Published in final edited form as:

Behav Brain Res. 2013 June 15; 247: 117–124. doi:10.1016/j.bbr.2013.02.041.

Links between breeding readiness, opioid immunolabeling, and the affective state induced by hearing male courtship song in female European starlings (*Sturnus vulgaris*)

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Abstract

Male courtship vocalizations represent a potent signal designed to attract females; however, not all females find male signals equally attractive. We explored the possibility that the affective state induced by hearing courtship vocalizations depends on the motivational state of a receiver. We used a conditioned place preference test of reward to determine the extent to which the rewarding properties of hearing male courtship song differed in female European starlings categorized as nest box owners (a sign of breeding readiness) or non-owners. Nest box owners developed a preference for a chamber in which they previously heard male courtship song. Non-owners displayed no preference for a chamber in which they previously heard song. Positive correlations were identified between the preference a female developed for the song-paired chamber and female nesting and dominance behaviors observed prior to conditioning (indices of the motivation to breed). Immunolabeling for met-enkephalin (an opioid neuropeptide involved in reward) in the medial preoptic nucleus, ventromedial nucleus of the hypothalamus, nucleus accumbens, and periaqueductal gray was higher in females with compared to those without nest boxes. Both nest box entries and song-induced place preference also correlated positively with met-enkephalin labeling in the ventromedial nucleus of the hypothalamus. These findings indicate that the reward value of vocal signals is linked to individual differences in motivational state; and that differences in enkephalin activity may play a role in modifying an individual's motivational state and/or the reward value of song.

Keywords

communication; reinforcement; social behavior; mate choice; motivation; birdsong

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1. Introduction

Vocal signals influence the behavior of conspecific receivers [1]. One mechanism by which vocal signals can influence behavior is by modifying the affective or reward state of the receiver. Male courtship vocalizations in songbirds represent a potent vocal signal designed to attract females [2]. However, not all females find male signals equally attractive (e.g., [3–5]). One possibility is that the affective/reward state induced by hearing song differs across individual receivers of the vocal signal.

Studies in songbirds indicate that hearing conspecific song can be rewarding. For example, isolated juvenile male zebra finches can be trained to peck a key to hear playback of conspecific song [6, 7]. Studies in which songbirds are required to land on a perch to hear playback of conspecific song also indicate that song can serve to reinforce behavior (e.g., [8–11]). These studies indicate that hearing song can be rewarding, but they do not address individual differences observed in responses to song. We propose that such differences may relate to differences in the affective or reward state induced by hearing song.

Reward is linked to an individual's motivational state. For example, the presence of rat pups is rewarding to female rats that have just given birth but not to virgin females [12]. Specifically, postpartum but not virgin female rats developed a preference for a location previously associated with rat pups (a conditioned place preference). This indicates that the reward value of a rat pup is tied to a female's motivation to parent. Similarly, food is more rewarding to hungry than to sated animals (reviewed in [13, 14]), demonstrating that the rewarding properties of food are influenced by the motivation to feed. Across songbird species, females are attracted to certain parameters of song (e.g., bout length or repertoire size and complexity [15]), and males emphasize these when singing to females (e.g., [16–18]); however, the affective impact of a social signal may also depend on the motivational state of the receiver. For example if a female is motivated to breed, male courtship songs may induce a positive affective state in the female. If not, the same songs may induce negative affect or possess no valence.

Dopamine is well-known for its role in anticipatory, motivated, reward-directed behaviors [19–21]. Dopamine in the nucleus accumbens (Ac) rises in female rats presented with a sexual partner, a finding that was interpreted to support the hypothesis that dopamine in Ac motivates a female to seek sexual contact [22]. Consistent with these studies, in female starlings we found that labeling for tyrosine hydroxylase (a rate limiting enzyme for dopamine synthesis) in the nucleus accumbens (Ac) correlated positively with nesting behavior (which we consider a reflection of the motivation to breed) [23].

Although dopamine is central for directing motivated behavioral responses towards rewarding stimuli, other neurochemicals, including opioid neuropeptides, are found to underlie the pleasure/reward associated with behaviors ranging from sexual activity, feeding, and the use of drugs of abuse [24–31]. Central opioid infusion in female white-crowned sparrows suppresses sexually-motivated responses to male song playback [32], a finding consistent with other studies showing that opioids induce sexual reward and a temporary suppression of sexually-motivated behaviors (possibly by inducing a state of satiety) [33]. Site-specific manipulations in female rats show that blocking opioid receptors in the ventromedial nucleus of the hypothalamus (VMH) or the medial preoptic nucleus (often abbreviated POM in birds), but not Ac, blocks reward induced by paced-mating activity [28]. If hearing male courtship song is rewarding to sexually-motivated female starlings, it is possible that the VMH and POM may be target sites of opioid activity.

Enkephalin opioids are speculated to play a role in reward associated with the act of song production in male songbirds [34–37]. Links between enkephalin opioids and behavioral

responses to vocal signals however have not been examined. We expect that enkephalin opioid innervation may differ in females that find hearing male song rewarding compared to those that do not, a finding that would suggest a role for opioids in modification of motivational state and possibly the rewarding properties of hearing song.

The goals of the present study were 1) to test the hypothesis that individual differences in female breeding readiness relate to differences in the affective state induced by hearing male courtship song, and 2) to provide insight into possible links between enkephalin activity in brain regions involved in reward and the extent to which females find male song rewarding.

2. Methods

All animals were treated in accordance with the Guidelines of the National Institutes of Health for the Care and Use of Laboratory Animals under a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee. Twenty-six adult female European starlings (*Sturnus vulgaris*) were trapped on a farm in Madison, WI and housed indoors in groups of up to five birds in stainless steel cages (91 cm × 47 cm × 47 cm). Each female received a unique combination of colored leg bands for identification. Females were exposed to a photoperiod of 18h light:6h dark for six weeks followed by 8h light:16h dark for six weeks. These are standard photoperiod manipulations that place female starlings in a state of photosensitivity, which is a physiological state observed in females at the onset of the spring breeding season [38].

2.1 Estradiol implants

When exposed to daylengths longer than 11h light, starlings enter a state of photorefractoriness in which they cease breeding activities (e.g., [39]). To promote breeding activities while avoiding the possibility that exposure to long daylengths would induce refractoriness we treated females with estradiol, which facilitates breeding behaviors in photosensitive females (e.g., [23]). Specifically, after the 6 weeks of exposure to 8h light (described above), each female received subcutaneous estradiol implants to facilitate breeding activities in captivity. Females were anesthetized with isoflurane gas and implanted just posterior to the last rib with two 18 mm silastic implants (1.47 mm i.d., 1.96 mm o.d.; Dow Corning, Midland, MI) filled with approximately 14 mm of 17-beta-estradiol.

2.2 Pre-conditioning behavioral observations

Immediately after receiving implants, females were housed in flocks of 4 birds in indoor observation aviaries on photoperiods of 11h light: 13h dark (a photoperiod that will not induce photorefractoriness in starlings (e.g., [40])). Each aviary contained 4 nest boxes and several perches. Food and water were available *ad libitum*. Aviaries contained a speaker mounted on the ceiling for playback of male song (below). Each aviary was equipped with a one-way glass concealing the observer.

Behavioral observations began 7 days after introduction of the females to the aviaries. Behavior during playback of recordings of male starling courtship song was observed for 15 min on each of 4 days prior to conditioning. During each observation period the numbers of times females entered nest boxes and displaced other females (measures of breeding readiness), fed and preened (general behavioral measures), and whether or not a female occupied (i.e., remained near and repeatedly entered) a nest box were recorded.

2.3 Conditioned place preference procedure

The conditioned place preference (CPP) method is commonly used to evaluate the rewarding properties of stimuli such as food or drugs or sexual behavior (e.g., [41–44]). This

method has also been used to examine reward associated with sexual behavior in birds [45]. CPP involves associating the primary rewarding properties of a specific unconditioned reward stimulus (e.g., food or drugs, or in the present study hearing male courtship song) with a previously neutral stimulus (e.g., a distinct chamber in a place preference apparatus). After pairing the unconditioned stimulus (e.g., the affective state induced by hearing male courtship song) with a neutral stimulus (e.g., a distinct chamber), the neutral stimulus then becomes a conditioned stimulus and can elicit an approach response (if the properties associated with the unconditioned stimulus [e.g., hearing male courtship song] are rewarding) or avoidance (if the properties associated with the unconditioned stimulus are aversive).

2.3.1 Habituation—One day after completion of the behavioral observations and one day prior to conditioning, each female was placed individually into a CPP apparatus for 15 min. The apparatus consisted of a stainless steel bird cage (91 cm × 47 cm × 47 cm). One side of the cage was decorated with red construction paper on three walls, the floor and the ceiling, the other side with blue. The color of each side was counterbalanced across the CPP apparatuses. Each side of the cage contained a single perch. During this phase females had access to both sides of the apparatus and the amount of time a female spent on each side was recorded to obtain a baseline preference.

2.3.2 Conditioning—The day after habituation, females were conditioned. An opaque partition was inserted into the CPP apparatus to divide the two distinct halves. Each side of the partition was covered in the appropriate side-specific color. Each female was placed individually into one side of a CPP apparatus (the same CPP apparatus to which she was habituated the previous day) for 15 min. One half of the females were exposed to song first for 15 min on one side of the CPP apparatus. They were then returned to the home aviary. Approximately 4 hours later the same females were placed on the other side of the same CPP apparatus and exposed to silence (i.e., no song playback) for 15 min. During the 15 min of song exposure, recordings of male starling courtship song (details of playback stimulus below) were played for 15 min from a playback speaker that was mounted on the ceiling of the room above the observation blind. The other half of the females were first exposed to silence (i.e., no playback) for 15 min on one side of the CPP apparatus, returned to their home cages, and then approximately 4 hours later they were placed on the opposite side of the CPP apparatus and exposed to song playback for 15 min. Side color and the order of song or silence presentation were fully counterbalanced across females.

The song playback stimulus consisted of recordings from 5 male starlings. Briefly, each male was placed singly in a small cage within a larger room. A female (not a female in the present study) was released into the room outside the male's cage. Male song was recorded from a microphone attached through an M-Audio MobilePre pre-amplifier (Irwindale, CA) to a computer with Avisoft Recorder software (Berlin, Germany) by an observer separated from the birds by a one-way mirror. Song was recorded both in the presence of the female and also just after her removal. Only song files containing bouts of full song were used as song stimuli (i.e., song containing introductory whistles, variable song types, rattles, and high frequency end notes [46]. Mean song duration was 51.76 secs + sem 0.99 (additional details in [47]).

2.3.4 Test day—The day after conditioning, the partition separating the 2 sides of the CPP apparatus was removed. Each female was placed for 15 min back into the apparatus in which she was conditioned the previous day. The total amount of time each female spent on each side of the apparatus in the absence of song playback was recorded.

2.4 Immunolabeling for met-enkephalin

The day after CPP testing, brains from the last 12 females observed were collected, fixed in acrolein (5%) overnight, cryoprotected in 30% sucrose, frozen on dry ice, and stored at -80°C until processing. We did not collect brains from the first 14 females because these females were used in additional pilot tests to determine whether additional conditioning days would strengthen the CPP response (they did not). All estradiol implants for the full group of 26 birds were checked at the end of the study and found to be present.

Brains were sectioned at 40 micrometers in a coronal plane using a cryostat. Every 3rd section was collected and immunohistochemistry performed to identify the opioid met-enkephalin, following a protocol detailed in [48]. The primary antibody was anti-met-enkephalin antibody made in rabbit (1:1000; Immunostar Cat.# 20065). The secondary was biotinylated goat anti-rabbit (1:250; Vector Laboratories Cat.# BA-1000).

2.5 Quantification of immunolabeling

Immunolabeling for mENK was examined on digital images captured using a Spot camera mounted to a Nikon Microscope. Metavue software (Molecular Devices, Sunnyvale, CA) was used to quantify optical density and the pixel area covered by labeled fibers. No significant effects were observed for optical density measures so these are not discussed further. The pixel area measures were converted into mm^2 for each brain region of interest. Metavue makes these calculations based upon a threshold, which a blind observer confirmed was set specifically to highlight labeled fibers and cells. The same threshold was used within a brain region for each individual. Labeling was quantified in POM, VMH, a putative location for the avian Ac [49, 50], and additionally the periaqueductal gray (PAG) (locations identified as in [48] and [23]). The POM and VMH were selected based on studies in female rats showing opioids in these areas to reward mating activities [28]. Ac in contrast may not be a site in which opioids reward female mating activity [28]; however opioids in Ac and the PAG (in addition to POM and VMH) have been implicated in reward [24, 25, 28, 51, 52]. The size of regions in which measurements were taken was as follows: POM, oval, 0.092 mm^2 ; VMH, rectangle, 0.228 mm^2 ; Ac, 0.159 mm^2 ; PAG, summed rectangles, 0.124 mm^2 . All regions were measured at regions at $20\times$. Left and right hemispheres of three serial sections were quantified. The mean area in mm^2 covered by labeled fibers was calculated for each individual for each brain region and used for analysis.

3. Results

3.1 Behavioral measures

We considered females that acquired and defended nest boxes to be in a heightened state of breeding readiness compared to females who did not occupy nest boxes. Based on the tendency to remain perched at the entrance and to enter a nest box, females could easily be identified as nest box owners ($n = 13$) or non-owners ($n = 13$), with ownership operationally defined as a bird observed to enter a nest box on at least 2 observation days. A repeated measures ANOVA with nest box ownership entered as an independent variable and the 4 behaviors (mean log transformed feeding, preening, displacing, and entering the nest box) entered as dependent variables revealed significant main effects for nest box ownership ($F_{1,24} = 24.76$, $p = 0.00004$) and a behavior $\times$ nest box ownership interaction ($F_{3,72} = 9.70$, $p = 0.00002$). Lilliefors tests revealed data to be non-normal for displacements and nest box entries. Data were therefore log transformed to improve normality. As expected given that birds were categorized based on whether or not they owned/defended a nest box, Fisher's posthoc analyses indicated that females with nest boxes displaced ($p = 0.003$) and entered nest boxes ($p = 0.000001$) more than females without nest boxes (Figure 1A and B). More

importantly, females with nest boxes did not differ from females without boxes on measures of general behavior (feeding: $p = 0.841$; preening: $p = 0.462$; Figure 1C and D).

3.2 Conditioned place preference

A repeated measures ANOVA was first run to compare the amount of time females with and without nest boxes spent on the future song-paired side of the CPP apparatus (measured during the habituation test period) and the amount of time they spent on the same side after conditioning (measured during the post-conditioning test period). This analysis revealed a significant nest box ownership $\times$ test period interaction ($F_{1,24} = 11.16$, $p = 0.003$; Table 1). Fishers' LSD post hoc comparisons revealed that females with nest boxes spent significantly more time on the song-paired side of the apparatus after compared to prior to conditioning ($p = 0.001$). No significant difference was found for females without nest boxes ($p = 0.282$).

Song-induced place preference (CPP) was then calculated as the time spent on the previously song-paired side of the CPP apparatus minus the baseline preference for the same side. A Student's t -test indicated that females with and without nest boxes differed significantly in the amount of time (above baseline preference) they spent on the two sides of the chamber ($t_{24} = 3.34$, $p = 0.003$; Figure 2). A song-induced place preference would be revealed if females spent significantly more time on the side of the chamber previously paired with male song playback than they did during baseline testing (reflected in a preference score significantly above zero). A place aversion would be revealed if females spent significantly less time (compared to the baseline preference) on this side of the chamber (reflected in a preference score significantly below zero). If song playback had no valence one would predict that the time females spent on either side of the apparatus would not differ from baseline preferences (reflected in a preference score that did not differ significantly from zero). We ran single means t -tests to determine whether preference scores for each group differed significantly from zero. A single means t -test showed that the time females with nest boxes spent on the side of the apparatus in which they previously heard song was significantly above a zero preference score (i.e., these females increased their preference for that side of the apparatus; $t_{12} = 3.36$, $p = 0.006$). In contrast, the same test in females without nest boxes showed that the amount of time females spent on the side of the apparatus in which they previously heard song did not differ significantly from a zero preference score (i.e., they did not develop a significant preference or aversion for that side of the apparatus; $t_{12} = 1.20$, $p = 0.253$).

A forward stepwise multiple regression analysis of females with nest boxes with the measure of song-induced place preference entered as a dependent variable and the 4 behavioral measures entered as predictor variables was significant ($\text{adj}R^2 = 0.65$, $p = 0.0012$). Both entering the nest box ($\beta = 0.55$, std error of $\beta = 0.17$, $t_{11} = 3.28$, $p = 0.0073$) and displacement ($\beta = 0.53$; std error of $\beta = 0.17$, $t_{11} = 3.16$, $p = 0.0090$) statistically explained variance in CPP (Figure 3), but feeding and preening did not.

3.3 Met-enkephalin immunolabeling

For mENK analyses one of the 12 females was dropped due to inconsistent and light labeling, resulting in 5 females without nest boxes and 6 females with nest boxes. An ANOVA with nest box ownership entered as a between subjects variable and the area covered by mENK labeling in each brain region (VMH, POM, Ac, and PAG) entered as a non-independent repeated measures variable revealed a significant main effect for nest box ownership ($F_{1,9} = 16.37$, $p = 0.003$; Figure 4). The ownership by brain region interaction was not significant ($F_{3,27} = 1.23$, $p = 0.317$). Post hoc Fisher's LSD tests revealed that the area covered by mENK labeling was significantly denser in nest box owners compared to non-owners ($p = 0.003$).

Forward stepwise multiple regression analyses were performed to evaluate the extent to which CPP (time on the song-paired side minus the baseline preference) and other behaviors statistically explained variance in mENK labeling in each brain region. For each analysis, mENK labeling in the brain region of interest was entered as a dependent variable and the measures of song-induced CPP (time on the song-paired side minus baseline), displacement behavior, feeding, and preening were entered as predictor variables. (The number of nest box entries was not included as a variable in these analyses as it was highly correlated with both CPP [$r = 0.74$] and displacement [$r = 0.65$] and thus inappropriate for inclusion in this analysis). Lilliefors tests were used to test the assumption of normality, and non-normal data (i.e., data for displacements) were log transformed. Individuals were considered outliers if they were identified as such using both deleted residuals and Cook's distances analyses. Outliers were dropped from analyses, resulting in removal of one individual from the PAG analysis.

For VMH results of a forward multiple regression analysis were significant ($\text{adj}R^2 = 0.35$, $p = 0.032$; Figure 5) with CPP found to contribute statistically to variance in mENK labeling ($\text{Beta} = 0.64$, $\text{sem of Beta} = 0.25$, $t_9 = 2.53$, $p = 0.032$). Results of overall forward multiple regression analyses were not significant for POM ($\text{adj}R^2 = 0.05$, $p = 0.296$) or PAG ($\text{adj}R^2 = 0.018$, $p = 0.313$), and no significant model returned for Ac.

As indicated above, the number of nest box entries was not included as a variable in the multiple regression analyses as it was highly correlated with both CPP and displacement. Separate correlation analyses were used to examine potential links between mENK in each brain region and nest box entries. A Lilliefors test indicated that nest box entries were not normally distributed so this variable was log transformed. Nest box entries correlated positively with mENK labeling in VMH ($r = 0.77$, $p = 0.005$; Figure 6A) and was just shy of significance for POM ($r = 0.59$, $p = 0.057$; Figure 6B). No significant correlations or trends were found for either Ac ($r = 0.27$, $p = 0.426$) or PAG ($r = 0.41$, $p = 0.209$).

4. Discussion

The results of this study are the first to link individual motivational state to the reward value of hearing vocal signals. We found that only females that possessed and defended nesting sites developed a CPP for a compartment in which they previously heard male courtship song. In contrast, females that did not occupy or show interest in nesting did not develop a preference (or a significant aversion) for the side of the apparatus in which they previously heard song. This indicates that hearing song induced a positive affective state in breeding-ready females, but had no valence for females that were not prepared to breed. Furthermore, behavioral measures indicative of breeding readiness (nest box entries and social dominance behaviors [displacement of other females]) related positively to CPP in females with nest boxes. Non-specific behaviors (feeding and preening) did not. This suggests a one-to-one relationship between female motivational state and the affective state induced by hearing song. These findings are consistent with past studies showing that the reward value of a stimulus depends upon the motivational state of the receiver (e.g., food is more rewarding in hungry than sated rats and offspring are more rewarding in postpartum than virgin females [12, 14]). We now show that this also applies to the reward value of vocal signals.

Coupling the reward value of vocal signals to motivational state ensures that signals are relevant only to females that are prepared to respond. For a female that is prepared to breed, a positive response to male courtship song can result in successful breeding. For a female that is not prepared to breed, a positive response to male song could result in wasted energy engaging in breeding activities that are likely to fail. Thus the evolution of neural mechanisms that limit reward induced by hearing song to individuals that are prepared to

breed can serve to coordinate breeding behaviors to maximize the likelihood of a successful reproductive outcome.

4.1 Possible factors influencing breeding readiness and song-induced reward

Why some females differ from others in terms of the tendency to acquire and defend a nest box (which we consider a reflection of breeding readiness) is not clear. In this study, we controlled both the attractiveness of song (all females received a song stimulus consisting of recordings of the same males from their natal region) and estradiol concentrations (via implants), both factors that can influence female interest in song [9, 15, 53–57]. Factors such as age, general health, parasite load or individual social status may influence a female's readiness to breed. It is also possible that all females in the present study would be prepared to breed if allowed access to a nest box but that some females were prevented from obtaining nest boxes by older or more aggressive individuals. Although this may be a factor in determining nest box ownership, in each aviary there were unclaimed nest boxes, indicating that all females in the study at least had the potential to acquire a box.

4.2 Met-enkephalin label was densest in females with nest boxes

Past studies implicate opioids, among other neurochemicals, in motivation and social reward [58–60]. Here we found that the area covered by labeling for the opioid met-enkephalin was greater for VMH, POM, Ac and PAG in females with nest boxes. We do not suggest that these differences were induced by hearing male song but that they reflect stable differences in females with and without nest boxes. Opioids in the VMH and POM, and PAG modulate production of sexually motivated behaviors [61–68]. Enkephalin opioids in the POM [24] and morphine in the PAG induce reward (i.e., a place preference) [25] in rodents, and mu opioid receptors in Ac are involved in social reward in rats [58]. As reviewed in the introduction, opioid receptors in the POM and the VMH (but not Ac) underlie reward induced by mating behavior (as measured using a CPP test) [28]. In the present study the finding that nest box entries correlated positively with mENK in VMH, and nearly with POM ($p = 0.057$) is consistent with the possibility that opioids in VMH and POM (but not Ac) may underlie reward induced by hearing male song in females with nest boxes. The strongest support for this hypothesis is the positive correlation identified between mENK labeling in VMH and female CPP. Overall, the results of our study are consistent with the possibility that stable differences in enkephalin opioids in the VMH, POM, Ac and/or PAG underlie differences in the motivational state of females with and without nest boxes. The results are also consistent with the hypothesis that opioids in VMH (and possibly POM) contribute to individual differences in the reward state induced in by hearing male courtship song.

Finally, although the rationale for examining opioids in the present study is based on the role of these neuropeptides in reward, it is important to note that opioids are complex modulators of behavior that influence many potentially socially-relevant processes other than pleasure/reward. These include (among others) learning, memory, stress, and social status, (reviewed in [69, 70]). The next step in this line of research therefore will be to combine measures of song-induced reward using CPP with site-specific manipulations of opioid receptors in VMH and POM to test the hypothesis the song-induced opioid release in these regions modulates the rewarding properties of hearing male song. Given that opioids also interact with dopamine to regulate sexually motivated behaviors [71, 72], future studies will also involve examination of contributions of both dopamine and opioids in specific brain regions to motivation and reward states underlying female responses to male song.

4.3 Utility of the CPP approach for evaluating female responses to male song

In past work, attractive properties of hearing male song have been assessed primarily using measures of female copulation solicitation [18, 73–75], approach responses to song playback [5, 76, 77], or operantly triggered playback of male song (reviewed in the introduction). These studies provide strong evidence that song can be reinforcing; however, these methods are labor intensive and females of many species either do not produce copulation solicitation displays or do so rarely in a laboratory setting [74]. The CPP procedure described here requires minimal training of subjects and does not involve difficult or unreliable measures of appetitive female behavioral responses to song. Many conditioned place preference studies involve multiple UCS-side pairings (for reviews of the procedure see [41, 43, 44]); however, in the present study we observed conditioning after only a single song-side pairing. This suggests that it may be important for females to rapidly learn and remember locations from which they previously heard a singing male, perhaps to enhance the likelihood of mating success. Similar rapidly acquired place preferences are observed in association with other highly biologically relevant rewards necessary for reproductive success, including copulation [78] and maternal rat exposure to pups [12]. The CPP method can thus provide an efficient method for evaluating rewarding properties of different song stimuli (e.g., courtship song versus non-breeding song, previously learned versus unlearned songs, local versus foreign song dialects) and can be used to assess the extent to which rewarding properties of song differ as a function of sex and breeding condition.

The CPP method has many strengths, but as with any technique there are weaknesses as well (reviewed by [41]). In the present study we presented females with song and silence, and it is thus possible that our results are not specific to song (i.e., it is possible that any sound would have produced the same effect). Although we think this is unlikely given that only breeding-ready females responded to the song, future studies are now needed to test different acoustic stimuli and to refine and expand upon this CPP paradigm. With the present design we are limited to evaluating the rewarding properties of only two stimuli at a time. Whether multiple CPP tests can be run in the same individual or whether CPP apparatuses with more than two distinct chambers can be used to effectively evaluate the relative rewarding properties of multiple song stimuli must be evaluated. Furthermore, the use of this paradigm to evaluate the rewarding properties of acoustic stimuli has not been tested in other species. However, several studies show that birds in addition to starlings can develop place preferences for rewarding stimuli. For example, male zebra finches and starlings can develop a place preference associated with the act of producing song [79], adult Japanese quail can develop a place preference for sexual stimuli [45], and quail as young as 12 days of age can develop a preference for food [80]. Notably the latter study also showed that place preferences can be established after only a single pairing with yet another biologically relevant stimulus necessary for survival. It is thus likely that this paradigm can be used to evaluate vocal signal-induced reward in a variety of avian species.

4.4 Conclusions

The present study supports the hypothesis that differences in the extent to which females find male courtship song rewarding are linked to individual differences in female motivational state. Results raise the possibility that persistent differences in enkephalin opioid activity in the VMH, POM, Ac, and PAG in females with and without nest boxes play a role in motivational state. The strong link between mENK in VMH and song-induced CPP identifies the VMH as a potential site in which opioid release may induce reward in response to hearing male courtship song. Finally song-induced CPP provides a novel method for assaying individual preferences for vocal signals.

Acknowledgments

We gratefully acknowledge support from the National Institutes of Health (R01 MH080225) to LVR and the Michael Guyer Postdoctoral Fellowship to JMSE. We also thank Chris Elliott for animal care.

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Research highlights

- Behavioral responses to male courtship song differ among female starlings
- Breeding-ready females preferred a chamber in which they previously heard male song
- Non-breeding-ready females did not develop a similar place preference for song
- In breeding-ready females mENK label was highest in VMH, mPOA, Ac, and PAG
- The reward value of male song is linked to motivational state and opioid label

Figure 1.

Females with nest boxes (filled bars) displayed higher rates of behaviors indicative of breeding readiness but not general behaviors compared to females without nest boxes (open bars). Mean (+ sem) untransformed number of times females A) entered a nest box, B) displaced other females and engaged in bouts of C) feeding and D) preening. * = $p < 0.05$

Figure 2.

Song induced CPP in females with nest boxes but not females without nest boxes. Mean (+sem) time spent (in the absence of song playback) on the side of the CPP apparatus previously paired with song (minus the baseline preference for that side during habitation) in females without (open bar) and with (filled bar) nest boxes. Zero on the y axis indicates no preference. * = $p < 0.05$.

Figure 3.

Scatterplots showing positive correlations between CPP in nest box owners and mean untransformed indices of breeding readiness (entering a nest box (A) and displacing other females (B)). Zero on the x axis indicates no preference. Each dot represents data from a single individual. Significant betas from multiple regression analysis are show in bottom right hand corner of each figure. See text for additional statistical details.

Figure 4. Mean mm² area covered by mENK labeling (+sem) in A) VMH, B) POM, C) Ac, and D) PAG in females without (open bars) and with (filled bars) nest boxes. See text for abbreviations and statistical details. * indicates a significant main effect for nest box ownership with mENK labeling found to be significantly higher across brain regions in birds with compared to without nest boxes.

Figure 5.

Scatterplot showing a positive correlation between CPP in females without (open dot) and with (filled dots) nest boxes and the mean mm² area covered by mENK labeling in VMH. Zero on the x axis indicates no preference. Each dot represents data from a single individual. Significant beta from multiple regression analysis is shown in the bottom right hand corner. See text for additional statistical details.

Figure 6.

Scatterplots showing correlations between numbers of nest box entries in females without (open dots) and with (filled dots) nest boxes and the mean mm² area covered by mENK labeling in A) VMH and B) POM. Each dot represents data from a single individual. Results of correlational analyses are shown in bottom right hand corner of each figure. See text for additional statistical details.

Table 1

Mean time in secs (sem) out of 15 min that females spent on the song-paired side of the CPP apparatus

Nest box owner?	Prior to conditioning	After conditioning
No (n = 13)	373.00 (57.22)	291.54 (72.52)
Yes (n = 13)	220.00 (57.22)	488.31 (72.52)