Nesting strategy shapes territorial aggression but not testosterone: a comparative approach in female and male birds

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Our understanding of the proximate and ultimate mechanisms shaping competitive phenotypes primarily stems from research on male-male competition for mates, even though female-female competition is also widespread. Obligate secondary cavity-nesting has evolved repeatedly across avian lineages, providing a useful comparative context to explore how competition over limited nest cavities shapes aggression and its underlying mechanisms across species. Although evidence from one or another cavity-nesting species suggests that territorial aggression is adaptive in both females and males, this has not yet been tested in a comparative framework. We tested the hypothesis that cavity-nesting generates more robust territorial aggression, in comparison to close relatives with less restrictive nesting strategies. Our focal species were two obligate secondary cavity-nesting species and two related species with more flexible nesting strategies in the same avian family: tree swallow (*Tachycineta bicolor*) vs. barn swallow (*Hirundo rustica*); Eastern bluebird (*Sialia sialis*) vs. American robin (*Turdus migratorius*). We assayed conspecific territorial aggression, and found that cavity-nesting species physically attacked a simulated intruder more often than their close relatives. This pattern held for both females and males. Because territorial aggression is often associated with elevated testosterone, we also hypothesized that cavity-nesting species would exhibit higher testosterone levels in circulation. However, cavity-nesting species did not have higher testosterone in circulation for either sex, despite some correlative evidence that testosterone is associated with higher rates of physical attack in female tree swallows. Our focus on a competitive context that is relevant to both sexes – competition over essential breeding resources – provides a useful comparative framework for co-consideration of proximate and ultimate drivers of reproductive competition in females and males.

Keywords: challenge hypothesis, evolutionary endocrinology, intrasexual selection, androgens, agonistic behavior

Introduction

How do competitive traits evolve? In male animals, ornaments, armaments, and intense aggressive behavior are thought to be primarily driven by mating competition; indeed, variation in competitive traits in males largely maps on to interspecific variation in sexual selection and mating systems (Bro-Jørgensen, 2007; Cooney et al., 2019; Emlen and Oring, 1977; Göran, 1998; Miles et al., 2018). For females, early hypotheses considered female competitive traits as byproducts of correlated selection on male traits (Darwin, 1871; Lande, 1980). The alternative hypothesis, that female-female competition directly shapes the evolution of competitive traits in females, has since received abundant evidence (Clutton-Brock, 2009; Hare and Simmons, 2019). Across the tree of life, females engage in social competition and receive fitness benefits (Boersma et al., 2020; Bro-Jørgensen, 2002; Krieg and Getty, 2020; Rosvall, 2011, 2008; Sandell, 1998; Slagsvold and Lifjeld, 1994; Stockley and Bro-Jørgensen, 2011; Wu et al., 2018). It is no longer in question that intrasexual competition among females is adaptive. However, debate remains as to how exactly selection shapes the evolution of competitive traits in females, in part because researchers often focus on different modes of selection in the two sexes (i.e. sexual vs. social selection, Cain and Rosvall, 2014; Carranza, 2009; Clutton-Brock, 2009; Price, 2015; Riebel et al., 2019; Tobias et al.,

2012; West-Eberhard, 1983). In essence, we need a unified framework on the evolution of competitive traits that applies to both sexes (sensu Emlen and Oring 1977).

This issue also comes into focus in evolutionary endocrinology, which has grappled with a mechanistic framework for the evolution of testosterone in females. Thirty years ago, the challenge hypothesis provided such a framework in males (Wingfield et al., 1990). One key component of this hypothesis is that interspecific variation in testosterone secretion is shaped by territorial aggression and trade-offs with parental care. Subsequent work has revealed higher levels of testosterone in species with enhanced territorial aggression or stronger degree of mating competition among males, at least in some vertebrate taxa (Garamszegi et al., 2005; Hirschenhauser et al., 2003; Mank, 2007; Marler and Trainor, 2020), but see (Goymann et al., 2019; Husak and Lovern, 2014; Moore et al., 2020). Research on the relationship between testosterone and female competition has accumulated more recently (Goymann and Wingfield, 2014; Ketterson et al., 2005; Rosvall et al., 2020). Despite the fact that testosterone is a natural and important part of female physiology (Drummond, 2006; Staub and De Beer, 1997), females tend to have lower levels of testosterone in circulation than males (Adkins-Regan, 2005), potentially due to sex-specific constraints on egg production and parental care, which could in turn influence male testosterone levels via intersexual coevolutionary processes (Ketterson et al., 2005). In females, macro-evolutionary patterns of testosterone are unrelated to mating system, degree of sexual dimorphism, and other metrics typically associated with male competition (Garamszegi, 2014; Goymann and Wingfield, 2014). Likewise, the correlative link between individual differences in testosterone and aggression is not well supported in females (reviewed in Rosvall et al., 2020), even in socially polyandrous species where females compete for mates (Lipshutz and Rosvall, 2020). However, exogenous testosterone may *experimentally* affect female aggression (Rosvall et al., 2020). In essence, there is some evidence linking testosterone to female aggression, but the application of hypotheses developed for males has not been fruitful in understanding how female aggression and its underlying mechanisms evolve (Rosvall et al., 2020, see also Duque-Wilckens and Trainor, 2017; Goymann and Wingfield, 2014). These observations highlight the need to test general principles on the evolution of competitive phenotypes using a framework that applies to both sexes.

One approach is to examine how behavior and physiology evolve when both sexes face strong selective pressures related to reproductive competition. We propose nesting strategy as a framework for the evolution of competitive phenotypes, because these resources are required for reproduction, and species vary in the flexibility vs. limited nature of this resource. A particularly limiting strategy is obligate secondary cavity-nesting, for which individuals must acquire natural or abandoned holes in order to nest and rear offspring (Newton, 1994; Nilsson, 1984; Zarnowitz and Manuwal, 1985). Cavity availability can be severely restricted, especially for obligate secondary cavity nesters who have no reproductive alternatives if they cannot maintain a pre-made cavity (Bunnell, 2013; Ibarra et al., 2017). Cavity-nesting has arisen independently at least 39 times in birds (Collias, 1997; Davidson et al., 2017), providing a useful comparative framework for testing behavioral and physiological hypotheses across multiple species. However, to our knowledge, there has not been a comparative evaluation of how cavity nesting shapes conspecific aggression, despite several lines of evidence that it should (elaborated below).

Here, we examine patterns of territorial aggression and testosterone secretion in 2 well studied obligate secondary cavity-nesting bird species in North America. We compare them each to a related species from the same avian family that has less restrictive nesting strategies, but otherwise similar life histories and ecologies. These non-cavity-nesting relatives utilize nest sites

that are more broadly distributed in their environment, which presumably reduces selection to compete over nest sites. Focusing on the early spring period of territorial establishment, we hypothesize that competition for a limited cavity resource drives higher territorial aggression. This hypothesis stems from species-specific observations that aggressive competition over nesting cavities can escalate to injury or death (Duckworth, 2008; Leffelaar and Robertson, 1985), presumably because aggression has some adaptive value in obtaining or maintaining access to limited cavities (Albers et al., 2017; Duckworth and Badyaev, 2007; Krieg and Getty, 2018; Rosvall, 2008; Sandell and Smith, 1997; Szász et al., 2019). One prediction is that cavity nesters will have higher aggression and elevated testosterone secretion, regardless of sex. Alternatively, nesting strategy may better predict trait variation in one sex than the other. For instance, cavity-nesting may primarily differentiate female behavior, assuming that males (but not females) in non-cavity nesting species also experience strong competition for territories and/or mates. Or, testosterone may track nesting strategy, but only in males, if this hormone does not generate interspecific variation in female aggression. The specific constellation of outcomes will yield important insights into the evolution of competitive traits.

Materials and Methods

Study system

We focus on 4 extremely well-studied songbird species, with abundant information on their life history. In the Turdidae family, Eastern bluebirds (*Sialia sialis*) nest inside cavities (Gowaty and Plissner, 2020) and their relatives, American robins (*Turdus migratorius*), build their nests within a variety of trees and shrubs (Vanderhoff et al., 2020). In the Hirundinidae family, tree swallows (*Tachycineta bicolor*) nest inside cavities (Winkler et al., 2020), and their relatives, barn swallows (*Hirundo rustica*), build their nests inside barns (Brown and Brown, 2020). Except for nesting strategy, each within-family species pair is ecologically similar with regard to mating system, parental care, foraging strategy, migration, etc., and evolutionary distances are roughly similar within each pair ~18-20 million years (Kumar et al., 2017).

We conducted fieldwork around Bloomington, IN (39.142 N, 86.602 W), Lexington, KY (38.104 N, 84.489 W) and Monticello, IL (40.028 N, 88.573 W). In many songbirds, aggression and testosterone tends to peak during the pre-breeding period of territorial establishment and mate acquisition prior to egg laying (George and Rosvall, 2018; Ketterson et al., 2005; Slagsvold and Lifjeld, 1994). Therefore, we collected data during the territorial establishment phase of breeding but before egg-laying, which ranged mid-March to early May from 2018 – 2020, depending on species-specific phenology. We monitored eBird and checked nest boxes daily as males and females arrived from migration, formed territorial pairs, and defended territories by displacing intruders. We determined breeding status based on the stage of nest development. For American robins, we observed pairs for several hours to determine territorial boundaries. Barn swallows reuse clay nests across years, so we monitored which nests were currently claimed as individuals flew in and out of the barn between foraging bouts. Though some criteria used to determine breeding stage vary in a species-specific manner, our observations collectively suggest that all individuals were within the territorial establishment phase. Methods were approved by Indiana University IACUC #18-004 and all relevant federal and state permits.

Simulated territorial intrusion

We simulated territorial intrusion in males and females using randomized combinations of 3-4 male or female taxidermy mounts and 3-5 conspecific vocalizations. In total, we assayed aggression in 116 individuals; see Table 1 for sample sizes specific to species and sexes. We began trials with a conspecific vocal lure to ensure the focal individuals noticed the mount and waited 30 seconds

before beginning the trial. Over the span of 5 minutes, we measured a suite of aggressive behaviors involved in territorial defense, including physical contact with the mount and average distance from the mount. We ultimately focused on physical contact as our key aggressive behavior, because overt expressions of aggression like physical attacks represent the culmination of competitive interactions. We counted the number of 5-second intervals that contained physical contact with the mount, totaling to a maximum attack score of 60. We also analyzed distance from the mount to confirm that all individuals were present and engaged with the simulated territorial intrusion, whether or not they were aggressively attacking. We did not evaluate other aggressive behaviors or signals of aggressive intent, such as flyovers or songs, because these traits may not be expressed uniformly between the sexes or between the two avian families.

Capture techniques and plasma collection

Our goal was to evaluate baseline levels of testosterone in circulation, rather than testosterone's response to social stimulation, particularly in light of evidence that testosterone rarely elevates following a social challenge birds (Goymann et al., 2019). We achieved this goal in three ways: 1) by sampling individuals without a simulated territorial intrusion, i.e. passive collection ($n = 68$). 2) by sampling individuals immediately after a short simulated territorial intrusion ($n = 16$), and 3) by sampling individuals several days after a simulated territorial intrusion ($n = 14$). Working under the assumption that any social elevation of testosterone takes at least 15 minutes and peaks closer to 30 or 45 minutes after initial activation of the HPG axis (Jawor et al., 2006; Rosvall et al., 2016), we aimed to sample blood within 15 min from the beginning of the territorial intrusion. Capture latencies in the immediate group averaged $16 \text{ min } 5 \text{ sec} \pm 1 \text{ min } 14 \text{ sec}$, from intrusion start to blood sampling. Within the immediate collection group, there was also no relationship between testosterone and latency to sampling (Pearson's correlation: $r = 0.10$, $p = 0.73$). There were also no differences in testosterone levels between individuals that were collected passively, immediately post-STI, or delayed post-STI for females (ANOVA: $F_{2,45} = 0.44$, $p = 0.66$), nor for males (ANOVA: $F_{2,46} = 1.31$, $p = 0.28$). As a consequence, we combined these groups for further analysis. In sum, we measured testosterone in 98 individuals – see Table 1 for sample sizes specific to each species and sex.

We captured individuals using a mist-net or a nestbox trap. Most animals were terminally collected for an ongoing study on comparative neurogenomics. We used an anaesthetic overdose of isoflurane, followed by decapitation and collection of trunk blood. We confirmed breeding status by examining whether females had small white follicles, and males had enlarged, white testes. Due to initially lower sample sizes, we obtained non-terminal collection from the brachial vein for some additional barn swallows, and we found no significant differences in testosterone across the two sampling types for this species ($t = -0.93$, $df = 21.56$, $p = 0.36$). We collected whole blood into heparinized BD Microtainers (product #365965) or heparinized microcapillary tubes and stored on an ice pack until we separated plasma by centrifuging for 10 minutes at 10,000 rpm. We stored plasma at -20°C for later testosterone assays.

Table 1: Sample sizes for territorial intrusion (aggression) and plasma collection (testosterone).

	Eastern Bluebird		American Robin		Tree Swallow		Barn Swallow	
	F	M	F	M	F	M	F	M
Aggression	17	20	9	11	14	24	10	11
Testosterone	15	15	7	12	14	8	13	14

Testosterone enzyme immunoassay

We extracted steroids from plasma samples using diethyl ether (3x extractions) and reconstituted in 250µL assay buffer. We measured testosterone using a High Sensitivity Testosterone Enzyme Immuno- Assay kit (Enzo #ADI-900-176, Farmingdale, NY, USA) following methods described in George and Rosvall (2018). We used 50µL plasma from females, and 10µL from males. We calculated T concentration by comparing sample absorbance with the absorbance of the assay's standard curve (Gen5 curve-fitting software, Biotek EPOCH plate reader, Winooski, VT, USA). Samples from 3 females and 2 males initially showed greater than 80% maximum binding, so we re-ran these samples at 20µL plasma to obtain values in the most sensitive part of the curve. Samples from 12 males initially showed less than 20% maximum binding, so we re-ran these samples with 10µL plasma reconstituted in 500µL assay buffer. In the case of plasma volume being insufficient (i.e. 18-19µL instead of 20 µL for two males, and 42µL instead of 50µL for one female), we added the remaining of water and calculated testosterone concentration accordingly; these samples still fell in the most sensitive part of the curve. We ran all samples in duplicate (duplicate CV = 4.0% ± 0.45). Each plate contained 3 duplicates of an extracted plasma pool, used to calculate the within and among plates variability. Intra-assay CV was 6.9% ± 0.36 and inter-plate CV was 13.3%. We suspect that this elevated inter-plate CV stems from the use of 3 different kit lots across the 3 years of this study, though we note that we balanced species and sexes within a plate each year.

Statistical analyses

We conducted all statistics in R version 3.3.2 (R-Core-Team, 2016). We examined normality using a Shapiro-Wilk test, visualized distributions with histograms, and examined outliers using a Grubbs test in the R package 'outliers'.

To evaluate whether nesting strategy predicted territorial aggression, our model included physical attacks as the response variable, with nest type, sex, and their interaction as fixed effects, and family as a random effect. Our dataset included many individuals that did not attack, resulting in excess zeros, so we ran a GLMM with zero-inflated Poisson distribution using the 'mixed_model' function in the GLMMadaptive package (Pinheiro and Bates, 1995). We used AICc in the MuMIn package (Barton, 2020) to compare this model with another using a Poisson distribution without zero-inflation, which did not perform as well ($\Delta AIC = 468.98$). We used the package 'performance' to obtain R² for variance from fixed effects (marginal R²) or fixed plus random effects (conditional R²) (Nakagawa et al., 2017).

To test whether species differed in their engagement with the simulated intrusion, regardless of the degree of their physical attack response, we compared average distance from the taxidermy mount. Our model included average distance from the mount as the response variable, with nest type, sex, and their interaction as fixed effects, and family as a random effect. Average

distance was normally distributed, so we ran a LMM with the 'lmer' function in the lme4 package (Bates et al., 2015).

As a secondary goal, we evaluated sex differences in territorial aggression within each species. For each species, we compared males and females in their rate of physical attacks using Wilcoxon-tests. We also used Wilcoxon-tests to evaluate whether aggression was influenced by the sex of the taxidermy mount, i.e. whether females more aggressive towards female intruders and vice versa.

To compare baseline levels of testosterone in circulation across species, we ran a LMM with testosterone as the response variable, nest type, sex, and their interaction as fixed effects, and family as a random effect. We normalized testosterone level using a log scale transformation.

For a subset of tree swallows ($n = 7$ females, $n = 5$ males), we were able to assess variation in aggression and circulating testosterone for the same individuals. We used spearman correlations to evaluate the relationship between % of time attacking the mount and baseline testosterone in circulation.

Results

Cavity-nesting species had higher territorial aggression

Nesting strategy significantly predicted the number of physical attacks, such that cavity-nesting species attacked the taxidermy mount significantly more ($\beta = 3.82$, $SE = 1.36$, $z = 2.82$, $p = 0.0049$; overall model: conditional $R^2 = 0.97$, marginal $R^2 = 0.97$; Fig. 1). Sex was not a significant predictor of physical attack ($\beta = 1.07$, $SE = 1.31$, $z = 0.81$, $p = 0.42$), nor was the interaction between nesting strategy and sex ($\beta = -1.21$, $SE = 1.31$, $z = -0.92$, $p = 0.36$), indicating that higher aggression in cavity-nesting species was similar for both females and males.

We did not find a significant difference in average distance from the taxidermy mount, either based on nesting strategy, ($\beta = -1.69$, $SE = 1.86$, $t = -0.91$, $p = 0.36$; overall model: conditional $R^2 = 0.12$, marginal $R^2 = 0.03$; Fig. 2), sex ($\beta = 0.98$, $SE = 2.02$, $t = 0.49$, $p = 0.63$) nor the interaction between the two ($\beta = 1.50$, $SE = 2.51$, $t = 0.60$, $p = 0.55$). This suggests that mount placement was a salient stimulus, in that individuals from all species were responding to the simulated intruder in one way or another, regardless of whether the taxidermy mount was at a nest box.

Sex differences in territorial aggression

For most species females and males attacked at similar rates, including for Eastern bluebirds ($W = 133.5$, $p = 0.22$), American robins ($W = 40.5$, $p = 0.40$), and barn swallows ($W = 55.5$, $p = 1$). However, in tree swallows, females attacked significantly more than males ($W = 235.5$, $p = 0.0082$). Territorial aggression was not related to the sex of the mount, except in Eastern bluebirds, males attacked male mounts significantly more than female mounts ($W = 6.5$, $p = 0.008$).

Figure 1: Boxplots show median and interquartile ranges for percent of time females (left panel) males (right panel) spent physically attacking taxidermy mount during simulated territorial intrusion in cavity-nesting species (light beige) and non-cavity-nesting species (dark seafoam).

Figure 2: Boxplots show median and interquartile ranges for distance from taxidermy mount in females (left panel) and males (right panel) during simulated territorial intrusions in cavity-nesting species (light beige) and non-cavity-nesting species (dark seafoam).

Levels of testosterone did not relate to nesting strategy for either sex

Nesting strategy was not a significant predictor of testosterone in circulation ($\beta = 0.019$, $SE = 0.11$, $t = 0.16$, $p = 0.87$; conditional $R^2 = 0.66$, marginal $R^2 = 0.65$; Fig. 3). For all species, levels of testosterone in circulation were significantly higher in males than in females ($\beta = 1.06$, $SE = 0.12$, $t = 9.13$, $p < 0.0001$). There was no significant interaction between nesting strategy and sex ($\beta = 0.026$, $SE = 0.16$, $t = 0.16$, $p = 0.87$). Means and

standard errors were: 0.19 ± 0.077 ng/mL and 2.99 ± 0.62 ng/mL for female and male robins, 0.22 ± 0.046 ng/mL and 2.83 ± 0.52 ng/mL for female and male bluebirds, 0.29 ± 0.054 ng/mL and 3.10 ± 0.80 ng/mL for female and male barn swallows, and 0.32 ± 0.099 ng/mL and 3.52 ± 1.03 ng/mL for female and male tree swallows.

Tree swallows were the one species for which we had individually matched aggression and testosterone data for a subset of our study subjects. In females, the rate of physical attack was significantly, positively correlated with testosterone in circulation ($\rho = 0.96$, $p = 0.0028$). There was no significant relationship between physical aggression and testosterone in male tree swallows ($\rho = -0.35$, $p = 0.56$).

Figure 3: Levels of log testosterone (ng/mL) in circulation. Percent of time spent physically attacking taxidermy mount during simulated territorial intrusion. Females (light blue) males (dark blue). Cavity-nesters represented by nestbox, non-cavity-nesters represented by an open cup nest.

Discussion

When it comes to the evolution of competitive traits, behavioral ecologists and endocrinologists have grappled with a functional and mechanistic framework that is broadly applicable to both females and males (Goymann and Wingfield, 2014; Ketterson et al., 2005; Lipshutz and Rosvall, 2020; Tobias et al., 2012), in part due to the separate consideration of selective pressures for each sex (Cain and Rosvall, 2014). In this comparative analysis, we propose and test a solution to this problem by evaluating the hypothesis that obligate secondary cavity nesting enhances territorial aggression and circulating testosterone levels in both sexes. This hypothesis was previously untested across species, though it lies at the core of species-specific studies on the adaptive value of competition for nesting cavities in birds (Duckworth, 2008; Gustafsson, 1988; Krieg and Getty, 2020; Leffelaar and Robertson, 1985; Rosvall, 2008). It also dovetails with evidence across other vertebrate taxa suggesting that females as well as males compete for limited

breeding resources (Brandtmann et al., 1999; Hare and Simmons, 2019; Reedy et al., 2017; Stockley and Bro-Jørgensen, 2011; While et al., 2009; Wu et al., 2018), and that androgens may be involved (Cox et al., 2015; Davies et al., 2016; Desjardins et al., 2006; Woodley and Moore, 1999). We found that obligate secondary cavity-nesting is associated with higher conspecific aggression in two avian families, and this pattern applied to both females and males. Nesting strategy was not associated with higher levels of testosterone in circulation for either sex, despite some correlative evidence that female aggression is associated with higher testosterone in tree swallows. Our comparative approach focuses on reproductive competition for nesting sites, and in doing so, provides a useful framework for understanding proximate and ultimate drivers of competition in both sexes.

Obligate cavity nesting and the evolution of aggression

Whereas most research on competitive traits focuses on male-male competition for mates or female-female competition for breeding resources, we demonstrate that interspecific variation in territorial aggression tracks nesting strategy in both sexes. In the Turdidae and Hirundinidae families, obligate secondary cavity-nesting species spent more of the simulated territorial intrusion physically attacking a conspecific mount compared to their relatives that have less restrictive nesting strategies. Past work on one or another obligate cavity nesting bird species found that aggression is beneficial for obtaining and maintaining a nesting territory (Duckworth and Badyaev, 2007; Krieg and Getty, 2020; Rosvall, 2008; Sandell and Smith, 1997; Szász et al., 2019). Our study finds that this adaptive behavioral trait is enhanced in species that compete for cavities, suggesting that nesting strategy is a potentially unifying driver of competitive phenotypes. This result adds to prior studies indicating that cavity-nesting may influence the evolution of a number of traits, via effects from predation, light regimes, heterospecific competition, and sexual selection (Davidson et al., 2017; Drury et al., 2020; Miles et al., 2018). Comparative studies like ours progress towards bridging within-species microevolutionary processes of behavioral adaptation with larger-scale macroevolutionary patterns, which are needed for a full understanding of behavioral evolution (Price et al., 2011).

Intriguingly, we did not find a sex-by-nesting type interaction on aggression, meaning that competition for nesting cavities is not necessarily a stronger selective force in one sex than the other. Sex-specific selection is a commonly hypothesized driver of sexually dimorphic phenotypes (Bell and Zamudio, 2012; Janicke et al., 2016; Rubenstein and Lovette, 2009; Shultz and Burns, 2017; but see Price, 2015). Likewise, when selection acts similarly in the two sexes, sexual dimorphism is reduced or absent (West-Eberhard, 1983). Accordingly, we see mutual ornaments and monomorphic traits such as brightly colored plumage and complex songs in female and male tropical birds that defend year-round territories (Dale et al., 2015; Tobias et al., 2011), as well as dewlaps in female and male in anoles (Harrison and Poe, 2012). Of course, males and females often differ in the direction or magnitude of some selective pressures. For instance, male territorial defense facilitates access to mates and mate guarding (Harts et al., 2016), and these factors should apply comparably to the 4 species in our study, all of which form socially monogamous pairs. In cavity-nesting females, aggression may also serve a number of functions, including securing exclusive access to mates (Sandell, 1998; Slagsvold et al., 1999, 1993), defense from conspecific brood parasitism (Gowaty and Wagner, 1988), ovide avoidance (Krieg and Getty, 2020), and anti-predator defense (Winkler, 1992). Our comparative case study demonstrates that cavity-nesting rises above these potentially sex- and species-specific factors as an especially salient predictor of variation in aggression.

We found that in tree swallows, physical aggression was significantly higher in females than in males, regardless of the sex of the mount. This result is consistent with several lines of evidence that competition may be fierce for these females. In most tree swallow populations, nest sites are under threat of usurpation by an abundance of female floaters (Stutchbury and Robertson, 1987, 1985). Whereas male tree swallows may find breeding success through extra-pair copulations (Lifjeld et al., 1993), obtaining and maintaining cavity access is the only route to reproductive success in females because conspecific brood parasitism is rare (Barber and Robertson, 1999; Lombardo, 1988). In our examination of sex-specific aggression we also found that in Eastern bluebirds, males attacked male mounts significantly more than female mounts. A previous study found that bluebirds attacked in a sex-specific manner (Gowaty and Wagner, 1988), but this pattern was strongest during egg laying, when the risk of conspecific brood parasitism and extra-pair matings are highest. These two sex-specific results demonstrate that other factors may explain additional behavioral variance, beyond that which we linked to the shared drive to compete for cavities.

Does testosterone facilitate parallel evolution of behavior?

If aggressive behavior has evolved in response to competition for cavities, a key next question is: how was this achieved at a mechanistic level? Evolutionary endocrinology often turns to testosterone to address divergence in traits related to reproductive or mating competition (Cox et al., 2009; Hau, 2007; Ketterson et al., 2009; Rosvall et al., 2016). Indeed, the challenge hypothesis proposes that species with more intense territorial aggression should have higher testosterone secretion (Wingfield et al., 1990). Our application of this hypothesis to competition for nesting cavities found parallel increases in aggression in cavity-nesting species, but this was not associated with concomitantly higher baseline levels of testosterone in circulation. This null relationship is not a female-specific pattern; testosterone did not track interspecific variation in male aggression either, even though males were clearly in breeding condition based on large testes and testosterone levels in the expected range (3.06 ng/ml average for males; 0.26 ng/mL for females). Although more sampling at additional breeding stages (e.g. during female fertility) is required to fully reject these links between testosterone and competition for nesting territories, our results mirror recent reports that the challenge hypothesis is context-dependent (Wingfield et al., 2020, 2019). Whereas testosterone levels have been related to alternative reproductive tactics (Küpper et al., 2015; Oliveira et al., 2005), male-female interactions (Goymann et al., 2019) and parental investment within species (Goymann and Flores Dávila, 2017; McGlothlin et al., 2007; Rosvall, 2013), as well as mating system across species (Garamszegi et al., 2005; Hirschenhauser and Oliveira, 2006), our study adds to a collection of many others that failed to find a relationship between testosterone and competitive traits across species (Goymann and Wingfield, 2014; Husak and Lovern, 2014). Uniquely, our study applies this null relationship in an appropriate context for both sexes: reproductive competition over breeding resources is essential for both female and male cavity-nesting species, and it is entirely analogous to territorial competition as laid out in the challenge hypothesis. This comparative framework better positions us to reject the hypothesis that interspecific variation in testosterone tracks variation in aggression. This issue is especially critical in females, for which the association between testosterone and competition has rarely been assessed across species, let alone in a context related explicitly to female competition.

If interspecific variation in territorial aggression is not generated by differences in testosterone secretion at baseline levels, then is it time to leave testosterone behind? Mixed

results at different levels of analysis suggest that this approach may be premature. For instance, we found a positive correlation between baseline testosterone and territorial aggression in female tree swallows, the species for which we had the most complete sampling of aggression and testosterone in the same individual. Correlational studies in other species have found mixed evidence for such a relationship (reviewed in Kempenaers et al., 2008; Rosvall et al., 2020; Williams, 2008) but there is good evidence that exogenous testosterone increases aggression in females (Rosvall et al., 2020), including tree swallows (Rosvall, 2013). There is still much to understand about why correlational links between testosterone and competitive traits *within* a population differ from patterns that emerge over larger evolutionary scales (Lipshutz et al., 2019). These mismatches may stem from context-dependent processes like local adaptation and phenotypic plasticity that generate or erode functional variation at one scale and not another (Agrawal, 2020; Hau and Goymann, 2015; Wingfield et al., 1997). Exploration of these processes in evolutionary endocrinology is promising (Cox, 2020; Lema, 2020), and more comparative approaches are needed. Levels of testosterone in circulation must also be considered within the entire sex steroid signaling system, which provides a diversity of routes to increase aggression without a change in testosterone (Ball and Balthazart, 2019; de Bournonville et al., 2020; Fuxjager and Schuppe, 2018; Schuppe and Fuxjager, 2019). Furthermore, aggression is regulated by many other mechanisms beyond testosterone, including arginine vasotocin, vasoactive intestinal peptide, serotonin, and progesterone (Goodson, 2005; Goodson et al., 2012; Goymann et al., 2008; Lischinsky and Lin, 2020; Nelson and Chiavegatto, 2001). As evolutionary behavioral endocrinology continues to embrace complexity, application of this comparative framework to more species will unveil the shared vs. diverse neuroendocrine mechanisms that facilitate behavioral evolution.

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