

Operant conditioning and copulation solicitation display assays reveal a stable preference for local song by female swamp sparrows *Melospiza georgiana*

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Abstract Operant conditioning assays are increasingly being used to test mating signal preferences in female birds. Operant behavior may be seen as farther removed from mate choice behavior as compared to other methods for measuring mating signal preferences, which could limit the evolutionary interpretation of operant results. I compared the song preferences of female swamp sparrows (*Melospiza georgiana*) as measured both by a copulation solicitation display assay and by an operant preference test. Both methods revealed a strong preference for songs from the females' Conneaut Marsh breeding population over songs from a Millbrook population over 500 km distant, which remained stable after extensive exposure to Millbrook songs. Further, there was a striking congruence in results on an individual level from the two assays. These findings support the conclusion that operant methods reveal evolutionarily significant mate choice preferences, providing the opportunity to study such preferences under circumstances when the copulation solicitation assay is less practical.

Keywords Female mate choice · Mating preferences · Operant conditioning · Copulation solicitation display · Geographic song discrimination · *Melospiza georgiana* · Swamp sparrow

Introduction

Sexual selection on a trait is measured most directly by mapping variation in reproductive success onto variation in the expression of the trait (Andersson 1994). Directly measuring reproductive success is not practical in some systems, in which case, proxies of reproductive success are used instead. In the field, proxies such as time to obtain a mate, the number of mates obtained, or the number of matings observed might be used. In captivity, assays that measure time spent in close proximity to potential mates, or the number of sexual displays given, can be used as proxies for mate choice preferences. In songbirds, a standard method for measuring female preferences for male song traits is the copulation solicitation display assay (King and West 1977; Searcy 1992; Searcy and Marler 1981). Sexually receptive female songbirds, when exposed to playback of male songs, will respond with a pre-copulatory display. The display varies across species, but in general, the female crouches forward, raises her head and tail, shivers her wings and gives copulation calls. The number and intensity of displays that a female gives in response to standardized song playback can be used to quantify response to different song traits.

An advantage of the solicitation assay is that the behavior being measured is linked closely to copulation and, thus, convincingly reflects mate preferences (Searcy 1992). This interpretation is supported by evidence that, in at least some species, solicitation displays correspond well to actual mate choice decisions made by females (Catchpole et al. 1984; West et al. 1981). In some species, however, females will not perform displays reliably in captivity, and in other species, females reared in the laboratory fail to respond at all (Searcy 1992). This raises the need for another type of captive preference assay. Other

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methods used to test song preferences in female captives include measures of affiliative approach in response to song playback (phonotaxis) (e.g., Clayton 1988; Holveck and Riebel 2007), phonotaxis in response to live males (e.g., Forstmeier and Birkhead 2004; Holveck and Riebel 2007), heart rate monitoring during song playbacks (e.g., Dooling and Searcy 1980; Ikebuchi et al. 2003; Zimmer 1982), and vocal responses to song playbacks (Nagle et al. 2002; Nelson and Marler 1993).

Success with operant conditioning provides an alternative method for testing song preferences in female songbirds. Male song stimulates females and is inherently rewarding in an operant paradigm (Stevenson 1967, 1969). Thus, female songbirds can be conditioned to perform an operant task, such as pecking a key or hopping on a perch, with song as the sole reward (Adret 1993; Collins 1999; Riebel 2000; Riebel and Slater 1998; Stevenson 1967; ten Cate 1991). When presented with a choice between two song exemplars, subjects demonstrate their preference by activating playback of one song more frequently than the other (e.g., Collins 1999; Riebel and Slater 1998). Operant behavior may be considered a less direct measure of mate choice than is solicitation display, however, making it necessary to validate the use of operant techniques for assessing female preferences. To this end, I compared the responses of the same group of female swamp sparrows using both the solicitation display assay and an operant assay.

In many songbirds, songs vary geographically (reviewed in Catchpole and Slater 2008; Podos and Warren 2007) and females have been shown to prefer songs from their own region over songs from a foreign region (white-crowned sparrow: Baker et al. 1981; Baker 1983; yellowhammer: Baker et al. 1987; swamp sparrow: Balaban 1988a, b; zebra finch: Clayton 1990; red-winged blackbird: Searcy 1990; song sparrow: Searcy et al. 1997, 2002). Thus, in a first experiment, I asked if female swamp sparrows from a population in Conneaut Marsh, PA, USA, discriminate against the songs of males from Millbrook, NY, USA, some 540 km distant. I tested the same females using both a copulation solicitation display assay and an operant assay in order to assess the consistency of the results between the two methods.

In a second experiment, I asked whether preference for local song can be modified by experience with foreign song variants in adult females. In the first round of operant and solicitation display testing, females recently exposed to Conneaut Marsh song variants in the field prior to capture were further exposed to Conneaut Marsh songs during the 14 days of operant training prior to testing. This leaves open the possibility that the preferences shown by the females in this study were due simply to familiarity or recency effects. Such effects have been shown to influence

song preferences in some studies (Clayton 1988; Collins 1995; MacDougall-Shakelton et al. 2001; Nagle and Krutzer 1997; O'Loughlen and Beecher 1999; Riebel 2000), but not in others (Cynx and Nottebohm 1992; Riebel and Slater 1998).

Materials and methods

Swamp sparrow songs are composed of a single syllable, composed of one to five notes, repeated in a continuous trill about 2–3 s in duration (Fig. 1). Individual males produce two to five song types, which they learn through exposure and imitation early in life (Marler and Peters 1988). Swamp sparrow songs include a limited number of note-type categories. Earlier work suggested that note-type categories are the same across the species' range, with populations differing in the ordering of notes within syllables (Clark et al. 1987; Balaban 1988b; Marler and Pickert 1984). However, recent work demonstrates that the structure of note-type categories also differs among populations (Prather et al. 2009; Lachlan et al. 2009).

Experiment 1

Solicitation display assay

In July 2007, I captured adult female swamp sparrows ($N=8$) from a population at Conneaut Marsh, Crawford County, PA, USA. I transported them to Duke University and housed them in individual cages (dimensions $46 \times 22 \times$

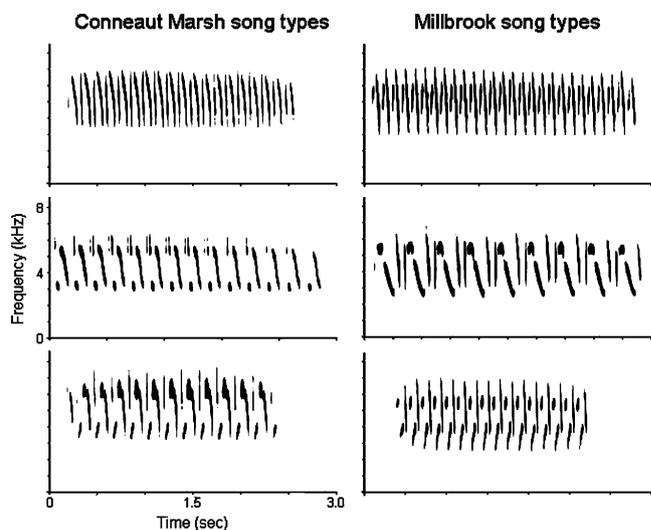


Fig. 1 Examples of Conneaut Marsh, PA, and Millbrook, NY, song exemplars used as stimuli in the copulation solicitation and operant assays. Songs were sampled at 44,100 pts/s. Spectrograms were made using Signal 4.0 software (172.3 Hz frequency resolution, 5.8 ms time resolution, 256 Fast Fourier Transform size, Hanning window)

26 cm) for 18 days prior to testing. I kept the light cycle at long daylength (15L:9D) until the first round of testing was completed in August 2007. Seven days prior to testing, I gave each female a subcutaneous implant of 17- β -estradiol in silastic tubing (1.96 outside diameter, 12 mm total length, 7–8 mm of hormone), which I inserted under the skin after application of a topical anesthetic. Estradiol implants prime female swamp sparrows to respond to song with solicitation displays, and are necessary to ensure that most study subjects give displays during testing (Searcy and Marler 1981; Searcy 1992; personal observation).

To test for preference between local and foreign songs, I first used a standard copulation solicitation assay (e.g., Nowicki et al. 2001, 2002; Ballentine et al. 2004). I tested females in individual sound isolation chambers (Industrial Acoustics model AC-1) and played song stimuli at mean amplitude 75 ± 2 dB SPL through a Realistic 4 \times 6 full-range speaker (model 40-1298C) mounted to the top center of each test chamber. Song playback amplitudes were measured at the center of each chamber using a RadioShack sound-level meter (model 33-2055) set to Fast Response and C-weighting. I presented females with two song treatments: local song exemplars recorded from males in the Conneaut Marsh population and foreign song exemplars recorded from males in a Millbrook, NY, population. The two populations are separated by approximately 540 km.

I tested each female on two consecutive days with a given pair of stimuli, the first day with presentation order counterbalanced across subjects, and the second day with the order reversed. I performed two trials on each day, one trial in the morning and one in the afternoon, with the two trials separated by 4 h to minimize habituation from the first trial to the second. In each trial, I presented the female with nine repetitions of a single song, repeated at a rate of one song per 10 s, followed by nine repetitions of a second song exemplar in the same category (either Conneaut Marsh or Millbrook). No female was tested with songs heard by another female. I matched pairs of song stimuli for vocal trill performance, a measure of how physically challenging a song is to produce (Podos 1997), and a song feature known to affect female preference in this species (Ballentine et al. 2004). I videotaped the females during trials and viewed the responses on a monitor hidden from the birds' view. The number of solicitation displays given during playback of each stimulus type was the sole measure of response (King and West 1977; Searcy et al. 1997; Nowicki et al. 2001).

Operant assay

One week after completion of the solicitation display trials, I tested the same group of females for local vs foreign song preference using an operant conditioning assay. This assay

was similar to that used by Riebel and colleagues with other species (Riebel and Slater 1998; Riebel 2000; Holveck and Riebel 2007). Females were housed singly (cage dimensions 46 \times 22 \times 26 cm) and had access to food and water ad libitum. Each cage contained two regular perches (20 cm long) and two smaller operant perches (15 cm long) attached to the front interior of the cage approximately 15 cm apart. The operant perches were affixed to lever microswitches that were connected to a PC computer running custom software. Each hop on an operant perch was logged automatically on the computer, and triggered playback of one song.

I did not remove the estradiol implants for operant testing to ensure that the females had similar hormone levels during both response assays. It is not known whether hormone implants are necessary to evoke responsiveness in the operant assay. However, there is good reason to presume that hormone could substantially affect operant measures since the operant reward in this case, male song, should be most rewarding to females in breeding condition.

The eight females were first exposed to the operant perches for a period of 14 days to learn that they could elicit song playback by landing on the perches. During training, the operant perches were active for 10 h per day, from 0600–1600 hours, and the perches remained in the cages when they were inactive. When active, one perch triggered a single Conneaut Marsh song, and the other perch triggered no sound. I switched the perch that triggered song from one side to the other (left or right side) every 2 days to ensure that the females did not associate song with one side of the cage, and to guard against generalized side biases.

After the 14-day learning period, I tested the females during 5-h blocks each day for eight consecutive days. The operant computer software could test only four females simultaneously, so on each day, I tested four females in the morning (approx. 0600–1100) and the other four in the afternoon (approx. 1200–1700). I alternated the test order between morning and afternoon on consecutive days so that each female was tested in total four times in the morning and four times in the afternoon. During a test, one perch elicited a Conneaut Marsh song exemplar and the second perch elicited a Millbrook song exemplar. Over the eight days, I tested each female with four pairs of Conneaut Marsh and Millbrook songs. I presented each song pair twice on consecutive days, once in the afternoon and once the following morning, and the assignment of song treatment to perch was reversed for each new song pair as a guard against side bias.

After completing the first round of operant and solicitation display assays, I removed the estradiol implants and housed the females in individual cages in an indoor aviary from 20 August 2007–1 February 2008. During this period,

the females had no exposure to song, and the room was gradually shifted to a 9L:15D light schedule (short day-length) to simulate the fall and winter months.

Experiment 2

I performed a second round of preference assays with the same cohort of females in the spring of 2008 to test whether exposure to foreign Millbrook songs altered preference for local Conneaut Marsh songs as measured by either or both of the two assays. On 2 February, I moved the individually housed females into a large sound attenuation chamber and gradually increased the light schedule to 15L:9D (long daylengths). After approximately 2 weeks of increasing daylength, I began exposing the females to playback of songs from Millbrook males. I played two sessions of song each day, one 2-h session in the morning and another in the afternoon, for 8 weeks. The playbacks comprised 26 different exemplars of 21 song types. Song types were played in bouts of ten repetitions at 18-s intervals. During week 7 of playback, I again implanted the females with 17- β estradiol. Beginning on 8 April 2008, I tested the song preferences of the females once again using the copulation solicitation assay. Each female heard four Conneaut Marsh songs, each paired with a different Millbrook song. The sequence and timing of song presentation were as in the 2007 experiment, but the songs used were novel.

Operant testing began 10 days after the completion of the solicitation tests. The females were first trained for 5 days with perches that triggered either a Conneaut Marsh song or silence, with the song perch switched between the left and right sides every other day. Seven of the eight females showed strong preference for song over silence by day 5, so I began testing on day 6. The testing procedure replicated the first round of operant tests the previous season, except that all females were tested for 5 h each day from 0600–1100, rather than testing blocks during both the morning and afternoon. I altered the protocol in this way because I could now test all eight females simultaneously using Sound Analysis Pro software (Ofer Tchernichovski, <http://ofer.sci.cuny.cuny.edu>) to manage song playbacks and log hop events. Also, it was obvious from the first round of operant tests that some females were more active and hopped on the operant perches more often in the morning than in the afternoon. Here again, the song stimuli were four Conneaut Marsh songs, each paired with a different Millbrook song.

All aspects of this study were performed in accordance with protocols approved by the Duke University Animal Care and Use Committee, and with animal collection and land use permits from both federal and state agencies.

Analysis

For the solicitation display assay, I compared the number of displays within subjects (i.e., in a paired design) using the Wilcoxon matched-pairs signed-ranks test using SYSTAT v.11.0 (Systat Software). I made the same within-subjects paired comparison for the operant data, comparing the number of hops on perches that triggered Conneaut Marsh songs or Millbrook songs. In addition, because there is often large variation in individual performance in operant assays, the percentage of responses for a given treatment, rather than the number of responses, is tested for deviation against 0.5 using a one-sample *t* test (Collins 1999; Riebel 2000; Riebel et al. 2002; Leitao et al. 2006). I first calculated the percentage of hops on Conneaut Marsh song perches for each bird and then normalized the data using the arcsine transformation (Sokal and Rohlf 1995). For all tests, $\alpha=0.05$.

Results

Female swamp sparrows from Conneaut Marsh showed a significant preference for their local population's songs over songs from a foreign population when assayed with both the copulation solicitation test and the operant test. On day 1 of the solicitation test, four of six displaying females gave more displays to Conneaut Marsh songs than to Millbrook songs (two females gave no displays), and on day 2, five of five displaying females gave more displays to Conneaut Marsh (three females gave no displays). Overall, subjects gave a significantly greater number of displays to Conneaut Marsh songs (total=72, mean=9.0) than to Millbrook songs (total=18, mean=2.3; Wilcoxon test, $z_{n=8}=-2.63$, $p=0.009$; Fig. 2a).

Over the 8 days of operant testing, seven of eight females hopped more often on perches playing Conneaut Marsh songs than on perches playing Millbrook songs (mean \pm SE hops for Conneaut Marsh=2,699 \pm 780 vs 1,310 \pm 267 hops for Millbrook; $z=2.24$, $p=0.025$; Fig. 2b). The overall preference score for Conneaut Marsh songs (mean \pm SE=0.66 \pm 0.054) deviated significantly from a chance ratio of 0.5, demonstrating an overall preference for Conneaut Marsh songs over Millbrook songs (one-sample *t* test, $df=7$, $t=2.98$, $p=0.02$).

In general, females that expressed strong preferences for Conneaut Marsh songs during the solicitation assay by giving more copulation solicitation displays in response to Conneaut Marsh songs also showed this strong preference by hopping on Conneaut Marsh song perches more often than they hopped on Millbrook song perches (Fig. 3; females 2, 5, 6, and 7). Similarly, within subjects, weak discrimination between song treatments (female 8) and a

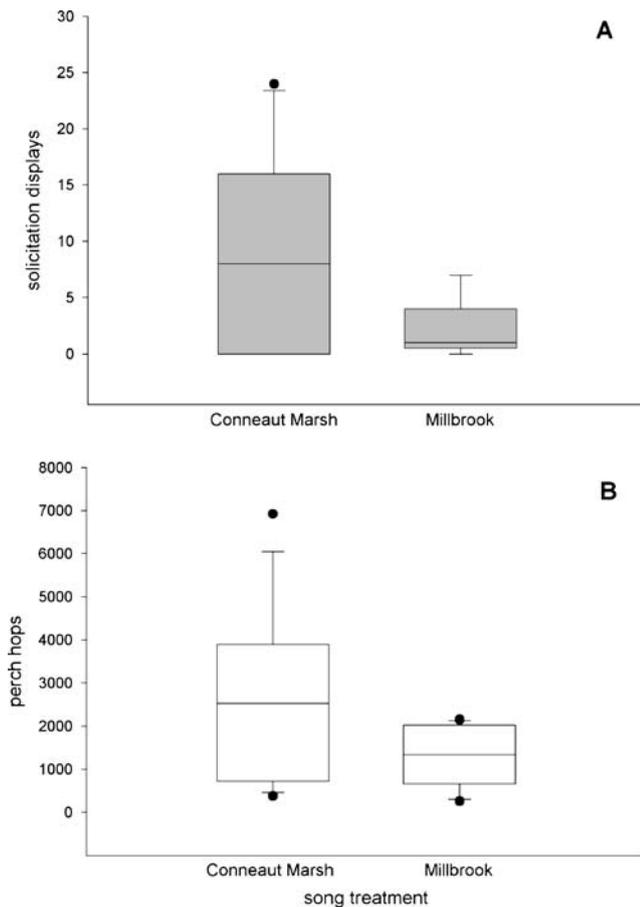


Fig. 2 The number of copulation solicitation displays (*gray boxes, a*), and perch hops (*white boxes, b*) in response to Conneaut Marsh, PA, songs and Millbrook, NY, songs in experiment 1 tests. The same eight females were the subjects in both tests. The *box boundaries* indicate the 25th and 75th percentiles, the *lines* within the boxes mark the medians, *whiskers* above and below boxes indicate the 90th and 10th percentiles, and *circles* mark outlying points

weak preference for Millbrook songs (female 3) were shown by both the solicitation and operant methods. The operant procedure revealed preferences held by females that gave no copulation solicitation displays: two females gave no displays in response to either Conneaut Marsh or Millbrook songs, but when assayed with the operant test, both females showed preferences for Conneaut Marsh songs (females 1 and 4). Overall, operant activity levels related positively to the number of solicitation displays performed (Spearman rank correlation, $\rho=0.57$, $p=0.03$). For example, the three females that gave the fewest number of solicitation displays (females 1, 3, and 4) also performed the fewest number of perch hops.

In the second round of preference testing in 2008, following over 200 h of exposure to Millbrook songs, the females retained their preference for Conneaut Marsh songs. In the solicitation display assay, six of seven displaying females gave more displays to familiar Conneaut

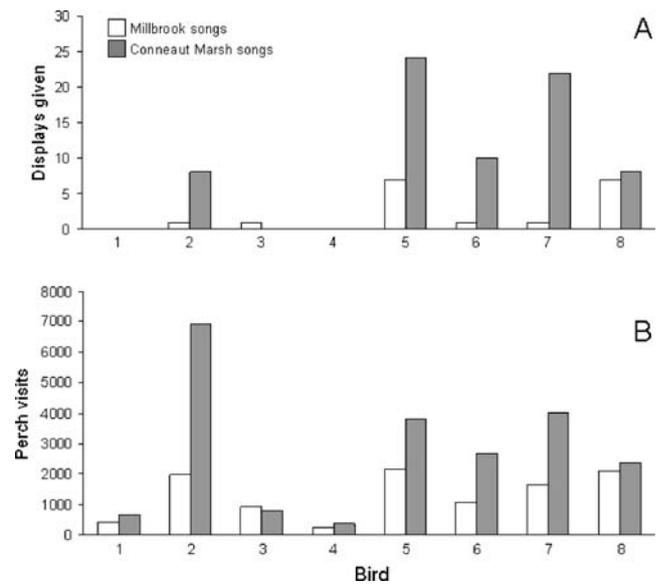


Fig. 3 Total numbers of copulation solicitation displays (**a**) and perch hops (**b**) that each of the eight females gave in response to Millbrook, NY, songs and Conneaut Marsh, PA, songs. Data are from experiment 1

Marsh songs than to familiar Millbrook songs, despite not having heard the former in over 5 months. On day 2, all five displaying females gave more displays to novel Conneaut Marsh songs than to novel Millbrook songs. Overall, the subjects gave a significantly greater number of displays to Conneaut Marsh songs (total=52, mean=6.5) than to Millbrook songs (total=6, mean=0.8) (Wilcoxon $z_{n=8}=-2.851$, $p=0.004$) (Fig. 4a).

Over the 8 days of operant testing, subjects made a total of 22,100 visits to Conneaut Marsh song perches and 10,954 visits to Millbrook song perches. The mean \pm SE number of visits to Conneaut Marsh song perches was $2,762.5 \pm 680$, vs $1,369.3 \pm 286$ visits to Millbrook perches (Wilcoxon $z_{n=8}=2.52$, $p=0.012$; Fig. 4b). The overall preference score for Conneaut Marsh songs (mean \pm SD = 0.65 ± 0.10) was significantly greater than chance, demonstrating an overall preference for Conneaut Marsh songs over Millbrook songs (one-sample t test, $df=7$, $t=4.06$, $p=0.005$).

Discussion

Finding efficient and reliable ways to assess female mating preferences is central to further understanding variation in female mate choice and its impact on sexual selection (Jennions and Petrie 1997; Wagner 1998). Here, I compared the song preferences of a single group of female swamp sparrows using two behavioral assays and found that the results obtained by operant testing mirror the preferences obtained using the copulation solicitation assay, an estab-

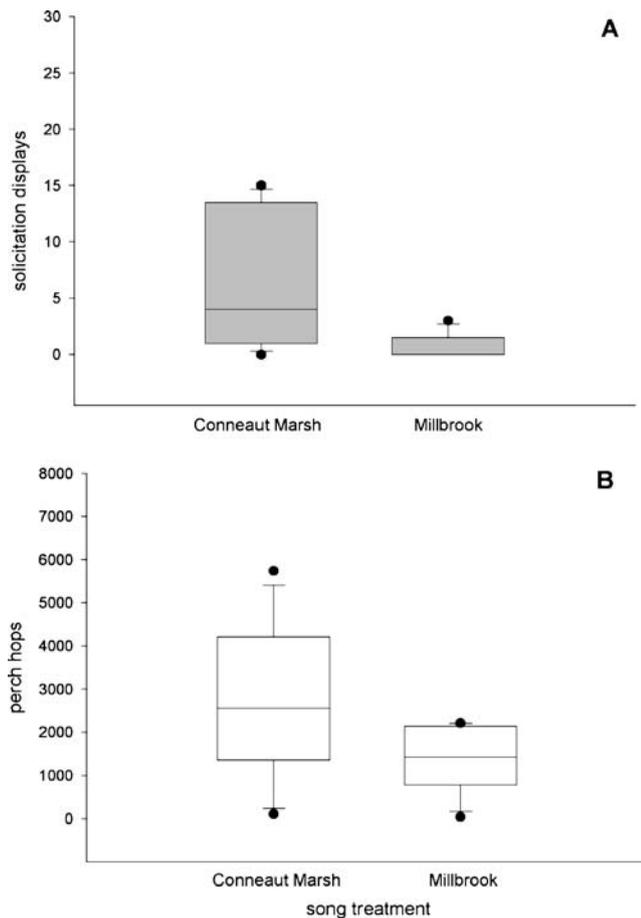


Fig. 4 The number of copulation solicitation displays (*gray bars, a*), and perch hops (*white bars, b*) in response to Conneaut Marsh, PA, songs and Millbrook, NY, songs in experiment 2. The *box boundaries* indicate the 25th and 75th percentiles, the *lines* within the boxes mark the medians, *whiskers* above and below boxes indicate the 90th and 10th percentiles, and *circles* mark outlying points

lished method for testing song preferences in female song birds. These results support the conclusion that operant methods provide a reliable means for revealing evolutionarily significant mate choice preferences. In addition, this study contributes to a growing body of evidence that different laboratory behavioral assays of female mating preference give congruent results (Holveck and Riebel 2007; but see Rutstein et al. 2007).

A potential benefit of operant methods is that they can reveal preferences in females that do not perform in the solicitation assay. Females of some species fail to perform displays at all in captivity when tested using the copulation solicitation assay (Searcy 1992), while in other species, certain classes of females do not display, such as those reared in captivity. Even within classes of females that typically perform well in the solicitation assay, including swamp sparrow females captured from the wild as adults, some number of subjects fail to perform displays. These females are excluded from analysis because one cannot

determine whether a female that does not display to any stimulus has no preference or is unresponsive because of some other factor such as low motivation. In this study, females that were unresponsive in the solicitation assay (two of eight) did learn the operant behavior and showed preferences for Conneaut Marsh songs in the operant assay. One possible explanation for the greater efficacy of the operant method is that the measured response is an *active* choice by the female that is continuously rewarded, in contrast to the *reactive* response to song measured in the solicitation assay.

Operant methods also confer the advantage of allowing long-term or repeated testing, presenting females multiple opportunities to choose between stimuli. Repeated testing is not possible with the solicitation assay because, at least in sparrows, the response decreases within days despite presentation of novel stimuli. This decrease in response has not been observed using the operant assay described here (Anderson, unpublished data). Thus, operant methods allow females to assess and choose between stimulus pairs over days and weeks, decreasing the restrictions on the number of tests that can be done. In addition, operant methods may provide a tool with which to measure social and song preferences in developing individuals, both males and females, perhaps well before they would exhibit such preferences in the context of mate choice or other breeding behavior.

In this study, swamp sparrow females from Conneaut Marsh preferred songs from their local population over songs from the Millbrook population, 540 km away. Geographic song discrimination and preference for local variants has been shown for several species (Baker 1983; Baker et al. 1987; Clayton 1990; Freeberg et al. 2001; King et al. 1980; Searcy 1990; Searcy et al. 1997, 2002). However, discrimination in the swamp sparrow has been shown previously only for two populations in Minnesota and New York (roughly 1,500 km apart) that have distinct differences in song syntax (Balaban 1988a, b). Song discrimination between the Conneaut Marsh and Millbrook populations is somewhat surprising in light of previous work comparing song similarity among swamp sparrow populations in the central and eastern USA. Marler and Pickert (1984) and Clark et al. (1987) first suggested that swamp sparrow songs include a limited number of note type categories, and that these categories are the same across the species' range. Balaban (1988a) then compared three measures of song similarity among seven populations and showed distinct variation between some populations (e.g., Lake Itasca, MN, and The Great Vly, NY) but minor variation between others, including the Conneaut Marsh population and a population at the Great Vly Swamp, NY. However, recent work demonstrates that the structure of note type categories differs among populations (Lachlan et

al. 2009) and that this variation is salient to males (Prather et al. 2009), suggesting a possible basis for the discrimination of geographic song variants shown by female swamp sparrows in this study.

Females retained their preference for local Conneaut Marsh songs despite extensive exposure to Millbrook songs, suggesting that preference for local song in this study cannot be simply explained by a preference for songs that are familiar. The Millbrook songs I used were almost certainly novel to the Conneaut Marsh females at the start of testing; however, they were certainly familiar to the females after 200 h of exposure. This result contrasts with studies in other species demonstrating that learned preference for familiar song can be modified by experience with unfamiliar song. Zebra finch females have been shown to prefer songs heard when young over unfamiliar songs, regardless of whether the songs were heard from live males or from playbacks (Clayton 1988; Miller 1979; Riebel et al. 2002). However, zebra finch females can modify their preferences to include songs heard later in life in addition to songs heard when young (Clayton 1988). In the canary, females that showed a preference for songs heard during tape tutoring later modified their preferences to include the songs of unfamiliar live males that they paired and bred with (Nagle and Kreutzer 1997). Thus, while familiarity influences the song preferences shown by some female songbirds, in the swamp sparrow at least, increased familiarity does not appear to increase the attractiveness of foreign songs or weaken local–foreign discrimination.

Although the preference for local song was not modified after extensive exposure to foreign song in this study, this experiment was not a definitive test of whether song preferences can be modified by experience. Several studies suggest the importance of social factors in the plasticity of preferences (King and West 1987; Freeberg 1998; Freeberg et al. 2001; King et al. 2003). For example, early work with female cowbirds (*Molothus ater*) found no evidence that experience was necessary to elicit local geographic preferences (e.g., King and West 1983a, b; King and West 1990; West et al. 1998), but subsequent work challenged this view by showing that females housed in aviaries with exposure to live foreign males broadened their preferences to include foreign songs (West et al. 2006). Such studies provide evidence that, although acoustic experience alone may not be sufficient to alter song preferences, exposure to live males singing foreign song variants may do so.

The success of the operant conditioning method described here has implications for the study of song discrimination learning and the development of song preferences in female songbirds. Because sexual selection involves both male signals and female signal preferences, understanding how female preferences originate and how they are affected by learning and experience could greatly

impact our understanding of signal evolution. For example, West and King (1986) highlighted the importance of understanding the role of female receivers in the study of signal variation, and coined the term “diacept” to discuss how population differences in female song preferences can influence the development of male song (King and West 1989; Smith et al. 2000; West and King 1988). In songbirds, we know much about how young males learn to produce song (Konishi and Nottebohm 1969; Marler and Peters 1981) and about how variation in song traits affects female mate choice and male reproductive success (Searcy and Andersson 1986; Searcy and Nowicki 2005). Studies with swamp sparrows and other closely related species, for example, have highlighted the song qualities that are preferred by females and influence mate choice decisions (e.g., Ballentine et al. 2004; Nowicki et al. 2002; Podos et al. 1999). How female swamp sparrows learn to discriminate song qualities, develop preferences, and apply these preferences to assessing potential mates, however, remains largely unknown. Operant methods provide a useful tool for addressing such gaps in knowledge in the swamp sparrow, and likely in other wild songbird populations, and are well suited to playing a role in advancing our understanding of the co-evolution of male song qualities and female song preferences.

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