Male song sparrows have elevated testosterone in response to neighbors versus strangers

Christopher Moser-Purdy [a,*], Scott A. MacDougall-Shackleton [b], Frances Bonier [c], Brendan A. Graham [a], Andrea C. Boyer [b], Daniel J. Mennill [a,*]

[a] Department of Biological Sciences, University of Windsor, Windsor, ON N9B 3P4, Canada
[b] Department of Biology, Western University, London, ON N6G 1G9, Canada
[c] Biology Department, Queen's University, Kingston, ON K7L 3N6, Canada

ARTICLE INFO

ABSTRACT

Upon hearing a conspecific signal, animals must assess their relationship with the signaller and respond appropriately. Territorial animals usually respond more aggressively to strangers than neighbors in a phenomenon known as the "dear enemy effect". This phenomenon likely evolved because strangers represent a threat to an animal's territory tenure and parentage, whereas neighbors only represent a threat to an animal's parentage because they already possess a territory (providing territory boundaries are established and stable). Although the dear enemy effect has been widely documented using behavioral response variables, little research has been conducted on the physiological responses of animals to neighbors versus strangers. We sought to investigate whether the dear enemy effect is observed physiologically by exposing territorial male song sparrows (*Melospiza melodia*) to playback simulating a neighbor or a stranger, and then collecting blood samples to measure plasma testosterone levels. We predicted that song sparrows would exhibit increased testosterone levels after exposure to stranger playback compared to neighbor playback, due to the role testosterone plays in regulating aggression. Contrary to our prediction, we found that song sparrows had higher testosterone levels after exposure to neighbor playback compared to stranger playback. We discuss several explanations for our result, notably that corticosterone may regulate the dear enemy effect in male song sparrows and this may inhibit plasma testosterone. Future studies will benefit from examining corticosterone in addition to testosterone, to better understand the hormonal underpinnings of the dear enemy effect.

© 2017 Elsevier Inc. All rights reserved.

1. Introduction

Territorial animals interact with many types of conspecific individuals. Upon encountering a conspecific individual, an animal must assess its threat level and respond appropriately. Across a wide range of animal taxa, territorial interactions are more intense when a resident animal encounters an unfamiliar stranger versus a familiar neighbor (Temeles, 1994). This phenomenon is known as the "dear enemy effect" (Fisher, 1954). Strangers are thought to pose a higher threat of territory takeover than neighbors who, by definition, already possess a territory and therefore might be less likely to usurp the resident animal's territory (Getty, 1987). Because neighbors may be less of a threat, the establishment of a less aggressive relationship between neighbors allows individuals to maximize their fitness by focusing effort on activities such as foraging and provisioning for young instead of costly aggressive

encounters. The dear enemy effect has been reported in many animal taxa including birds (e.g. Hardouin et al., 2006), mammals (e.g. Rosell et al., 2008), insects (e.g. Langen et al., 2000), frogs (e.g. Lesbarrères and Lodé, 2002), lizards (e.g. Whiting, 1999), fish, (e.g. McGregor and Westby, 1992), and crustaceans (e.g. Booksmythe et al., 2010).

The dear enemy effect has been well studied using physical and acoustic measures of behavioral response (Temeles, 1994). In contrast, physiological responses to neighbor and stranger stimuli are almost completely unknown (but see Aires et al., 2015). Under the Challenge Hypothesis, territorial male animals are predicted to increase their testosterone levels in response to a territorial challenge (Wingfield et al., 1990). Testosterone is a steroid hormone that often regulates aggression in vertebrates. For example, experimentally increased testosterone causes increased aggression toward conspecific individuals (e.g. Wingfield, 1994; Mougeot et al., 2005) and testosterone levels often increase during simulated territorial intrusions (e.g. Hau et al., 2000; Wingfield and Wada, 1989). Given that testosterone can modulate responses to territorial challenges, and given that strangers might pose a heightened threat in comparison to neighbors, we predicted that

individuals will increase circulating testosterone levels when exposed to a stranger more than when exposed to a neighbor. This increase in testosterone may mediate the behavioral responses observed in dear enemy effect studies. Only one study has previously examined this potential hormonal mechanism of the dear enemy effect by examining a tropical fish species. Mozambique tilapia (*Oreochromis mossambicusi*) showed an increased level of 11-ketotestosterone in response to presentation of a stranger in comparison to a neighbor (Aires et al., 2015). It is unknown whether any other vertebrates have a similar endocrine response to challenges from neighbors versus strangers.

We used song playback to study the role of testosterone in mediating the dear enemy effect in territorial male song sparrows (*Melospiza melodia*). We exposed territorial males to playback simulating either an unfamiliar stranger or an established territorial neighbor and then sampled their testosterone levels, to test the prediction that birds would show heightened testosterone during confrontation with a stranger. Song sparrows are an ideal study system for this experiment given that multiple previous studies show that they display the behavioral response predicted by the dear enemy hypothesis (Harris and Lemon, 1976; Kroodsma, 1976; Moser-Purdy et al., 2017; Stoddard et al., 1990; Stoddard et al., 1991) and because they increase testosterone when challenged by conspecifics during breeding (Wingfield and Wada, 1989).

2. Methods

2.1. Study site and species

We presented territorial male song sparrows with experimental playback of recordings of conspecific song at the Queen's University Biological Station (44° 34′ N, 76° 19′ W; Ontario, Canada). During April of 2016 we recorded birds and mapped territories; we spent ≥90 min observing each bird, over multiple days, and we logged each of his song posts using a Global Positioning System (Garmin 60CS). From May 4 to May 19, 2016 we conducted playback experiments on 22 paired male song sparrows. All birds had established territories and were paired at the time of playback (i.e., each male was observed affiliating with a female over extended periods, regularly foraging together and moving around their territory together), as determined during observations in April and early May. Four birds were banded from studies in previous years with unique combinations of colored bands and a Canadian Wildlife Service numbered band. In addition, we banded 12 of the birds we caught in 2016; these birds were banded after the post-playback blood collection, rather than before playback. We did not want to capture the birds prior to playback, exposing our subjects to a potentially stressful event and reducing our ability to capture them a second time for blood collection after playback. We had no difficulty identifying birds, regardless of whether or not they were banded, based on location, behavior, and individually distinctive songs (song sharing is low in Eastern populations of song sparrows; Hughes et al., 2007; Stewart and

MacDougall-Shackleton, 2008; although see Foote and Barber, 2007). Testosterone levels did not differ among birds from different banding cohorts (i.e., banded in 2015, banded in 2016, or unbanded; Spearman's rank correlation: rho = 0.30, $p = 0.18$, $N = 22$).

2.2. Playback stimuli

To construct playback stimuli we recorded spontaneous bouts of singing from male song sparrows. We used an omni-directional microphone (model: Sennheiser ME62/K6) mounted inside of a parabolic reflector (model: Telinga MK2) connected to a solid-state digital recorder (model: Marantz PMD660, 44.1 kHz sampling rate, 16-bit encoding, WAVE format). We collected five song types from each male to use in constructing playback stimuli. We isolated five songs that showed no overlapping background noise (assessed visually based on spectrograms generated in Audition 3.0, Adobe, San Jose, CA), and we normalized each song to −1 dB using the "normalize" feature of Audition 3.0. Each stimulus was 30 min in length and consisted of five song types played at a rate of one song every 10 s (this is a natural singing rate for this population based on our preliminary observations). One song type was broadcast for 3 min before switching to a different song type (this too was within the range of natural song type switching in our population). We cycled through each of the five song types twice for a total of 30 min (Fig. 1). Each stimulus set was composed of different song types from the same bird.

To create neighbor stimuli, we collected a recording of the neighbor from a bird adjacent to each playback subject. To create stranger stimuli, we collected a recording from a bird at least 2 km away from the focal bird. We had 14 stimuli in total: four were used twice (three were used once as a neighbor and once as a stranger, one was used twice as a stranger) and two were used three times (twice as a neighbor and once as a stranger).

In addition to neighbor and stranger stimuli used during the playback phase of the experiment, we also created a capture stimulus for the capture phase of the experiment (see below). A separate stimulus was necessary because the subjects' responses to neighbor playback did not incite sufficiently close approaches to permit capture. Songs for the capture stimulus were collected from a bird >5 km away from all of our playback subjects. We designed the capture stimulus to be highly aggressive and thereby incite rapid, close approach to the loudspeaker so that we could capture birds in a mist net. The stimulus consisted of five song types from the same bird, each played five times separated by 2 s of silence, and with 8 s of song sparrow calls inserted prior to each change in song type. We used the same capture stimulus for all birds.

2.3. Playback experiment

We presented each of the 22 subjects with 30 min of stranger playback ($N = 10$) or neighbor playback ($N = 12$) (Fig. 1). After this 30-min
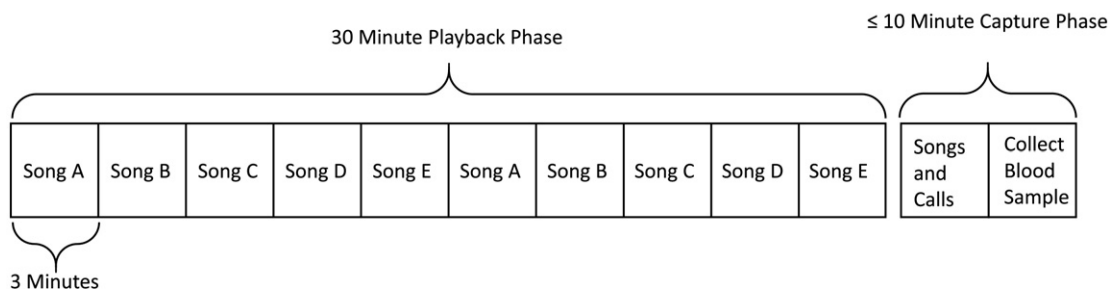

Fig. 1. Schematic representation of the experimental procedure used to study androgen responses to neighbor or stranger playback in song sparrows. Birds received 30 min of playback consisting of either neighbor or stranger songs, with each song type played for a 3-minute bout. Five song types were used for each playback stimulus and a bout of each song type was repeated once. Following the 30-minute playback phase we began the capture phase. During the capture phrase, we played a capture stimulus to facilitate rapid capture of the subject, followed by the collection of a blood sample within 10 min (except in three cases, see *Hormone Analysis* for details).

"playback phase" we captured the focal bird using playback of the capture stimulus, i.e. the "capture phase", which were broadcast for as long as necessary to capture the bird: on average the capture stimulus was played for 3.7 ± 0.6 min (mean $\pm$ SE; range: 0.6–9.0 min). The length of time the capture stimulus was played did not differ between neighbor versus stranger trials (Exact Wilcoxon Rank Sum Test: $W = 63$, $p = 0.86$, $N = 22$). We collected a blood sample for testosterone analysis following capture (see *Hormone analysis* section below for details on time course of testosterone sampling). Each subject received either a stranger or a neighbor playback; i.e. we followed a between-subjects design). We played either neighbor or stranger playback from the loudspeaker, determined randomly based on an initial coin toss followed by alternation of neighbor and stranger playback.

All playback trials took place between 0630 and 1130 h. Before starting our playback trials, we set up a mist net within the subject's territory but kept it closed until the capture phase. For both neighbor and stranger trials we set up a loudspeaker (model: Scorpion TX200, FoxPro Inc.), which we positioned 10 m from the edge of the territory of the focal bird (as determined during territory mapping), near the boundary of its neighbor. During the capture phase of our experiment, we placed the loudspeaker in the middle of our focal male's territory in order to incite an aggressive response for rapid capture. We placed the loudspeaker within a sound baffle constructed from a 20-in. diameter plastic parabola with a 2-in. thick lining of foam covered by camouflage fabric. This baffle appreciably reduced noise behind the speaker with the aim to reduce interference from neighbors. We did not observe any interactions between neighbors and focal birds, and neighbors never appeared to respond to playback. We attempted to minimize the effect of breeding stage on playback responses by conducting playback experiments during a short time span (15 days) when we expected that many of the pairs in the population were incubating eggs. We confirmed that two pairs were incubating eggs by finding their nests. We also captured two additional females after playback and found they had an edematous brood patch, indicating that these pairs were also incubating. Furthermore, our observations of males prior to playback trials further supports this idea: males were traveling around their territories independently, suggesting that females were incubating. Song sparrows breed fairly synchronously within years (Wilson & Arcese 2006; Wilson et al., 2000) so it is likely that other pairs for which we did not find nests or catch females were also incubating.

We measured the birds' aggressive responses to playback by assigning each individual an aggression score (Emlen, 1971; Wunderle, 1978) between 1 and 5. A higher score was assigned to more aggressive responses (i.e. spending time near the speaker, making a high number of flights around the speaker, spending more time responding to the stimulus, singing more soft songs) and a low score was assigned to a less aggressive response (see Table 1). Although in most cases the latency of response and duration of response was associated with the same aggression score, in cases where they differed we took the highest aggression score associated with the subject's latency or duration of response. Although this method of quantifying behavioral

response is qualitative, it helped us to determine whether a dear enemy effect was observed across our trials as has been found in several other studies of song sparrows (Harris and Lemon, 1976; Kroodsma, 1976; Stoddard et al., 1990; Stoddard et al., 1991) including a study on birds within the same population during incubation (Moser-Purdy et al., 2017). We validated our qualitative aggression score by applying it to quantitative data we collected during a separate neighbor-stranger experiment (Moser-Purdy et al., 2017). One of the observers of the current experiment (C M-P) conducted an analysis blind to the neighbor or stranger treatment in the other experiment, and assigned an aggression score to each trial from the previous experiment ($N = 52$) based on the collected dataset. The qualitative aggression scores were highly correlated with the quantitative principal components scores representing aggressive response from the previous dataset (Spearman's rank correlation; rho $= 0.93$; $p < 0.00001$; Moser-Purdy et al., 2017).

This study was approved by the University of Windsor Animal Care Committee under AUPP-16-01 and by the Queen's University Animal Care Committee under permit 2016–1652.

2.4. Hormone analysis

We collected blood samples for hormone analysis immediately upon extracting birds from mist nets during the capture phase. We collected up to 75 μL of blood from the brachial vein using a 26-gauge needle and heparinized microcapillary tubes. Blood samples were immediately stored on ice until they were centrifuged and the plasma was harvested within 5 h of blood collection. Separated plasma samples were stored at -20 °C until they were assayed 4–5 weeks later. Blood samples were collected on average within 7.8 ± 0.5 min (mean $\pm$ SE; range: 3.6–13.0 min) of the end of the playback phase and on average 4.0 ± 0.4 min (mean $\pm$ SE; range: 1.5–7.3 min) following capture. The amount of time to collect blood samples did not differ between neighbor and stranger trials (Student's *t*-test: $t = 0.76$, $p = 0.46$, $N = 22$). Previous research shows that testosterone does not show an increase in song sparrows until exposure to at least 10 min of playback (Wingfield and Wada, 1989). We collected blood from only three birds after 10 min (two birds caught after 10.5 min and one bird caught after 13 min) but found no effect of time before blood collection on plasma testosterone (Spearman's rank correlation: rho $= -0.16$, $p = 0.48$). Exclusion of the three data points collected after 10 min provided the same result as those presented below, and therefore we retained them in our final analysis.

We used a commercially available enzyme immunoassay kit to quantify plasma testosterone (1–2403, Salimetrics, State College, PA, U.S.A.) that had been previously validated for song sparrows in our study population (see Schmidt et al., 2014). The antibody of this kit has some cross reactivity with androgens, thus hereafter we refer to "androgen levels" rather than "testosterone levels". We used assay buffer to dilute plasma 1:12. We then analyzed samples in duplicate, using 25 μL of the diluted plasma in each well. All samples were run in one assay. The intra-assay coefficient of variation was 13.2% for a low control (250 pg/mL) and 1.5% for a high control (2500 pg/mL). The total intra-assay coefficient of variation was 4.9%. Androgen levels of all samples fell within the range of the standard curve (6.1–600 pg/mL).

2.5. Statistical analysis

We used R (version 3.2.3, R Development Core Team, 2015) to analyze our data. Because the androgen data were not normally distributed (Shapiro-Wilk Normality Test: $W = 0.69$, $p < 0.0001$, $N = 22$), we used a Wilcoxon rank sum test to analyze the data, with treatment group as a predictor variable. Similarly, because the aggressive response data were not normally distributed (Shapiro-Wilk Normality Test: $W = 0.83$, $p = 0.001$, $N = 22$) and because some individuals shared the same aggression scores, we used an exact Wilcoxon rank sum test using the package exactRankTests (Hothorn and Hornik, 2015) to

Table 1
Explanation of the aggression scores assigned to playback subjects on the basis of their latency to respond to playback and the duration of their response to playback.

Aggression score	Behavior observed
1	No response to playback
2	High latency to respond to playback (>15 m); short duration (<1 min) spent near the speaker
3	Moderate latency to respond to playback (10–15 min); moderate duration spent near the speaker (1–10 min)
4	Low latency to respond to playback (1–10 min); high duration of time spent within 15 m of the speaker (10–20 min)
5	Immediate response to playback (<1 min); very high duration (>20 mins) spent within 15 m of the speaker

analyze the data with treatment group as a predictor variable. Additionally, we calculated effect size (i.e. Cohen's d) for all of these analyses using an online effect size calculator (http://www.campbellcollaboration.org/escalc/html/EffectSizeCalculator-SMD1.php). Lastly, due to the non-normality of our data, we conducted a Spearman's rank correlation to determine if there was a correlation between plasma androgens and aggression score.

3. Results

Song sparrows had higher plasma androgens when exposed to neighbor playback than when exposed to stranger playback (Wilcoxon rank sum test: W = 100, $p = 0.007$; Cohen's d = 0.81; Fig. 2). Plasma androgen levels were more than twice as high, on average, among birds that received playback simulating a neighbor versus birds that received playback simulating a stranger.

Song sparrows showed higher aggression scores when exposed to stranger playback than when exposed to neighbor playback (exact Wilcoxon rank sum test: W = 7.5, $p = 0.003$; Cohen's d = 1.4; Fig. 3), consistent with the dear enemy effect, and consistent with the results of all previous behavioral studies of neighbor-stranger discrimination in song sparrows (Harris and Lemon, 1976; Kroodsma, 1976; Moser-Purdy et al., 2017; Stoddard et al., 1990; Stoddard et al., 1991).

Across treatment groups, song sparrows with lower plasma androgens had higher aggression scores (Spearman's rank correlation: rho = −0.50, $p = 0.02$).

4. Discussion

We found that territorial male song sparrows exhibited lower androgen levels in response to playback of a stranger versus playback of a neighbor. Similar to previous work on song sparrows (Harris and Lemon, 1976; Kroodsma, 1976; Moser-Purdy et al., 2017; Stoddard et al., 1990; Stoddard et al., 1991), we observed a behavioral dear enemy effect in our study, where males responded more strongly to playback of strangers versus neighbors. Androgen levels and behavioral responses were negatively correlated with each other, indicating that song sparrows that displayed higher levels of aggression had decreased levels of testosterone. This finding stands in direct contrast to our prediction that strangers, which might be perceived as more threatening rivals, would induce higher testosterone levels than neighbors. Strangers should pose a higher threat than neighbors because they theoretically
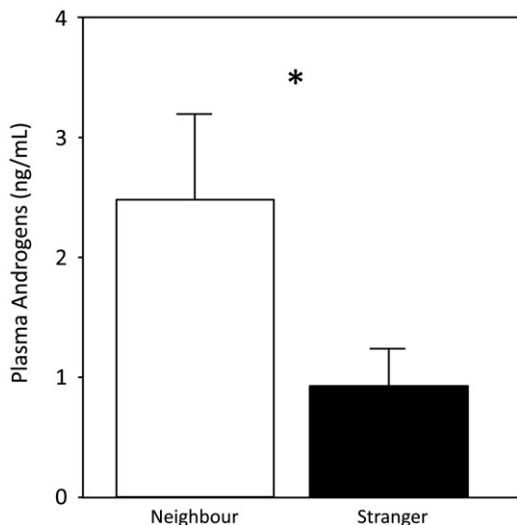

Fig. 2. Territorial male song sparrows had higher levels of plasma androgens after 30 min of playback of recorded songs of a neighboring male ($N = 12$) compared to 30 min of playback of recorded songs of an unfamiliar stranger ($N = 10$) (Wilcoxon rank sum test; $p = 0.007$). Data are presented as means + SE.
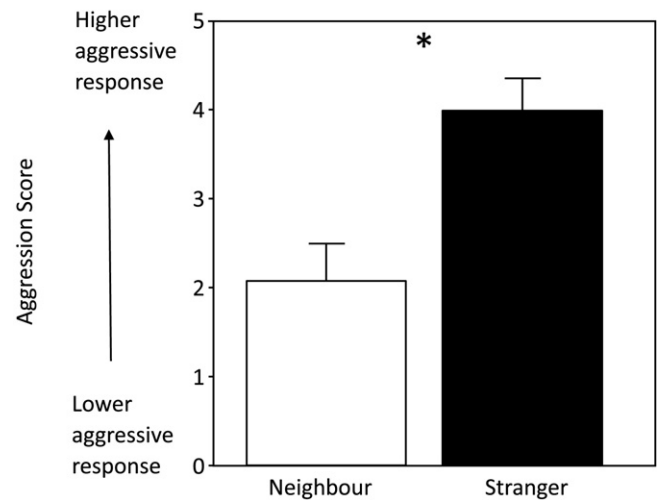

Fig. 3. Territorial male song sparrows showed higher aggression scores (a qualitative measurement of birds' responses to playback) when exposed to playback of recorded songs of a stranger male ($N = 12$) compared to playback of recorded songs of a neighboring male ($N = 10$), consistent with the dear enemy effect (exact Wilcoxon rank sum test; $p = 0.003$). Data are presented as means + SE.

represent a higher threat to territory takeover than neighbors (Temeles, 1994). Here we discuss this counter-intuitive finding, we explore alternative explanations for why androgens would be higher in response to playback of strangers, and we draw connections to other studies that show lower androgens in aggressive situations.

The Challenge Hypothesis (Wingfield et al., 1990) predicts that territorial animals exposed to a social challenge should increase plasma testosterone to regulate aggression. However, due to the negative effects of testosterone (e.g. reduced parental care; Wingfield et al., 2001), animals may benefit from increased testosterone levels only when the benefits outweigh the costs. Across the literature, some studies report an increase in testosterone during simulated intrusions (e.g. Wikelski et al., 1999; Wingfield and Hahn, 1994; Wingfield and Wada, 1989). Other studies, however, report an absence of testosterone response during simulated intrusions (e.g. Deviche et al., 2014; Gill et al., 2008; Landys et al., 2010; Rosvall et al., 2012) or a decrease in plasma testosterone during simulated intrusions (e.g. Landys et al., 2007; Van Duyse et al., 2004) possibly due to the detrimental effects of testosterone on breeding effort through reduced parental care. We found that exposure to a higher threat (i.e. playback simulating a stranger) resulted in lower plasma androgens than exposure to a lower threat (i.e. playback simulating a neighbor) during a period when the playback subjects were incubating. This suggests that plasma androgens do not play a role in dear enemy effect expression during incubation in song sparrows, and that song sparrows may benefit from avoiding the detrimental effects of testosterone during this time period. Indeed, plasma testosterone appears to be lower during incubation in song sparrows (Wingfield and Hahn, 1994), and plasma androgens following either playback in our study are relatively low compared to plasma testosterone following challenges during the pre-nesting phase in other studies on song sparrows (Wingfield, 1985; Wingfield and Wada, 1989).

Given that higher testosterone is associated with reduced parental care (Schoech et al., 1998; Silverin, 1980; Van Roo, 2004), song sparrows may mount an aggressive response through mechanisms other than testosterone during their incubation and nestling provisioning stages. Wingfield (1984) investigated whether male song sparrows showed elevated testosterone levels when inhabiting a territory next to a neighbor that had a testosterone implant. During incubation and provisioning, song sparrows with testosterone-implanted neighbors did not have increased testosterone levels, whereas in other breeding stages song sparrows with testosterone-implanted neighbors had elevated testosterone levels. This finding suggests that during incubation

and provisioning, testosterone plays less of a role in interactions with aggressive conspecific rivals. In addition to reducing parental care, increased testosterone levels can be associated with reduced immune function, reduced fat stores, and increased mortality (reviewed in Wingfield et al., 2001), which song sparrows would benefit from avoiding. It is possible that instead of testosterone, corticosterone drives dear enemy effect expression in song sparrows during incubation. Corticosterone is the major glucocorticoid in birds and plays a role in regulating stress responses through the mobilization of resources such as glucose to cope with energy-demanding activities (Romero, 2002), which may include intense response to conspecific stimulus.

Landys et al. (2007), Van Duyse et al. (2004), and Sorenson et al. (1997) found that upon exposure to conspecific stimuli, their playback subjects (birds in all cases) responded with increased aggression and corticosterone levels, but decreased testosterone levels. Additionally, female song sparrows exposed to a simulated intrusion had decreased testosterone levels compared to controls (Elekonich and Wingfield, 2000) and male song sparrows increased corticosterone when exposed to a simulated territorial intrusion in the mid-to-late breeding season (Newman and Soma, 2011; but see Wingfield and Hahn, 1994). Corticosterone is well established as a suppressor of the hypothalamic-pituitary-gonadal (HPG) axis and can have an inhibitory effect on plasma testosterone (Deviche et al., 2010; Dong et al., 2004; James et al., 2008; McGuire and Bentley, 2010; Monder et al., 1994). Landys et al. (2007) suggest that corticosterone may suppress testosterone levels during challenges in order to ensure testosterone levels remain low and do not interfere with parental care, although they only apply this hypothesis to birds that raise a single brood of offspring each year, whereas song sparrows at our field site often produce multiple broods. Unfortunately, we were unable to test whether corticosterone was associated with the dear enemy effect in our study due to lack of samples collected under 3 min from capture (corticosterone sampling requires blood samples to be collected within this timeframe; e.g. Romero and Reed, 2005) and limited amounts of blood plasma following testosterone analyses. Future studies may benefit from including corticosterone levels as well as testosterone levels when examining the physiological responses associated with the dear enemy effect. Future studies may also benefit from conducting experiments during different breeding stages.

An alternative to the idea that corticosterone mediates the dear enemy effect is that dehydropiandrosterone (DHEA) may instead regulate the dear enemy effect. DHEA is a steroid hormone and is metabolized to testosterone or 17-β-estradiol (E2) in the brain (Heimovics et al., 2016). DHEA has been associated with aggression in non-breeding song sparrows despite the suppression of testosterone during this time period (Newman and Soma, 2011; Soma et al., 2002) suggesting that it may mediate behavioral responses to conspecific challenges. Furthermore, E2 has been shown to regulate aggression in response to simulated territorial intrusions in non-breeding male song sparrows (Soma et al., 2000). However, this hypothesis fails to explain why plasma androgens in response to strangers were lower than in response to neighbors because there is no evidence that DHEA or E2 suppress plasma testosterone, and increased DHEA may be associated with increased plasma testosterone (Soma et al., 2002). Nevertheless, it may be beneficial to include neurosteroids such as DHEA and E2 in future studies investigating the hormonal correlates of the dear enemy effect alongside corticosterone.

Another possible explanation for our results is that during neighbor playback, subjects may have had visual contact with their neighbor, whereas during stranger playback subjects would not have seen the simulated intruder. Wingfield and Wada (1989) showed that song sparrows increase testosterone levels most when an acoustic stimulus is paired with a visual stimulus. Song sparrows in our study that were exposed to neighbor playback may have had both an acoustic stimulus (i.e. the playback) and a visual stimulus (i.e. the actual neighbor) whereas subjects exposed to stranger playback only had exposure to an acoustic

stimulus. We did not observe any interactions between focal birds and neighbors during our trials, however we were not able to continuously track the focal bird's location if the bird temporarily left the playback area. Furthermore, Wingfield and Wada (1989) found an increase in plasma testosterone after only 10 min of playback; our study measured plasma androgens after roughly 40 min from the beginning of playback. It is possible that song sparrows had increased levels of plasma androgens during their initial response to stranger playback but decreased plasma androgens when no stranger was observed visually. However, during neighbor playback visual confirmation of the neighboring male may have caused higher androgen levels to persist. Additionally, due to the length of our playback period it is possible that there may have been negative feedback on androgen release during stranger trials. However, 30 min is a common playback length when measuring hormonal response to playback (e.g. Deviche et al., 2014; Gill et al., 2008), and we have no reason to expect that negative feedback would be enhanced in stranger versus neighbor trials. Future studies would benefit from either removing the neighboring song sparrows during neighbor playback trials or capturing neighbors and strangers to use as live decoys during playback trials, although the logistics of this approach may prove challenging.

Three final explanations for our unexpected finding that song sparrows show higher testosterone levels in response to playback of neighbors rather than strangers merit consideration. (1) Birds in our study population may find neighbors a higher threat than strangers. Several lines of evidence suggest this explanation is unlikely. Our behavioral score shows stronger responses to playback of strangers than neighbors, indicative of a higher threat level perceived in response to strangers. Furthermore, many other studies of song sparrows have clearly documented a dear enemy effect (Harris and Lemon, 1976; Kroodsma, 1976; Stoddard et al., 1990; Stoddard et al., 1991) including a study on song sparrows from our study population during incubation (Moser-Purdy et al., 2017). (2) Given that our playback experiment was conducted during the incubation period, several weeks after territory boundaries were established, our playback of 30 min of neighbor song may have simulated a resurgence of territorial activity by the neighbor and subsequently raised androgen levels. However, this does not explain why neighbor playback elicited increased androgen levels compared to stranger playback, given that both represent a threat to territory ownership under this explanation. (3) An increase in neighbor song may indicate that the neighbor's female is sexually receptive (Møller, 1991); thus upon hearing an increase in song output from their neighbor, an individual may increase androgens in preparation for extra-pair copulations (Wingfield and Monk, 1994). Song sparrows have considerable levels of extra-pair copulation (e.g. 36.1% of broods have at least one extra-pair offspring; Hill et al., 2011), and so males may find an advantage in attending to the fertility status of females in neighboring territories, possibly by monitoring changes in the song output of neighboring males. However, one previous study of song sparrows shows that males sing less when their partner is fertile (Turner and Barber, 2004), and therefore this explanation may also be unlikely.

5. Conclusions

Song sparrows increased their androgen levels more in response to neighbor playback than in response to stranger playback, despite displaying a dear enemy effect in their aggressive behavior. Our results suggest that androgens do not play a role in regulating the aggression observed during dear enemy effect expression in the incubation breeding stage in song sparrows. We speculate that the dear enemy effect may be mediated by corticosterone during this time.

Acknowledgements

References

R Development Core Team, 2015. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna https://www.R-project.org.

Aires, R.F., Oliveira, G.A., Oliveira, T.F., Ros, A.F.H., Oliveira, R.F., 2015. Dear enemies elicit lower androgen responses to territorial challenges than unfamiliar intruders in a cichlid fish. PLoS One 10, e0137705.

Booksmythe, I., Jennions, M.D., Backwell, P.R.Y., 2010. Investigating the "dear enemy" phenomenon in the territory defence of the fiddler crab, Uca mjoebergi. Anim. Behav. 79, 419–423.

Deviche, P.J., Hurley, L.L., Fokidis, H.B., Lerbour, B., Silverin, B., Silverin, B., et al., 2010. Acute stress rapidly decreases plasma testosterone in a free-ranging male songbird: potential site of action and mechanism. Gen. Comp. Endocrinol. 169, 82–90.

Deviche, P., Beouche-Helias, B., Davies, S., Gao, S., Lane, S., Valle, S., 2014. Regulation of plasma testosterone, corticosterone, and metabolites in response to stress, reproductive stage, and social challenges in a desert male songbird. Gen. Comp. Endocrinol. 203, 120–131.

Dong, Q., Salva, A., Sottas, C.M., Niu, E., Holmes, M., Hardy, M.P., 2004. Rapid glucocorticoid mediation of suppressed testosterone biosynthesis in male mice subjected to immobilization stress. J. Androl. 25, 973–981.

Elekonich, M.M., Wingfield, J.C., 2000. Seasonality and hormonal control of territorial aggression in female song sparrows (Passeriformes Emberizidae Melospiza melodia). Ethology 106, 493–510.

Emlen, S.T., 1971. The role of song in individual recognition in the indigo bunting. Z. Tierpsychol. 28, 241–246.

Fisher, J., 1954. Evolution and bird sociality. In: Huxley, J., Hardy, A.C., Ford, E.B. (Eds.), Evolution as a Process. Allen & Unwin, London, pp. 71–83.

Foote, J.R., Barber, C.A., 2007. High level of song sharing in an eastern population of song sparrow (Melospiza melodia). Auk 124, 53–62.

Getty, T., 1987. Dear enemies and the prisoner's dilemma: why should territorial neighbors form defensive coalitions? Am. Zool. 27, 327–336.

Gill, S.A., Costa, L.M., Hau, M., 2008. Males of a single-brooded tropical bird species do not show increases in testosterone during social challenges. Horm. Behav. 54, 115–124.

Hardouin, L.A., Tabel, P., Bretagnolle, V., 2006. Neighbor–stranger discrimination in the little owl, Athene noctua. Anim. Behav. 72, 105–112.

Harris, M., Lemon, R., 1976. Responses of male song sparrows Melospiza melodia to neighbouring and non-neighbouring individuals. Ibis 118, 421–424.

Hau, M., Wikelski, M., Soma, K.K., Wingfield, J.C., 2000. Testosterone and year-round territorial aggression in a tropical bird. Gen. Comp. Endocrinol. 117, 20–33.

Heimovics, S.A., Prior, N.H., Ma, C., Soma, K.K., 2016. Rapid effects of an aggressive interaction on dehydroepiandrosterone, testosterone and oestradiol levels in the male song sparrow brain: a seasonal comparison. J. Neuroendocrinol. 28.

Hill, C.E., Akçay, Ç.C., Campbell, S.E., Beecher, M.D., 2011. Extrapair paternity, song, and genetic quality in song sparrows. Behav. Ecol. 22, 73–81.

Hothorn, T., Hornik, K., 2015. exactRankTests: Exact Distributions for Rank and Permutation Tests. R Package Version 0.8-28.

Hughes, M., Anderson, R.C., Searcy, W.A., Bottensek, L.M., Nowicki, S., 2007. Song type sharing and territory tenure in eastern song sparrows: implications for the evolution of song repertoires. Anim. Behav. 73, 701–710.

James, P., Rivier, C., Lee, S., Rivier, C., Clayton, T., 2008. Presence of corticotrophin-releasing factor and/or tyrosine hydroxylase in cells of a neural brain-testicular pathway that are labelled by a transganglionic tracer. J. Neuroendocrinol. 20, 173–181.

Kroodsma, D.E., 1976. The effect of large song repertoires on neighbor "recognition" in male song sparrows. Condor 78, 97–99.

Landys, M.M., Goymann, W., Raess, M., Slagsvold, T., Goymann, W., Raess, M., Slagsvold, T., 2007. Hormonal responses to male - male social challenge in the blue tit Cyanistes caeruleus: single - broodedness as an explanatory variable. Physiol. Biochem. Zool. 80, 228–240.

Landys, M.M., Goymann, W., Schwabl, I., Trapschuh, M., Slagsvold, T., 2010. Impact of season and social challenge on testosterone and corticosterone levels in a year-round territorial bird. Horm. Behav. 58, 317–325.

Lesbarrères, D., Lodé, T., 2002. Variations in male calls and responses to an unfamiliar advertisement call in a territorial breeding anuran, Rana dalmatina: evidence for a "dear enemy" effect. Ethol. Ecol. Evol. 14, 287–295.

McGregor, P.K., Westby, G.W., 1992. Discrimination of individually characteristic electric organ discharges by a weakly electric fish. Anim. Behav. 43, 977–986.

McGuire, N.L., Bentley, G.E., 2010. A functional neuropeptide system in vertebrate gonads: gonadotropin-inhibitory hormone and its receptor in testes of field-caught house sparrow (Passer domesticus). Gen. Comp. Endocrinol. 166, 565–572.

Møller, A.P., 1991. Why do songbirds sing so much: mate guarding and male announcement of mate fertility status. Am. Nat. 138, 994–1014.

Monder, C., Sakai, R.R., Miroff, Y., Blanchard, D.C., Blanchard, R.J., 1994. Reciprocal changes in plasma corticosterone and testosterone in stressed male rats maintained in a visible burrow system: evidence for a mediating role of testicular 11β-hydroxysteroid dehydrogenase. Endocrinology 134, 1193–1198.

Moser-Purdy, C., MacDougall-Shackleton, E.A., Mennill, D.J., 2017. Enemies aren't always dear: male song sparrows adjust dear enemy effect expression in response to female fertility. Anim. Behav. 126, 17–22.

Mougeot, F., Dawson, A., Redpath, S.M., Leckie, F., 2005. Testosterone and autumn territorial behavior in male red grouse Lagopus lagopus scoticus. Horm. Behav. 47, 576–584.

Newman, A.E.M., Soma, K.K., 2011. Aggressive interactions differentially modulate local and systemic levels of corticosterone and DHEA in a wild songbird. Horm. Behav. 60, 389–396.

Romero, L.M., 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. Gen. Comp. Endocrinol. 128, 1–24.

Romero, L.M., Reed, J.M., 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? Comp. Biochem. Physiol. A Mol. Integr. Physiol. 40, 73–79.

Rosell, F., Gundersen, G., Le Galliard, J.F., 2008. Territory ownership and familiarity status affect how much male root voles (Microtus oeconomus) invest in territory defence. Behav. Ecol. Sociobiol. 62, 1559–1568.

Rosvall, K.A., Reichard, D.G., Ferguson, S.M., Whittaker, D.J., Ketterson, E.D., 2012. Robust behavioral effects of song playback in the absence of testosterone or corticosterone release. Horm. Behav. 62, 418–425.

Schmidt, K.L., MacDougall-Shackleton, E.A., Soma, K.K., MacDougall-Shackleton, S.A., 2014. Developmental programming of the HPA and HPG axes by early-life stress in male and female song sparrows. Gen. Comp. Endocrinol. 196, 72–80.

Schoech, S.J., Ketterson, E.D., Nolan Jr., V., Sharp, P.J., Buntin, J.D., 1998. The effect of exogenous testosterone on parental behavior, plasma prolactin, and prolactin binding sites in dark-eyed juncos. Horm. Behav. 10, 1–10.

Silverin, B., 1980. Effects of long-acting testosterone treatment on free-living pied flycatchers, Ficedula hypoleuca, during the breeding period. Anim. Behav. 28, 906–912.

Soma, K.K., Tramontin, A.D., Wingfield, J.C., 2000. Oestrogen regulates male aggression in the non-breeding season. Proc. R. Biol. Sci. 267, 1089–1096.

Soma, K.K., Wissman, A.M., Brenowitz, E.A., Wingfield, J.C., 2002. Dehydroepiandrosterone (DHEA) increases territorial song and the size of an associated brain region in a male songbird. Horm. Behav. 41, 203–212.

Sorenson, L.G., Nolan, P.M., Brown, A.M., Derrickson, S.R., Monfort, S.L., 1997. Hormonal dynamics during mate choice in the northern pintail: a test of the "challenge" hypothesis. Anim. Behav. 54, 1117–1133.

Stewart, K.A., MacDougall-Shackleton, E.A., 2008. Local song elements indicate local genotypes and predict physiological condition in song sparrows Melospiza melodia. Biol. Lett. 4, 240–242.

Stoddard, P.K., Beecher, M.D., Horning, C.L., Willis, M.S., 1990. Strong neighbor-stranger discrimination in song sparrows. Condor 92, 1051–1056.

Stoddard, P.K., Beecher, M.D., Horning, C.L., Campbell, E.S., 1991. Recognition of individual by song in the song sparrow, a species with song repertoires. Behav. Ecol. Sociobiol. 29, 211–215.

Temeles, E.J., 1994. The role of neighbors in territorial systems: when are they "dear enemies"? Anim. Behav. 47, 339–350.

Langen, T.A., Tripet, F., Nonacs, P., 2000. The red and the black: habituation phenomenon in two desert Pheidole ants. Behav. Ecol. Sociobiol. 48, 285–292.

Turner, W.C., Barber, C.A., 2004. Male song sparrows Melospiza melodia do not announce their female's fertility. J. Avian Biol. 35, 483–486.

Van Duyse, E.L.S., Pinxten, R., Darras, V.M., Arckens, L., Eens, M., 2004. Opposite changes in plasma testosterone and corticosterone levels following a simulated challenge in male great tits. Behaviour 141, 451–467.

Van Roo, B.L., 2004. Exogenous testosterone inhibits several forms of male parental behavior and stimulates song in a monogamous songbird: the blue-headed vireo (Vireo solitarius). Horm. Behav. 46, 678–683.

Whiting, M.J., 1999. When to be neighbourly: differential agonistic responses in the lizard Platysaurus broadleyi. Behav. Ecol. Sociobiol. 46, 210–214.

Wikelski, M., Hau, M., Wingfield, J.C., 1999. Social instability increases plasma testosterone in a year-round territorial neotropical bird. Proc. R. Biol. Sci. 266, 551–556.

Wilson, S., Arcese, P., 2006. Nest depredation, brood parasitism, and reproductive variation in island populations of song sparrows (Melospiza melodia). Auk 123, 784–794.

Wilson, P.L., Towner, M.C., Vehrencamp, S.L., 2000. Survival and song-type sharing in a sedentary species of the song sparrow. Condor 102, 355–363.

Wingfield, J.C., 1984. Environmental and endocrine control of reproduction in the song sparrow, Melospiza melodia II. Agonistic interactions as environmental information stimulating secretion of testosterone. Gen. Comp. Endocrinol. 56, 417–424.

Wingfield, J.C., 1985. Short-term changes in plasma levels of hormones during establishment and defense of a breeding territory in male song sparrows, Melospiza melodia. Horm. Behav. 19, 174–187.

Wingfield, J.C., 1994. Regulation of territorial behavior in the sedentary song sparrow, Melospiza melodia morphna. Horm. Behav. 28, 1–15.

Wingfield, J.C., Hahn, T.P., 1994. Testosterone and territorial behavior in sedentary and migratory sparrows. Anim. Behav. 47, 77–89.

Wingfield, J.C., Monk, D., 1994. Behavioral and hormonal responses of male song sparrows to estradiol-treated females during the non-breeding season. Horm. Behav. 28, 146–154.

Wingfield, J., Wada, M., 1989. Changes in plasma levels of testosterone during male-male interactions in the song sparrow, Melospiza melodia: time course and specificity of response. J. Comp. Physiol. A. 166, 189–194.

Wingfield, J.C., Hegner, R.E., Dufty Jr., A.M., Ball, G.F., 1990. The "challenge hypothesis": theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. Am. Nat. 136, 829.

Wingfield, J.C., Lynn, S.E., Soma, K.K., 2001. Avoiding the "costs" of testosterone: ecological bases of hormone-behavior interactions. Brain Behav. Evol. 57, 239–251.

Wunderle, J.M.J., 1978. Differential response of territorial yellowthroats to the songs of neighbors and non-neighbors. Auk 95, 389–395.