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by

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Introduction

An animal's hormonal state can affect both the production and the perception of breeding signals. For example, previous research has shown that female songbirds show increased neuronal activity in response to their own species' song when they are in breeding condition (Maney and Pinaud 2011; Lattin et al. 2017). Sanford et al. (2010) found that the auditory forebrain was selective for conspecific song when plasma estradiol, the main female sex steroid, reached breeding levels, suggesting that processing of auditory conspecific signals is seasonally regulated by sex steroids. However, past studies have focused only on the comparisons between conspecific song and either neutral tones or the song of other songbirds. For instance, in the Sanford et al. (2010) study, after estradiol manipulation, female birds were presented with conspecific song, neutral tones, or no playback. What is not known is whether female songbirds also "tune out" the calls of predator species that present danger, such as hawks and owls.

There is evidence from behavior studies that birds may perceive predator calls differently than other types of sounds, for example by performing stress-induced behaviors when presented with a predator call. In response to predator playback, Congdon et al. (2016) found that chickadees often attempted to recruit nearby conspecifics to mob the predator. Another anti-predator response observed in this study was frequent moving from location to location, perhaps to prepare the bird to fight off the predator or to simply fly away. Finally, it was also noticed in this study that the chickadees did not ruffle their feathers in high-threat conditions, perhaps to avoid the risk of being seen by the predator. However, little is known about how an animal's hormonal state might affect these types of anti-predator behaviors.

Furthermore, we also know little about what parts of the songbird brain respond to predator vocalizations. However, we do know from past work that brain regions such as the

caudomedial nidopallium (NCM, involved in auditory perception), medial ventral arcopallium (AMV, involved in threat perception), caudal hippocampus (cHP, involved in integrating sensory and emotional responses), apical hyperpallium (HA, involved in behavioral flexibility), and caudolateral nidopallium (NCL, involved in decision making) can all show an increased response to aversive or threatening conditions (Marzluff et al. 2012; Cross et al. 2013; Kimball et al. 2022). This makes these likely candidate regions to be involved in responding to predator calls.

To fill these knowledge gaps, we conducted a lab study comparing protein expression of the immediate early gene (IEG) ZENK as a measure of neuronal activity in female sparrows exposed to predator calls or male conspecific song. Behavioral and neural responses to song in female birds have proven to be great models to study the effects of hormones on the brain and behavior (Maney and Pinaud 2011). IEGs like ZENK can be used to determine the intensity of neural activity over a period of time of constant exposure to stimuli ranging anywhere from minutes to hours (Guzowski et al. 2005). Previous research has demonstrated estradiol's ability to increase the range of frequency sensitivity with regards to the hearing organs of some animals, thus causing an enhanced perception of male vocalization frequencies (Sisneros and Bass 2003; Henry and Lucas 2009). With this in mind, we stimulated breeding condition in female house sparrows (*Passer domesticus*) by giving birds estradiol or empty implants because breeding state can affect neuronal activity in response to sound cues.

If female birds with estradiol implants "tune out" predator calls, they will show decreased ZENK expression in brain regions involved in auditory perception (e.g., NCM). compared to estradiol-treated females hearing sparrow calls. There should be no difference in ZENK expression in sparrows receiving empty implants, regardless of whether they hear sparrow or predator calls. Previous work has shown that the NCM region shows increased selectivity

towards conspecific communication signaling (Maney and Pinaud 2011). We also expect to see less ZENK expression in regions involved in aversive responses and threat perception in estradiol-treated birds listening to predator playback compared to females with empty implants. These regions specifically include the AMV, NCL, cHP, and apical hyperpallium. Finally, we also analyzed the behaviors of female sparrows while they were exposed to either of the auditory cues (Remage-Healey 2020). We expected to see increased freezing or stress-related behaviors in female sparrows exposed to predator calls compared to females exposed to male conspecific song. We also predicted we might see mating behaviors in female sparrows with estradiol implants when exposed to male conspecific song, and possibly fewer stress-related behaviors in response to predator calls, which might point to them being less aware of the predator vocalizations.

Methods

Study Subjects

Adult female house sparrows (n=32) were captured using mist nets at bird feeders in East Baton Rouge Parish between June and August 2020. Sparrows were doubly housed in cages in a vivarium at Louisiana State University with unlimited access to mixed seeds, grit, a vitamin-rich food supplement (Purina Lab Diet), and water. Birds also had access to a variety of perches and a dish of sand for dustbathing. Sparrows were maintained at natural day length (12L:12D) for a minimum of four weeks to acclimate to the captive environment before trials began. Animals were collected under a Louisiana State Scientific Collecting Permit and all experimental procedures approved by the Louisiana State University Institutional Animal Care and Use Committee under protocol 18-096. We used approved methods for bird capture, transport, and husbandry as specified in the Ornithological Council's Guidelines to the Use of Wild Birds in

Research (Fair et al. 2010), and approved methods of euthanasia for laboratory animals as specified in the 2020 American Veterinary Medical Association Guidelines for the Euthanasia of Animals.

Implant Surgeries

All female sparrows received subcutaneous implants in the skin of the back (n=16 estradiol, n=16 control). Estradiol implants consisted of silastic medical-grade tubing packed with crystalline 17-beta-estradiol (Sigma-Aldrich, St. Louis, MO, USA), sealed at both ends with a silicone adhesive. Control implants were empty. The size of implants (15 mm long, 2 mm outer diameter) was the same as used in a previous experiment (Lattin et al. 2017), and have been shown to significantly increase circulating estradiol concentrations and ovary size in female house sparrows compared to females without implants. For implant surgeries, sparrows were anesthetized with inhaled isoflurane (4%), and maintained at a surgical plane of anesthesia during the procedure (2-3.5%). Depth of anesthesia was assessed using toe pinch, breathing rate, and palpebral reflex, and a heating pad under a sterile surgical pad used to maintain body temperature. Birds were given subcutaneous ketoprofen (5 mg/kg) as a pre-emptive analgesic, then a small incision was made in cleaned and disinfected skin between the shoulder blades, an implant inserted, and the incision site closed with Vetbond (3M, Maplewood, MN, USA). The following day, all birds were monitored to ensure proper healing and check implant placement, and given a second dose of ketoprofen. We left implants in place for one week and then removed implants using a similar procedure to implant insertion: isoflurane anesthesia (4% induction, 3.5-2% maintenance), ketoprofen, an incision in cleaned and disinfected skin, removal of the implant using forceps, and the incision closed with Vetbond. We then waited four weeks before

conducting playback experiments. We used this time course because previous work saw a greater effects of estradiol treatment on brain responses to conspecific vs. heterospecific playback during this later time point than during the first week implants were in place (Lattin et al. 2017), suggesting the full effects of estradiol on the brain and auditory perception may take several weeks to fully develop.

Playback Trials

During playback trials, females were rapidly transported to an acoustically isolated testing room and exposed to either a playback of several different male house sparrows singing (n=8 control females, n=7 estradiol-treated females) or a mix of several different types of local predator calls, including red-tailed hawks, Loggerhead shrikes, Eastern screech owls, and Mississippi kites (n=8 control females, n=9 estradiol-treated females). Songs were obtained from the Macaulay Library (Cornell Lab of Ornithology, Ithaca, NY, USA) and the Borror Laboratory of Bioacoustics (The Ohio State University, Columbus, OH, USA). Each bird heard a unique playlist of different clips in a randomized order. Loudness was standardized to 60 dB from bird to speaker using a sound level pressure meter.

Females were exposed to a song stimulus for 30 min and their behavior video-recorded. Previous work in songbirds has shown that IEG proteins peak ~90 min after stimulus exposure (Goodson et al. 2005) Therefore, after the 30 min playback, females were transported to a dark quiet room for 60 min before being deeply anesthetized with ketamine and xylazine at doses appropriate for house sparrows (Muresan et al. 2008). Once animals were in a surgical plane of anesthesia, they were transcardially perfused with ice-cold heparinized saline, and tissue fixed with 0.1 M phosphate buffer containing 4% paraformaldehyde.

Behavioral Analysis

Thirty minute video recordings were analyzed using BORIS v 7.10.2. An ethogram was created to associate keys with point-type behaviors, which were discrete behaviors with no duration (movement, beak wiping, feather ruffling, and calling), and state-type, which were behaviors with duration (preening and feeding). Movement was classified as any time an individual would hop, fly, jump, or anytime both feet came off the ground. Behaviors like foot adjustments, shuffling, stretching, and head bobbing were not considered movements. Beak wiping refers to when an individual wiped its beak on an object in the cage. One bout of beak wiping was considered when at least 1 s occurred between wipes. Feather ruffling was classified as an event when an individual would puff up feathers and ruffle quickly. Calling was classified as any time when a bird would briefly open its beak to call back to the sound playback. Preening was classified as any time an individual would pull on their feathers with their beak and spread oil from their preen glands. Finally, feeding was classified as when the bird would perch on the food dish in the cage and feed. Total number of occurrences, means, and standard deviations were recorded for each behavior and total duration was recorded for each state-type behavior. All videos were watched with no sound so the observer was blind to playback type (predator vs. male sparrow). The observer was also blind to bird treatment (estradiol vs. empty implant), and all videos were watched by the same observer to ensure consistency (intra-observer coefficient of variation from watching 4 videos twice: movement=0.5%, beak wipes=5.1%, calls=28.3%, feeding duration=1.1%). Beak wiping and feather ruffling were infrequently observed (each occurred in only 25% of videos), so they were not included in the final behavior analysis.

Immunohistochemistry and ZENK Quantification

Brains were post-fixed in 4% paraformaldehyde phosphate buffer for 24 h at 4°C, then soaked in 0.1 M phosphate buffer containing 30% sucrose for cryoprotection. After sinking (~2 days), brains were flash-frozen in powdered dry ice and stored at -80°C until sectioning. Brains were cut at -20°C in the coronal plane in 40 µm sections using a ThermoFisher NX50 cryostat. Starting at striatum, triplicate sections were collected in wells containing cryoprotectant (0.2 M phosphate buffer, 15 M PVP, 1.5 M sucrose, and 0.5 M ethylene glycol in distilled water) and stored at -20°C until the day of immunohistochemistry.

Brain regions were identified based on visible landmarks. Apical hyperpallium (HA) sections were taken when Area X was still visible and approximately ~120 µm before the first appearance of the lateral septum. We used caudal dorsomedial hippocampal sections where the cerebellum first became visible and the mesopallium began to disappear. We targeted medial ventral arcopallium (AMV) based on the visibility of the cerebellum and arcopallium. Sections used for nidopallium caudolateral (NCL) were 40 µm after AMV sections, in a pallial area where we have confirmed the presence of dense basket fiber staining for tyrosine hydroxylase in house sparrows, consistent with NCL in other songbird species (von Eugen et al. 2020). Nidopallium caudomedial (NCM) was taken ~40-80 µm after the appearance of field L2. For each region we ran immunohistochemistry for all 22 animals in the same assay.

We measured immunopositive cell density for ZENK in all 5 regions of interest. We measured four sections per individual, with each section including right and left hemispheres unless the hemisphere was damaged during staining. Images of each region were captured using an Olympus TH4-100 microscope through a 20x objective lens with consistent lighting for each

photo. One to two images were captured for smaller regions (HA, NCM, NCL, and AMV), and 3 pictures were taken of the caudal hippocampus.

We used ImageJ (Schneider et al. 2012) to measure immunopositive cell density in each image using a procedure adapted from Mischler et al. (2017). All images were cropped to include only the region of interest, and the area of each region was measured. We then converted each image to 8-bit grayscale, increased the contrast, and used the threshold tool to make the immunopositive nuclei white against a black background. We then used the count function to quantify the number of immunopositive nuclei and calculated staining density. Image analysis was done by two observers blind to treatment (estradiol vs empty; predator vs sparrow).

Data Analyses

We used JMP Pro 16.0 (SAS Institute) for all behavior and IEG analyses, and all individuals were included in each analysis (n=32). We first ran models assessing the effect of hormone and sound treatments on behavior. Hops/flights, feed duration, and number of calls were all Box-Cox transformed to better fit a normal distribution using the following equations:

$$\frac{(\text{Hops\_Flights}+1)^{0.255}-1}{0.0088866448} \qquad \frac{(\text{Feed duration})^{-0.151}-1}{-0.013847945} \qquad \frac{(\text{Calls})^{-0.468}-1}{-0.090904934}$$

Box-Cox transformation cannot be run on values zero and below, therefore we added 1 to all data prior to transformation. We then performed three separate linear mixed model analyses with hops/flights, feed duration, or calls as dependent variables, and hormone treatment, sound treatment, and hormone treatment*sound treatment interaction as fixed effects, and bird ID as a random effect in each model. For all models we assessed normality of residuals using normal quantile plots. In cases where there was a significant interaction between hormone and sound

treatment, we compared treatment groups using ANOVA and Tukey's HSD tests. Bartlett's test indicated equal between-group variances.

We next ran models assessing the effect of hormone and sound treatments on ZENK IEG expression in all regions of interest. For all analyses we used ZENK density as the dependent variable, and brain region, hormone treatment, sound treatment, hormone treatment*sound treatment interaction, hormone treatment*brain region interaction, and sound treatment*brain region interaction as fixed effects, and bird ID as a random effect in each model. In cases where there was a significant interaction between brain region and hormone treatment, we compared treatment groups using ANOVA and Tukey's HSD tests. Bartlett's test indicated equal between-group variances.

Results

Behavior

In linear mixed models examining the effects of hormone and sound treatments on behavior, there was a significant overall effect of sound treatment on number of hops and flights (Figure 1a; $F_{1,28}$=9.70, p=0.0042). Female sparrows exposed to predator calls performed fewer hops and flights than females exposed to male sparrow calls (Figure 1b; ANOVA: F=9.71, p=0.004). For feeding duration, there was a significant overall effect of sound treatment (Figure 1c; $F_{1,28}$=27.16, p<0.0001) and a significant interaction between hormone and sound treatment (Figure 1c; $F_{1,28}$=9.40, p=0.0048). Females that had previously had estradiol implants and were exposed to sparrow calls spent more time feeding compared to all other treatment groups (Figure 1c; Tukey's HSD: all p<0.0079). There was also a trend towards an effect of sound treatment on calls (Figure 1d; $F_{1,28}$=3.40, p=0.076).

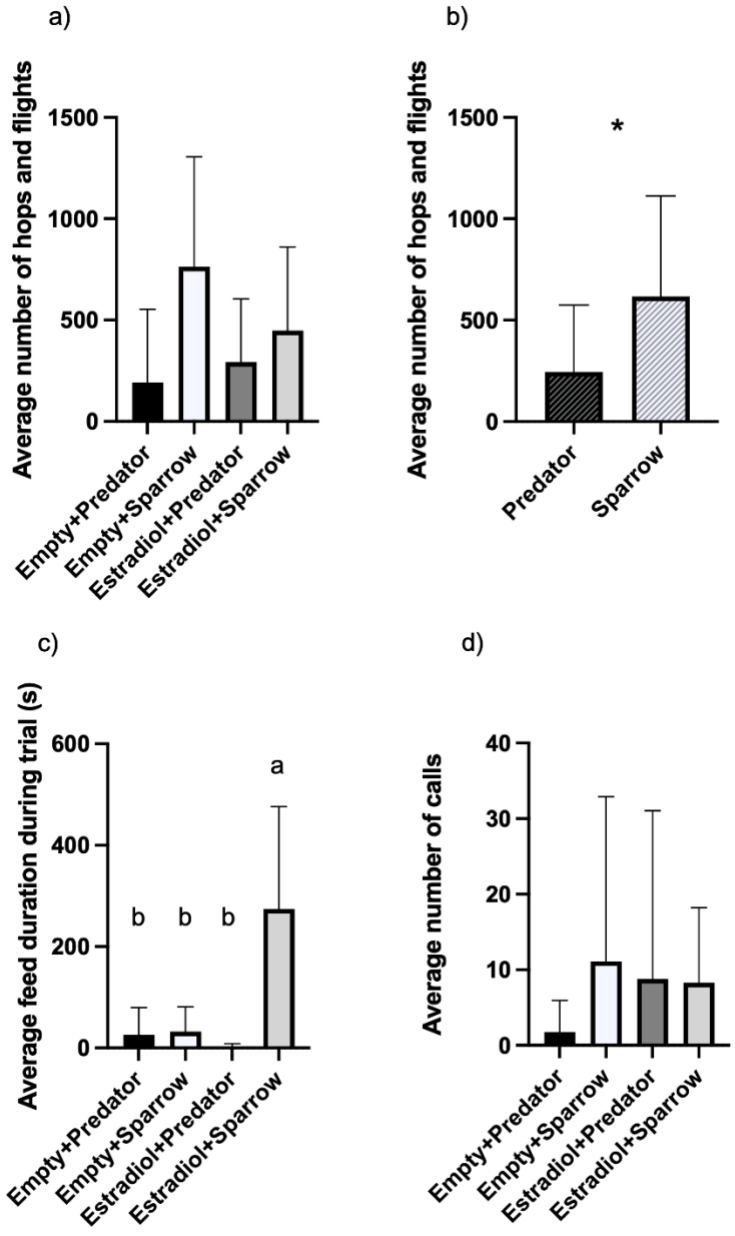

Figure 1. Behavior during playback trials. Sample sizes for panels a, c, and d were n=8 for Empty+Predator, n=8 for Empty+Sparrow, n=9 for Estradiol+Predator, and n=7 for Estradiol+Sparrow. In panel b, sample sizes were n=17 for Predator and n=15 for Sparrow sound treatment groups. In panel c, different letters indicate $p<0.05$. Error bars represent standard deviation. *$p<0.05$

Immediate early gene expression

In linear mixed models examining the effects of hormone and sound treatments on ZENK protein expression, we found significant overall effects of brain region ($F_{5,149}$=178.55, p<0.0001), hormone treatment ($F_{1,149}$=4.83, p=0.03), and a significant interaction between brain region and hormone treatment ($F_{5,149}$=2.75, p=0.02). However, we found no overall effect of sound treatment ($F_{1,149}$=0.63, p=0.43), or interactions between hormone and sound treatment ($F_{1,49}$=0.078, p=0.78), or sound treatment and brain region ($F_{5,149}$=0.34, p=0.89). Region by region post-hoc analyses revealed that there was increased ZENK density in the HA (Figure 2c; ANOVA: $F_{1,27}$=4.91, p=0.035) and NCL (Figure 2d; ANOVA: $F_{1,31}$=4.73, p=0.037) in sparrows who had previously had empty implants compared to those who had previously had estradiol implants.

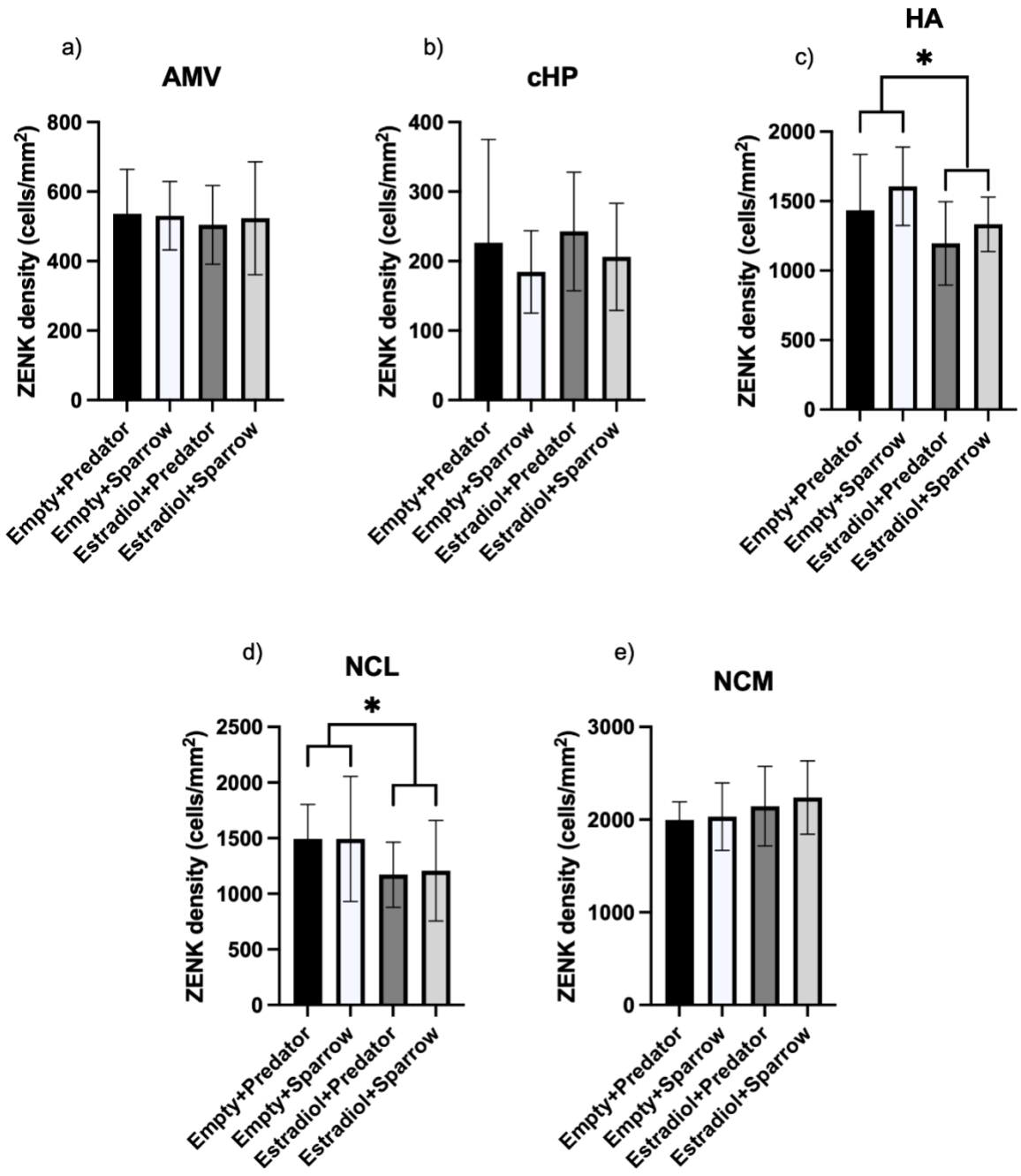

Figure 2. Protein expression of the immediate early gene ZENK (mean ± SD cell density) for each brain region of interest in female house sparrows. Sample sizes are n=8 for Empty+Predator, n=8 for Empty+Sparrow, n=9 for Estradiol+Predator, and n=8 for Estradiol+Predator, except for data point losses in the following regions: cHP: n=8 for Estradiol+Predator, n=7 for Estradiol+Predator; HA: n=7 for Empty+Predator, n=6 for Empty+Sparrow, n=8 for Estradiol+Predator; NCM: n=7 for Empty+Sparrow, n=8 for Estradiol+Predator, n=6 for Estradiol+Predator. AMV=medial ventral arcopallium, cHP=caudal

hippocampus, HA=apical hyperpallium, NCL=caudolateral nidopallium, and NCM= caudomedial nidopallium. Error bars represent standard deviation. *p<0.05

Discussion

The overall goal of this research was to understand the effects of hormonal state on behavior and brain activity in response to breeding and predation signals. We predicted that sparrows exposed to predator calls would show an increase in freezing or stress-related behaviors. Our findings support this hypothesis: female sparrows exposed to predator playback showed a decrease in hops and flights when compared to females hearing male sparrow song, regardless of hormone treatment. This is likely due to the sparrows performing freezing behaviors in order to not draw attention to themselves while a predator is nearby, as seen in previous avian studies (Borchelt and Ratner 1973; Quinn and Cresswell 2005). Freezing behaviors are a common antipredator strategy of many wild animals in response to direct predator signals or the alarm calls of conspecifics (Beani and Dessí-Fulgheri 1998; Rahlfs and Fichtel 2010; Bedore et al. 2015). Other antipredator behaviors include vigilance and escape; however, the type of antipredator behavior exhibited may depend on the type of predator signal. For example, in study of red-legged partridges that simulated terrestrial and aerial predators, freezing was the most common behavior in response to aerial predators, whereas vigilance was most common in response to terrestrial predators (Binazzi et al. 2011). It is possible that we observed freezing (characterized as less movement in our study) due to the inclusion of aerial predator calls in our recordings. We included both aerial and terrestrial predator calls in our predator playback, and future studies could investigate the context of type of predator call on behavior in breeding and non-breeding conditions.

We gave sparrows estradiol implants to stimulate breeding condition, which we predicted would increase mating behaviors. Although we did not observe copulation solicitation behaviors, one of the main mating behaviors studied in female birds, we found that sparrows with estradiol implants spent more time feeding when exposed to conspecific song. A study conducted on Holstein heifers reported that estradiol implants increased feeding by 2.4% (Lammers et al. 1999), and our previous work found that estradiol-treated female sparrows had increased circulating glucose levels, demonstrating that estradiol increases energy mobilization (Lattin et al. 2017). These studies suggest that there may be a direct relationship between increased estrogen levels and feeding behavior in some species. However, we only observed increased feeding behavior in estradiol-implanted females hearing conspecific song, not in those hearing predator calls. House sparrows have been shown to increase their latency to feed in the presence of a predator signal (Seress et al. 2011). Therefore, females in breeding condition may suppress feeding behavior in the presence of predator calls to increase vigilance. A trade-off between feeding and vigilance behavior is commonly observed across different species (Dill and Fraser 1984; Poysa 1987; Pueta et al. 2016).

In this experiment, we hypothesized female birds in breeding condition would selectively "tune out" predator calls, as they do neutral tones (Sanford et al. 2010) and the song of other songbird species (Lattin et al. 2017). Based on this hypothesis, we predicted that there would be decreased ZENK expression in brain regions involved in auditory perception, such as NCM, in estradiol-treated sparrows exposed to predator calls compared to estradiol-treated females hearing sparrow calls. In response to predator playback, we also predicted that there would be less ZENK expression in regions involved in aversive responses and threat perception in estradiol-treated birds compared to females with empty implants, specifically in the AMV, NCL,

caudal HP, and apical hyperpallium. We found that in the HA and NCL, sparrows who had received empty implants had increased ZENK expression compared to sparrows who had received estradiol implants. Estradiol has been shown to increase or decrease ZENK expression depending on brain region (Maney et al. 2008; Svec and Wade 2009). However, there was no effect of sound treatment on ZENK expression: there was no difference in neuronal activity in sparrows hearing predator calls and male sparrow song. These data suggest that female sparrows did not tune out the predator vocalizations while they were in breeding condition, and responded to each sound treatment with the same intensity regardless of the estradiol implant.

In conclusion, we found that female sparrows alter their behavior in response to breeding condition and predator calls, specifically showing increased freezing behavior and decreased feeding in response to potentially threatening sounds. Additionally, we found that estradiol-treated female sparrows do not selectively tune out predator calls in the same way that they have previously been shown to tune out neutral sounds like tones or other bird song (Sanford et al. 2010, Lattin et al. 2017). These behavioral and neural findings indicate that female sparrows are vigilant and responsive to the threat of predation at all times, regardless of their breeding condition.

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