor number or action are needed in this and other species to fully address these questions.

<H2>*Other Potential Hormones*

Lastly, alternate hormones, androgen precursors or androgen metabolites could have a role in the regulation of female aggressive behaviour (Wingfield, Moore, Goymann, Wacker, & Sperry, 2006). For example, dehydroepiandrosterone (DHEA; an androgen precursor that can be converted to T) has been reported to support territorial aggression during the nonbreeding season in male and female spotted antbirds (Hau et al., 2004) and may have a role in nonbreeding aggression and song in male song sparrows (Soma & Wingfield, 2001; Soma, Wissman, Brenowitz, & Wingfield, 2002). Furthermore, oestrogen (E2) is a metabolite of T that might also contribute to the regulation of aggression through either circulating concentrations or the conversion of T to E2 in the brain by the enzyme aromatase (Adkins-Regan, 2005). Aromatization of T into E2 has been shown to mediate aggression in male Japanese quail, *Coturnix japonica* (Schlinger & Callard 1990), but little is known of a similar relationship in female birds.

Oxytocin, which acts as a modulator of aggression and affiliative behaviours in birds, and which induces egg laying, might also be influential to aggression during incubation (Goodson, Schrock, & Kingsbury, 2015; Johnson, 2015; Kelly & Goodson, 2014). Although concentrations of oxytocin following the onset of incubation should decrease, activation of various brain nuclei following an increase and then a decrease in this hormone could be influential.

Alternatively, inhibitory effects of hormones, such as progesterone (P4), could influence aggressive behaviour in female cardinals. Initially discovered in some mammalian species, P4 can have a modulating effect on female aggression, with higher concentrations of the hormone inhibiting aggressive behaviour but lower concentrations allowing for its occurrence (e.g. Davis & Marler, 2003; de Sousa et al., 2010; Fraile, McEwen, & Pfaff, 1988; Kolhert & Meisel, 2001). Similar results were reported for a sex-role-reversed bird species, the African black coucal (Goymann et al., 2008). In a study by Goymann et al. (2008), P4 concentrations of female coucals were reported to be significantly lower in individuals engaged in aggressive behaviour than in females assessed in nonaggressive contexts. Although cardinals are not a sex-role-reversed species, they share some behavioural attributes with African black coucals in that females of both species perform male-typical behaviours, such as singing and territorial defence. Thus, it is plausible that circulating T, or changes in concentrations of T, are not necessary to support aggression in incubating females and that neurological changes as well as alternative hormones need investigation for their influences on this behaviour to be understood.

<H2>*Conclusions*

Our findings suggest that incubating female cardinals do not significantly elevate circulating concentrations of T following response to intrasexual, conspecific 'intruders' at the nest; nor does general circulating T correspond with the level of aggression shown. Considering our study is one of the few to investigate connections between endogenous circulating androgens and maternal aggression (not territorial aggression) in birds, it would be premature at this point to draw definitive conclusions concerning this hormone–behaviour relationship. Yet results of this study are similar to those that have examined relationships between circulating T and female territorial aggression in birds, which suggests that high concentrations of androgens might not be necessary to support aggressive behaviour in females. It is plausible that aggression in female birds is influenced by greater sensitivity to lower concentrations of T, androgen precursors and metabolites, or modulated by additional hormones to prevent the potentially negative impacts of elevated T on female reproduction (e.g. delayed ovulation, reduced egg production, negative maternal effects). However, until more research is conducted to investigate links between hormones and female behaviour, generalized relationships between T and female aggression cannot be formed.

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Table 1

Linear mixed model analysis assessing the impact of behavioural context (aggressive or nonaggressive), year, day of incubation, corticosterone (CORT) concentration and mass on testosterone (T) concentrations of incubating female cardinals

Fixed effects	Estimate	*df*	*F*	*P*
Behaviour context	–0.97	1, 19.67	0.11	0.74
Year		2, 19.66	1.09	0.36
Day of incubation		9, 19.48	1.29	0.30
CORT	0.07	1, 19.32	0.22	0.64
Mass	–0.01	1, 20.00	0.59	0.81

Table 2

Means and SEs of testosterone (T, ng/ml) and corticosterone (CORT, ng/ml), and ranges

of T and CORT of female cardinals captured in passive (control) and aggressive

(simulated nest intrusion, SNI) contexts

	T		CORT	
	Mean	Range	Mean	Range
SNI	0.96 (0.06)	0.35–1.65	8.67 (0.91)	1.00–21.1
Control	0.77 (0.08)	0.26–1.20	6.73 (0.94)	1.41–12.8

Figure 1. Relationship between testosterone (T) and corticosterone (CORT) concentrations of incubating female cardinals captured during simulated nest intrusions (filled circles) and nonaggressive contexts (open circles). Testosterone and CORT were not correlated (Pearson correlation coefficient: $r_{22} = 0.45$, $P = 0.78$).

Figure 2. Relationship between testosterone (T) concentration and time until capture of incubating female cardinals captured in aggressive contexts (simulated nest intrusions).

Figure 3. Relationship between corticosterone (CORT) concentrations and time until capture of incubating female cardinals captured in aggressive contexts (simulated nest intrusions).