

Recent experience modulates forebrain gene-expression in response to mate-choice cues in European starlings

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Mate-choice decisions can be experience dependent, but we know little about how the brain processes stimuli that release such decisions. Female European starlings (*Sturnus vulgaris*) prefer males with long-bout songs over males with short-bout songs, and show higher expression of the immediate early gene (IEG) ZENK in the auditory forebrain when exposed to long-bout songs than when exposed to short-bout songs. We exposed female starlings to a short-day photoperiod for one of three durations and then, on an increased photophase, exposed them to one week of long-bout or short-bout song experience. We then examined their IEG response to novel long-bout versus novel short-bout songs by quantifying ZENK protein in two song-processing areas: the caudo-medial hyperstriatum ventrale and the caudo-medial neostriatum. ZENK expression in both areas increased with tenure on short-day photoperiods, suggesting that short days sensitize females to song. The ZENK response bias toward long-bout songs was greater in females with long-bout experience than in females with short-bout experience, indicating that the forebrain response bias toward a preferred trait depends on recent experience with that category of trait. This surprising level of neuroplasticity is immediately relevant to the natural history and fitness of the organism, and may underlie a mechanism for optimizing mate-choice criteria amidst locally variable distributions of secondary sexual characteristics.

Keywords: bird song; female choice; immediate early gene; neuroplasticity; sexual selection; *Sturnus vulgaris*

1. INTRODUCTION

Animal mate-choice decisions can depend on the social environment and on recent experiences with the various phenotypes of potential mates (Alonzo & Sinervo 2001; MacDougall-Shackleton *et al.* 2001; Van Gossum *et al.* 2001). Such behavioural plasticity requires a correspondingly plastic nervous system (Tinbergen 1950) capable of integrating an individual's recent social experience and precipitating motor programmes that give rise to a mate-choice decision modulated by the experience. Despite the powerful selective pressures exerted by mate-choice preferences (Darwin 1871; Andersson 1994), however, we know little about the neural processing of cues that release such decisions (Wilczynski & Ryan 1992) and how recent experience modulates these neural responses.

Advances in molecular neuroscience have afforded ethologists some tools necessary to begin exploring the neural underpinnings of very complex behaviours (Ryan 1998). One such advance, particularly intriguing in light of Tinbergen's (1963) call 39 years ago for molecular explanations of complex behaviour, is the characterization and localization of specific transcription factors rapidly induced in the brain in response to behaviourally relevant stimuli (Clayton 2000). A large body of evidence links neuronal induction of these so-called immediate early genes (IEGs) to a variety of memory-dependent behavioural and perceptual phenomena, as experience-based

plasticity in mate choice might be considered. In fact, several IEGs have been implicated in the molecular cascade controlling the formation and stability of long-term memories (Clayton 2000). *Zif-268* is one such IEG (Jones *et al.* 2001), and, consequently, upregulation of its expression can serve as a marker for experience-dependent neuroplasticity.

Thus, IEG induction is likely to be involved in the molecular signal-transduction pathways underlying the types of neuroplasticity that experiential modulation of mate choice may require (Ball & Balthazart 2001). We demonstrate that a female songbird's IEG response bias in her auditory forebrain toward a preferred category of male song depends on the song category with which she has recent experience. These findings reveal a surprisingly subtle neuroplasticity and a potential neural correlate of the experience-dependent modulation of mate choice observed in a diversity of animals.

Like many songbird species (Catchpole & Slater 1995; Searcy & Yasukawa 1996), female European starlings (*Sturnus vulgaris*) choose mates based on acoustic variation among the songs of different males. In wild free-living starlings, song-bout length correlates positively with mating success, reproductive success, male immunocompetence and female choice (Eens *et al.* 1991; Mountjoy & Lemon 1996; Eens 1997; Duffy & Ball 2002). In contexts mimicking natural mate-choice situations, female starlings housed in laboratories demonstrate robust preferences for songs organized into long bouts over songs organized into short bouts, probably because of acoustic features in the long-bout songs not found in the short-bout songs

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(Gentner & Hulse 2000). The electrophysiological properties of two adjacent areas of the songbird auditory forebrain, the caudo-medial hyperstriatum ventrale (cmHV) and the caudo-medial neostriatum (NCM), suggest their high-order positioning within a processing hierarchy specialized for the acoustically complex features of songbird communication (Chew *et al.* 1995; Stripling *et al.* 1997; Ribeiro *et al.* 1998; Sen *et al.* 2001). Following exposure to a variety of complex acoustic stimuli, songbirds upregulate expression of the IEG ZENK (the avian homologue of, and an acronym for, *zif-268*, *egr-1*, *NGFI-A* and *Krox-24*) in the cmHV and NCM, and ZENK expression in these areas is selective for conspecific over heterospecific song (Mello *et al.* 1992). Interestingly, female starlings show higher ZENK expression in the NCM after exposure to 30 min of long-bout songs than after exposure to 30 min of short-bout songs, revealing a potential neural correlate of the female starling's behavioural preference for long-bout songs over short-bout songs (Gentner *et al.* 2000).

Experience early in life may modulate song-induced IEG expression in the auditory forebrain of adult songbirds (Bolhuis *et al.* 2000). However, is IEG expression sensitive to very recent adult experience, as might be necessary in the context of making rapid mate-choice decisions? Exploiting the starling's behavioural preference for and IEG response bias toward long-bout songs over short-bout songs, we determined whether adult females with recent short-bout experience differ from those with recent long-bout experience in their forebrain response bias toward novel long-bout songs. Stated more generally, we propose that the neural response bias toward a trait preferred in mate-choice contexts (Gentner & Hulse 2000) depends on recent experience with that category of trait. An IEG response bias dependent on prior experience with stimulus categories important in mate choice would necessitate a level of neuroplasticity not previously demonstrated—a level linked to categories of recent social experience and immediately relevant to the natural history and fitness of the organism.

2. MATERIAL AND METHODS

(a) *Experimental procedure*

During winter 2001, we captured adult European starlings on a farm near Baltimore, MD, USA, and housed them in single-sex cages under a daily photoperiod of 16 L : 8 D. Throughout the experiment, we provided birds with access to food and water *ad libitum*.

On 22 May 2001, we laparotomized 20 females, and found their regressed ovaries to be consistent with the reproductive state of photorefractoriness (Nicholls *et al.* 1988), confirming that these females were synchronous in the seasonal reproductive cycle at the start of our experiment. On 30 May, we transferred these females to a pre-experiment room, and housed them, four animals per cage, with a photoperiod of 8 L : 16 D to restore photosensitivity (Dawson 1991). We randomly assigned one female from each cage to each of four treatment groups: (i) long-bout experience, long-bout stimulus; (ii) long-bout experience, short-bout stimulus; (iii) short-bout experience, long-bout stimulus; (iv) short-bout experience, short-bout stimulus (see § 2b). Thus, there were five females per treatment level, counterbalanced within each cage.

On 13 July, we transferred eight 'session-1' females to four sound-attenuation chambers (51 cm × 91 cm × 47 cm), two per chamber. The next morning, we changed the photoperiod in these chambers to 11 L : 13 D, and, for 7 days, we played through a speaker either long-bout songs or short-bout songs for 5.5 h per day at partially randomized 30 min intervals during the photophase only (hereafter termed experience treatment). The first 30 min playback of each day always began at the onset of the photophase. No more than two 30 min playbacks occurred in a row (i.e. without at least one intervening 30 min silent period), and no playback occurred during each day's last 30 min of photophase. This resulted in the playbacks being dispersed throughout the photophase in an unpredictable pattern, and, together with our selection of the 11 L : 13 D photoperiod, was an attempt to mimic the experience of free-living females at the latitude of their capture when they would be making mate-choice decisions early in the spring. White noise broadcast in the room housing the chambers masked inter-chamber sound.

Near the end of the photophase on the seventh day of the experience treatment (after completion of the last playback), we transferred these females to eight sound-attenuation chambers (37 cm × 51 cm × 34 cm), where we held them in isolation on a photoperiod of 11 L : 13 D through the following day. Beginning 30 min after the onset of the photophase the subsequent day, we exposed one female of each pair in the experience treatment to novel long-bout songs and the other to novel short-bout songs for 30 min (hereafter termed stimulus treatment). We staggered, by 30 min, the stimulus treatment for the four pairs of session-1 females such that we had completed all stimulus exposures by 2.5 h and collected all brains (see below) by 3.5 h into the photophase.

At 90 min after stimulus onset, we anaesthetized females by intramuscular injection of 6.5 mg of secobarbital (Sigma), rapidly decapitated them and removed their brains. We blocked the brains in the coronal plane *ca.* 2 mm from their most anterior point and agitated them for 3 h in 5% acrolein (Polysciences) in phosphate buffer (pH of 7.2). We followed this with three 30 min washes in 100% phosphate buffer and then transferred the brains to 30% sucrose in phosphate buffer. After 3 days at 4 °C, we rapidly froze the saturated brains on dry ice and stored them at -70 °C.

At the time we transferred session-1 females from the experience-treatment to the stimulus-treatment chambers, we transferred eight session-2 females to the experience-treatment chambers, which we had returned to a photoperiod of 8 L : 16 D, and proceeded identically as for session 1 (changing the photoperiod to 11 L : 13 D the next day). Likewise, we paired the final four females of session 3 in two experience-treatment chambers the following week. Therefore, we counterbalanced each of the four treatment levels within the successive sessions, and we separated sessions by one week, a period that females not yet transferred to the experience-treatment chambers spent under an 8 L : 16 D photoperiod.

(b) *Song recordings*

Details of the song recordings have been described previously (Gentner & Hulse 2000; Gentner *et al.* 2000). Briefly, for the song sets used in the experience treatment, we recorded a large library of complete song bouts from a single laboratory-housed male directing song at a female. From these songs we selected 12 exemplars, which, based on bout length, we divided into two sets of six: a long-bout set and a short-bout set, with mean bout lengths of 55.2 and 26.0 s, respectively. We made the recordings

used for the stimulus treatment in the same manner, except that we recorded the initial library of songs from a different male. The mean bout lengths of the long-bout and short-bout sets used in the stimulus treatment were 55.6 and 25.4 s, respectively. Neither total song nor total silence duration differed between the long-bout and the short-bout sets. The two males' songs had very little in common (*ca.* 1% of their motifs), and females preferred the long-bout sets of both males (Gentner 1999; Gentner & Hulse 2000). Because we used songs from one male for both levels of the experience treatment and songs from another for both levels of the stimulus treatment, stimulus songs were always novel, even when the experience and stimulus categories were the same (e.g. preferred (long-bout) experience followed by preferred (long-bout) stimulus).

(c) Immunocytochemistry

We sectioned the brain tissue at 40 μm in the sagittal plane, and performed immunocytochemistry as previously described by Gentner *et al.* (2000) except that, initially, tissue was incubated in a 7.5% avidin blocking unit and then a 7.5% biotin blocking unit (Vector Laboratories), each for 15 min. We incubated the tissue in *egr-1* antibody (Santa Cruz Biotechnology, catalogue # sc-189) diluted 1 : 8000 in 0.01 M phosphate-buffered saline (pH of 7.5) with 0.3% Triton X-100 and 2% normal goat serum for *ca.* 40 h at 4 °C. We visualized the avidin–biotin horseradish peroxidase complex in a 0.025% diaminobenzidine tetrachloride solution containing 0.4% glucose (Sigma), 0.08% ammonium chloride (Sigma), 2.5% nickel ammonium sulphate (Sigma) and 0.003% glucose oxidase (Sigma). After 13 min, we stopped the reaction in 0.1 M acetate buffer. We processed all the tissue in two immunocytochemistry batches, counterbalancing the four treatment groups within each. To show that the antibody is specifically recognizing the *egr-1* protein, immunocytochemistry was performed using the brains from three photosensitive females exposed to song, with some sections from each brain incubated in *egr-1* antibody and others in a solution of *egr-1* antibody along with the *egr-1* peptide (Santa Cruz Biotechnology, catalogue # sc-189P). High levels of staining were observed in the sections exposed to the antibody only, whereas no staining was observed in the sections incubated with the antibody plus peptide.

(d) Quantification and statistical analyses

We conducted all quantification procedures blind to the experimental condition of each animal and using a method detailed previously (Gentner *et al.* 2000). Briefly, we sampled from the cmHV and NCM, for which the anatomical boundaries have been described (Gentner *et al.* 2000). The samples were 640 pixel \times 480 pixel 8-bit greyscale images captured with a video camera mounted on a microscope (magnification of *ca.* \times ; 412 μm \times 309 μm image area). We quantified every fourth section from the midline to 1120 μm bilaterally. For the cmHV, we placed the sampling window as dorso-caudally as possible (figure 1a). Owing to its larger size, we sampled from two locations in the NCM and then used the mean value of the two. For the dorsal sample, we placed the sampling window as ventro-caudally as possible in the dorsal half of the NCM. For the ventral sample, we placed the sampling window as ventrally as possible. We also sampled from the centre of the nucleus mesencephalicus lateralis pars dorsalis (MLd) and area X, auditory regions respectively, afferent and efferent, to the regions of primary interest.

We counted the number of ZENK-immunoreactive cells

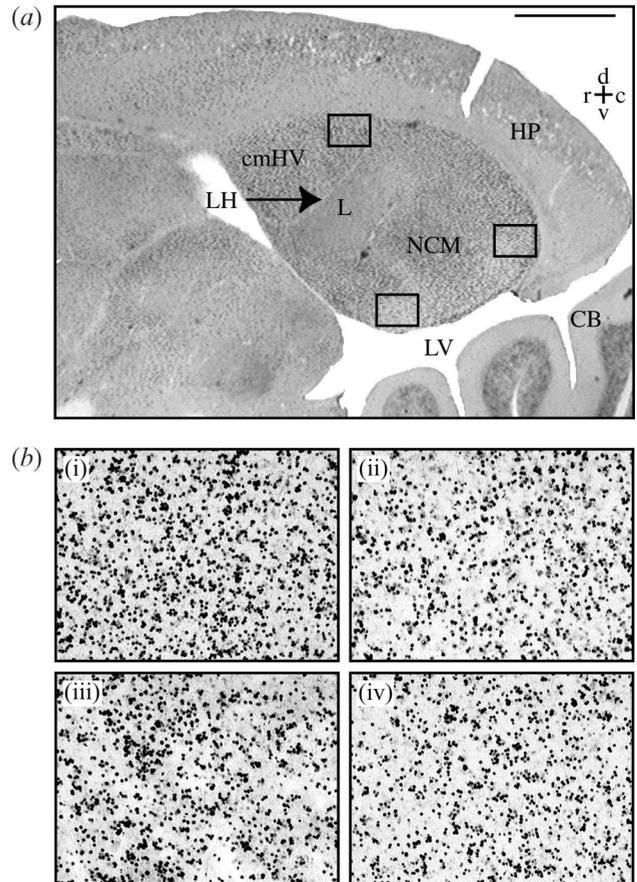


Figure 1. (a) Photomicrograph of a sagittal section of the female European starling brain at the level of the cmHV and NCM. Rectangles indicate the locations of images collected for quantification of ZENK-ir. CB, cerebellum; HP, hippocampus; L, field L; LH, lamina hyperstriatica; LV, lateral ventricle. Scale bar, 1 mm. (b) Representative photomicrographs (412 μm \times 309 μm) of ZENK-ir in the cmHV for each experimental treatment group: (i) long-bout experience, long-bout stimulus; (ii) long-bout experience, short-bout stimulus; (iii) short-bout experience, long-bout stimulus; and (iv) short-bout experience, short-bout stimulus.

(ZENK-ir) in each digitized image using an automated NIH Image (v1.62, National Institutes of Health) routine expressly written for this task (Gentner *et al.* 2000), and verified the accuracy by comparing automated with manual counts on a subset of images. We log-transformed (Mead 1988) the mean count of an image and its contralateral counterpart (figures depict untransformed data), and analysed the change in ZENK-ir in the cmHV and NCM using a single repeated-measures split-unit ANOVA with brain section (laterally from the midline) as one within-factor nested within brain area (cmHV, NCM) as another, experience as the main unit, stimulus as the split unit and session as a factor. We analysed change in ZENK-ir in the MLd and area X separately in two repeated-measures split-unit ANOVAs. We used linear contrasts and Fisher's protected least significant difference for post-hoc comparisons.

3. RESULTS

Regardless of experiment treatment, we observed ZENK-ir throughout much of the brain, particularly in the auditory forebrain areas of the cmHV and NCM

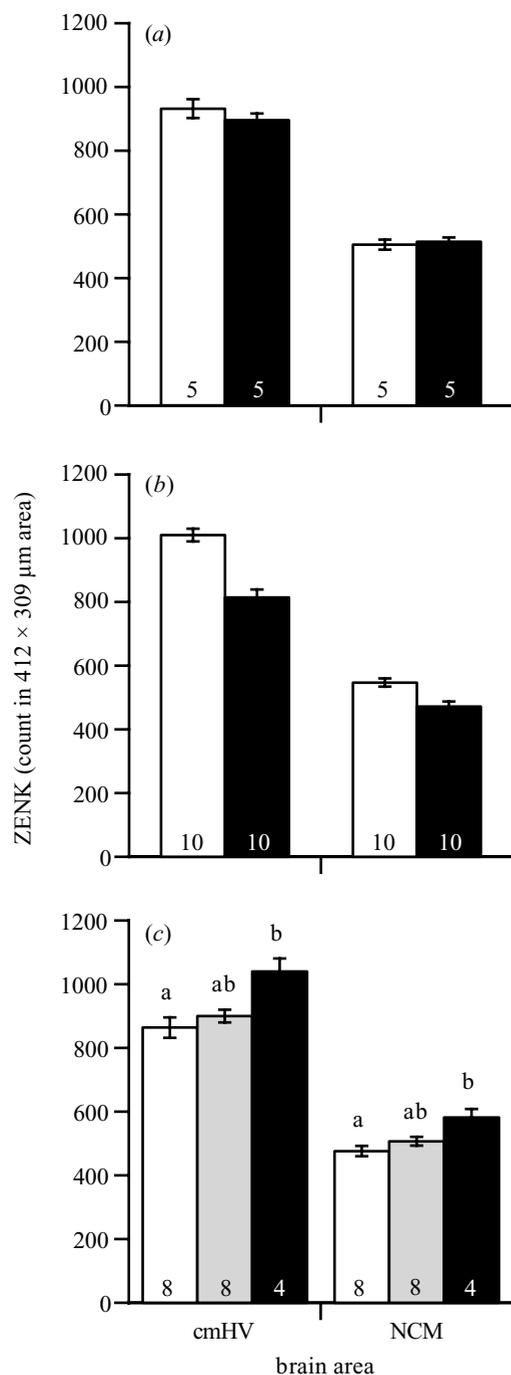


Figure 2. Effects of (a) bout-length experience (black bars, short bout; white bars, long bout), (b) bout-length stimulus (black bars, short bout; white bars, long bout) and (c) session number (white bars, session 1; grey bars, session 2; black bars, session 3) on ZENK-ir (mean cell count \pm s.e.m.) in the cmHV and NCM of the female European starling. Indicated for each treatment is the number of experimental units, based on a split-unit design. In (c), bars with a letter in common were statistically indistinguishable ($p \geq 0.05$) based on Fisher's protected least significant difference values (session 1 versus 2: $PLSD_7 = 0.032$, $p > 0.2$; session 1 versus 3: $PLSD_7 = 0.091$, $p = 0.025$; session 2 versus 3: $PLSD_7 = 0.058$, $p = 0.11$).

(figure 1). Recent song experience had no main effect on ZENK expression in the cmHV and NCM (figure 2a). That is, when we collapsed across session number and stimulus treatment, ZENK-ir in the cmHV and NCM did

not differ between females exposed to 7 days of long-bout songs and females exposed to 7 days of short-bout songs ($F_{1,4} < 0.01$, $p > 0.20$). However, the ZENK response in the cmHV and NCM was influenced by the 30 min song stimulus (figure 2b). When we collapsed across session number and experience treatment, ZENK-ir in females exposed to the long-bout stimulus was significantly greater than that in females exposed to the short-bout stimulus ($F_{1,7} = 14.20$, $p < 0.01$). Tenure on a short-day photoperiod also influenced ZENK-ir in the cmHV and NCM (figure 2c). When we collapsed across the experience and stimulus treatments, ZENK-ir increased significantly with session number ($F_{1,7} = 7.49$, $p < 0.05$).

Although recent song experience had no main effect on ZENK-ir in the cmHV and NCM, it did influence the ZENK response bias toward long-bout songs (figure 3). The effect of the interaction between experience and stimulus on ZENK-ir was significant, indicating that the stimulus effect depended on experience ($F_{1,7} = 5.75$, $p < 0.05$). Post-hoc linear contrasts indicated that the response bias toward long-bout songs in females with long-bout experience was greater than that in females with short-bout experience.

In general, ZENK-ir in the cmHV was higher than that in the NCM (figures 2 and 3). However, neither experience, stimulus nor session interacted with brain area to significantly alter ZENK-ir. That is, the effects (or lack thereof) on ZENK-ir described above did not differ between the cmHV and the NCM. In addition, the treatment effects in the auditory forebrain described above were not apparent in the MLD or area X. For neither the MLD (experience: $F_{1,4} = 0.63$, $p > 0.20$; stimulus: $F_{1,7} = 4.01$, $p = 0.09$; experience \times stimulus: $F_{1,7} = 3.31$, $p = 0.11$; session: $F_{1,7} = 2.37$, $p = 0.17$) nor area X (experience: $F_{1,4} = 0.65$, $p > 0.20$; stimulus: $F_{1,7} = 3.85$, $p = 0.09$; experience \times stimulus: $F_{1,7} = 0.30$, $p > 0.20$; session: $F_{1,7} = 0.05$, $p > 0.20$) were any of the main effects or interactions described above statistically significant. However, trends in the response of both the MLD and area X to song stimulus and to the interaction between experience and stimulus were similar to those in the cmHV and NCM.

4. DISCUSSION

Song-induced gene expression in the starling auditory forebrain was modulated by tenure on short-day photoperiods (session number), and this forebrain gene expression in response to the preferred song type varied according to the female's recent song-type experience. Thus, recent experience with a stimulus preferred by females in a mate-choice context can amplify subsequent perceptual responses to novel forms of that stimulus, but this amplification is itself selective for stimuli of the preferred type.

(a) Sensitization to song cues by a short-day photoperiod

The rise in ZENK expression with session number suggests that tenure on a short-day photoperiod influences the strength of the response to song stimulation. The observation of a session effect in brain areas involved in the high-order processing of song cues and not in the MLD

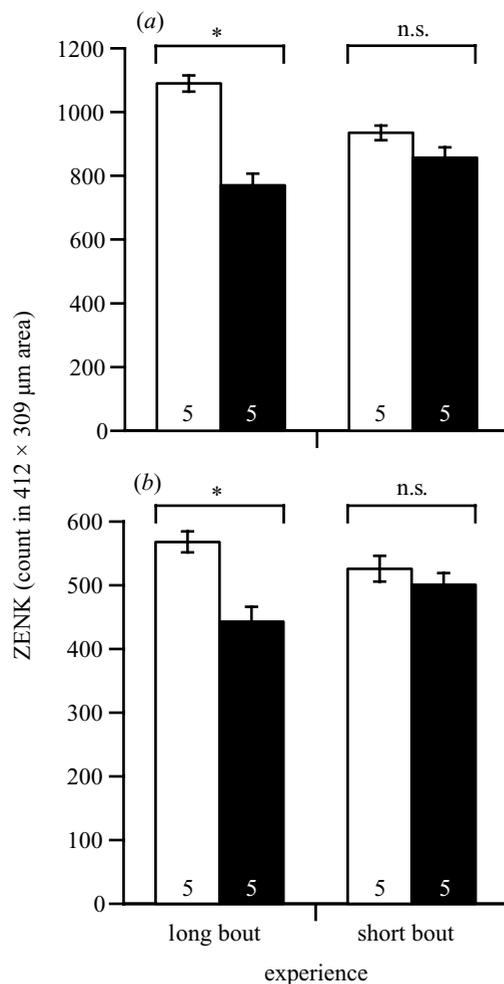


Figure 3. Experience-dependent ZENK response (mean immunoreactive cell count \pm s.e.m.) to song-type stimulus in (a) the cmHV and (b) the NCM of the female European starling. Black bars, short-bout stimulus; white bars, long-bout stimulus. Indicated for each treatment is the number of experimental units, based on a split-unit design. Asterisks indicate significant ($p < 0.05$) differences based on post-hoc linear contrasts (long-bout experience/long-bout stimulus versus long-bout experience/short-bout stimulus: $F_{1,7} = 19.01$, $p = 0.003$; short-bout experience/long-bout stimulus versus short-bout experience/short-bout stimulus: $F_{1,7} = 0.94$, $p > 0.2$).

and area X renders improbable the possibility that the effect was a response to something other than song exposure, such as an elevation in background ZENK expression.

In seasonally breeding animals, the long-term predictable cue of photoperiod (Rowan 1926) partially regulates the timing of seasonal breeding through its ability to stimulate or suppress neuroendocrine secretions necessary for the development and maintenance of the gonads (Ball & Bentley 2000). However, animals fine-tune reproduction to coincide with times when other environmental factors, such as ecological and social conditions, are favourable (Perrins 1970; Bronson 1989; Wilczynski *et al.* 1993). These fluctuating less predictable environmental cues, termed 'essential supplementary information' (Wingfield 1983), are integrated independently of predictive cues (Ball 1993) such that reproduction is

appropriately timed (Wingfield *et al.* 1992). One such supplementary cue in songbird species is conspecific male song, which can stimulate various aspects of reproductive development in females (Kroodsma 1976; Hinde & Steel 1978; Morton *et al.* 1985).

It is well established that exposure to short-day photoperiods sensitizes most bird species to the stimulatory effects of long-day photoperiods (Farner & Mewaldt 1955; Nicholls *et al.* 1988). However, what effect might short days have on sensitivity to supplementary cues, such as song? The session-number effect observed in the present study suggests that, in addition to their photosensitizing effects, short-day photoperiods can also sensitize a female bird to the stimulatory effects of song. Further studies are necessary to determine whether this perceptual response gives rise to a reproductive response and whether short-day photoperiods sensitize females to the stimulatory effects of supplementary cues involving other sensory modalities.

(b) Experience-dependent modulation of the neural response to mate-choice cues

Previous studies have demonstrated neural responses biased toward traits preferred in a mate-choice context (Ryan *et al.* 1990; Gentner *et al.* 2000). Consistent with these studies, we observed higher ZENK expression in the auditory forebrains of females exposed to the long-bout stimulus than in those of females exposed to the short-bout stimulus. Surprisingly, the strength of this response bias was dependent on the females' recent song experience. Whereas females with the long-bout experience showed a robust response bias, those with the short-bout experience did not. These results indicate that the neural response bias toward a preferred male trait can depend on recent adult experience with subtle variation in the social environment. Moreover, because the song sets comprising the experience and stimulus treatments were taken from different males, the songs varied between treatments in their spectro-temporal features while retaining their categorical distinctions as being preferred or not preferred. In light of this treatment design, our data reveal an experience-dependent response plasticity spanning the category into which the stimulus is organized.

In starlings, when a female's prior song experience is uncontrolled, exposure to longer song bouts elicits higher ZENK expression than does exposure to shorter song bouts (Gentner *et al.* 2000). Gentner *et al.* reasoned that this long-bout response bias was a consequence of the difference in motif repetition rates between the long-bout and short-bout songs. Because the long-bout songs have larger motif repertoires, they maximize the total exposure to song while minimizing the rate of motif repetition (Gentner & Hulse 2000). In the absence of any controlled song experience, this motif-habituation hypothesis is sufficient to explain the pattern of ZENK expression elicited by the long-bout and short-bout songs, as well as the results of earlier ZENK habituation studies (Mello *et al.* 1995). Similar repertoire-habituation hypotheses have been suggested in behavioural studies of female preferences in a variety of songbird species (e.g. Searcy 1992), but could habituation to our experience songs explain how birds responded to our stimulus songs? Despite the minimal motif sharing between the experience songs and the

stimulus songs (see § 2b), it is possible that they shared properties at the sub-motif level and that females habituated to areas of convergence in these songs. However, habituation resulting from song similarity would lead to a general reduction in forebrain ZENK expression (Mello *et al.* 1995), most probably in the long-long or short-short treatment group (or both), but could not account for the results of the present study, in which the females' experience modulated the *bias* for novel long bouts over novel short bouts. Such a perceptual sensitivity to song features that are both beyond the spectro-temporal characteristics of any one song or singer and invariant across the song category requires cognitive processes beyond simple habituation.

One interpretation of the data is that the perceptual and cognitive mechanisms underlying experience-dependent mate choice are based on plastic criteria for stimulus relevance. The data appear to support not a passive process, whereby neural habituation drives the behavioural preference, but rather an active process, in which experience with the long-bout songs establishes neural selectivity for acoustic signals not present in the short-bout songs. The net effect is to set a threshold for stimulation relative to the density of preferred characteristics during prior song exposure, thus providing for a neural system that can adjust the criteria for stimulus relevance based on experience. Because mate choice may have profound direct effects on fitness (Kirkpatrick & Ryan 1991), behaviours that derive from such a system would have tremendous adaptive benefits within mate-choice contexts where the goal is to optimize decisions with respect to locally variable distributions of secondary sexual characteristics.

The authors thank S. Burmeister, D. Duffy, E. Fortune, K. Hoke, D. Maney, M. McCarthy, K. Olesen, J. Sartor and A. Waters. Funding was provided by a NICHD Individual NRSA to K.W.S. and a NINDS R01 to G.F.B.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.