# Forebrain circuits underlying the social modulation of vocal communication signals

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Running title: Social context affects EGR-1 expression

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## ABSTRACT

Across vertebrate species, signalers alter the structure of their communication signals based on the social context. For example, male Bengalese finches produce faster and more stereotyped songs when directing song to females [female-directed (FD) song] than when singing in isolation [undirected (UD) song], and such changes have been found to increase the attractiveness of a male's song. Despite the importance of such social influences, little is known about the mechanisms underlying the social modulation of communication signals. To this end, we analyzed differences in immediate early gene (EGR-1) expression when Bengalese finches produced FD or UD song. Relative to silent birds, EGR-1 expression was elevated in birds producing either FD or UD song throughout vocal control circuitry, including the interface nucleus of the nidopallium (NIf), HVC, the robust nucleus of the arcopallium (RA), Area X, and the lateral magnocellular nucleus of the anterior nidopallium (LMAN). Moreover, EGR-1 expression was higher in HVC, RA, Area X, and LMAN in males producing UD song than in males producing FD song, indicating that social context modulated EGR-1 expression in these areas. However, EGR-1 expression was not significantly different between males producing FD or UD song in NIf, the primary vocal motor input into HVC, suggesting that context-dependent changes could arise *de novo* in HVC. The pattern of context-dependent differences in EGR-1 expression in the Bengalese finch was highly similar to that in the zebra finch and suggests that social context affects song structure by modulating activity throughout vocal control nuclei.


Keywords: Bengalese finch, birdsong, HVC, sequencing, social context

## INTRODUCTION

A number of species, including humans and songbirds, critically depend on vocal signals for social communication (Doupe and Kuhl, 1999; Catchpole and Slater, 2008). Furthermore, signalers alter the structure and content of their vocal signals depending on their social audience and context, and this modulation has been hypothesized to increase the salience and interpretability of communication signals (Kuhl, 2010; Seyfarth et al., 2010). For example, humans alter the structure of speech and language depending on the age, familiarity and social status of their audience (Fernald and Kuhl, 1987; Giles et al., 1987; Gudykunst and Kim, 2003), and the composition of vocalizations in rats, bats, and cetaceans changes depending on the presence and familiarity of conspecifics (Wright et al., 2010; Gadziola et a., 2012; Knörnschild et al., 2012; Bohn et al., 2013; King et al., 2013). Despite the prevalence and importance of such social influences, little is known about the mechanisms through which social context modulates communicative behaviors.

Songbirds offer a powerful model system to reveal neural mechanisms underlying the social modulation of communication. Song is controlled by specialized and discrete neural circuits ('song system') that are analogous to brain areas underlying vocal control and plasticity in mammals (Figure 1; Jarvis, 2004; Reiner et al., 2004a; Doupe et al., 2005; Mooney, 2009). Generally speaking, the song system consists of two main pathways: the vocal motor pathway (VMP) and the anterior forebrain pathway (AFP). The VMP encodes the motor commands for song and consists of forebrain areas like the nucleus interface of the nidopallium (NIf), HVC (used as proper name), and the robust nucleus of the arcopallium (RA). Neurons in RA project to hindbrain areas that control vocal musculature and respiration to regulate song production. The AFP is an avian forebrain-basal ganglia circuit homologous to cortical-basal ganglia circuits in mammals that consists of the basal ganglia nucleus Area X, the dorsolateral anterior thalamic nucleus (DLM), and the lateral magnocellular nucleus of the anterior nidopallium (LMAN; Doupe et al., 2005; Woolley and Kao, 2014). Like mammalian cortical-basal ganglia circuits, the

avian forebrain-basal ganglia pathway is important for vocal motor control and plasticity (Brainard and Doupe, 2013; Woolley and Kao, 2014).

As in humans, songbirds acutely alter the structure of their song depending on the social context (reviewed in Sakata and Vehrencamp, 2012; Woolley and Kao, 2014). For example, adult zebra finches produce faster songs that contain syllables with more stereotyped structure when directing songs to females (female-directed or FD songs) than when producing song in isolation (undirected or UD songs; Cooper and Goller, 2006; Kao and Brainard, 2006; Teramitsu and White, 2006; Stepanek and Doupe, 2010). Similarly, the FD songs of male Bengalese finches are faster and more stereotyped in structure than their UD songs (Sakata et al., 2008; Hampton et al., 2009; Sakata and Brainard, 2009; Dunning et al., 2014). In Bengalese finches but not zebra finches, the sequencing of syllables is also more stereotyped during FD song than UD song (Kao and Brainard, 2006; Sakata et al., 2008; Hampton et al., 2009; Sakata and Brainard, 2009; Heinig et al., 2014). Relatively little is known about how the nervous system generates the diversity of context-dependent changes to song, though there have been some insights into the neural mechanisms underlying the social modulation of syllable structure and song tempo in the zebra finch (e.g., Jarvis et al., 1998; Kao and Brainard, 2006; Kao et al., 2005, 2008; Hampton et al., 2009; Leblois et al., 2010; Stepanek and Doupe, 2010; Leblois and Perkel, 2012).

To gain insight into the neural circuits that could mediate social influences on song, we analyzed how social context affected the expression of the immediate early gene EGR-1 in forebrain and basal ganglia circuits. Immediate early genes like EGR-1 are cellular markers of activity used to map functional neural circuits in a range of species, including songbirds (reviewed in Guzowski et al., 2005; Clayton, 2013). Previous studies in the zebra finch have adopted this approach and found dramatic differences in EGR-1 mRNA and protein expression between birds producing FD and UD song (Jarvis et al., 1998; Castelino and Ball, 2005; Hara et al., 2007). Understanding how social context affects EGR-1 expression in the Bengalese finch is

important to assess the generality of findings in the zebra finch. Further, it will be informative to compare and contrast context-dependent changes to EGR-1 expression between Bengalese and zebra finches because of species similarities and differences in the effects of social context on song structure.

## MATERIALS AND METHODS

Animals:

Adult male Bengalese finches (>4 months post-hatch; n=30) were raised in our colony at McGill University or purchased from vendors (Exotic Wings and Things, Ontario, Canada). Birds were housed in all-male group cages before experimentation, and beginning at least two days before experimentation, birds were housed individually in sound-attenuating chambers ('soundboxes'; TRA Acoustics, Cornwall, Ontario, Canada). Birds were housed on a 14L:10D light cycle and provided food and water *ad libitum*. All procedures were approved by the McGill University Animal Care and Use Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Experimental conditions:

We examined how social context affected song organization and immediate early gene expression in the brain using an established experimental design (e.g., Jarvis et al., 1998; Castelino and Ball, 2005; Hara et al., 2007; Matheson and Sakata, 2015). In particular, we analyzed the degree to which song and EGR-1 expression differed depending on whether birds directed songs at females following exposure to females or produced undirected song in isolation. On the morning of the experiment, individual male Bengalese finches were allowed to eat and drink for 5-10 minutes after lights on then either exposed to females and allowed to produce only female-directed (FD) song ('FD birds'), kept in isolation and allowed to produce only undirected (UD) song ('UD birds'), or kept silent in isolation ('silent birds'). FD birds (n=11) were repeatedly (1-5 min intervals) and briefly (<30 sec) exposed to

females housed in separate cages to elicit FD song (e.g., Sakata et al., 2008). Female-directed songs were almost always produced soon after the introduction of the female and are readily distinguishable from UD songs because they are produced after a male approaches or faces the female, accompanied by a courtship dance (e.g., pivoting body from side to side), and associated with the fluffing of the male's plumage (Morris, 1954; Zann, 1996). Songs were considered as FD songs if they were accompanied by at least two of the above behaviors, and FD birds were confirmed to produce FD songs during female presentations. Similar to previous experiments, FD males were prevented from producing UD song between presentations of females by tapping on the soundbox whenever the male attempted to produce UD song (e.g., Jarvis et al., 1998; Hara et al., 2007; Castelino and Ball, 2005). UD birds (n=11) were left alone in their soundbox and allowed to produce spontaneous UD song. Silent birds (n=8) were prevented from singing by keeping the soundbox door ajar and by tapping on the soundbox whenever they attempted to produce song (e.g., Kimpo and Doupe, 1997; Jarvis et al., 1998; Castelino and Ball, 2005; Hara et al., 2007; Matheson and Sakata, 2015).

Song recording and analysis:

Song was recorded on control days preceding the experimental day as well as on the experimental day (see below) using an omnidirectional microphone positioned above the bird's cage (Countryman Associates, Menlo Park, CA). A computerized, song-activated recording system was used to detect and digitize song [Sound Analysis Pro v.2011.104 (http://ofer.sci.ccny.cuny.edu/html/sound_analysis.html); digitized at 44.1 kHz]. Recorded songs were digitally filtered at 0.3-10 kHz for analysis using software written in the Matlab programming language (The MathWorks, Natick, MA, USA).

Bengalese finch song consists of acoustically distinct elements ('syllables') separated by ≥10 ms of silence (Figure 2; Okanoya and Yamaguchi, 1997; Sakata and Brainard, 2006) and is performed in bouts that are preceded by soft, short, and simple vocal elements called 'introductory notes'. We manually labeled individual song syllables

based on visual inspection of spectrograms following amplitude-based syllable segmentation (e.g., Okanoya and Yamaguchi, 1997; Woolley and Rubel, 1997; Sakata and Brainard, 2006; Sakata et al., 2008; Warren et al., 2012). Individuals labeling songs (LEM, HS) were highly experienced with song labeling and blind to experimental condition. Detailed examination of syllable labeling (>6200 syllables) confirmed that labeling was highly reliable and accurate (>99.7% accuracy).

Adult Bengalese finch song consists of syllables arranged in stereotyped sequences as well as sequences in which syllable transitions vary from rendition to rendition ('branch points'; Sakata and Brainard, 2006; Jin, 2009; Warren et al., 2012). Stereotyped and branch point sequences were identified during manual labeling and confirmed using bigram plots (e.g., Okanoya and Yamaguchi, 1997; Kakishita et al., 2009; Fujimoto et al., 2012; Heinig et al., 2014). We analyzed the effects of social context on syllable sequencing at branch points by computing the probability of different syllable transitions (e.g., Sakata et al., 2008). For each branch point, this sequence variability was quantified as the transition entropy:

$$\text{transition entropy} = \Sigma\text{-}p_i * \log_2(p_i)$$

where the sum is over all transitions produced at the branch point, and $p_i$ is the probability of the $i^{th}$ transition across all renditions of the branch point. Branch points with transitions that are more variable (i.e., closer to uniform probability) have higher transition entropy scores. We paid close attention to long-range statistics (i.e., history dependence) in Bengalese finch song (e.g., Fujimoto et al. 2011; Katahira et al. 2011; Warren et al. 2012) and analyzed sequencing at branch points based on these sequence dependencies. For example, if a branch point sequence 'cd' was preceded either by an 'a' or a 'b', we analyzed 'acd' and 'bcd' sequences separately if transition probabilities were significantly different across sequence contexts (Likelihood ratio tests, $\alpha = 0.05$); otherwise, we pooled across sequence contexts for the analysis. Instances in which song was terminated immediately following the branch point were not included in the calculation of

entropy, and sequences in which the dominant transition occurred >95% of the time were not considered branch points. Only branch points that occurred at least 15 times were analyzed (n=104 branch points in 22 males).

The structure of individual song syllables varies across renditions, and this variability is influenced by the social context in which song is produced (e.g., Kao and Brainard, 2006; Sakata et al., 2008; Woolley and Doupe, 2008). We measured the fundamental frequency (FF) of syllables with relatively flat, harmonic structure by calculating the autocorrelation of a segment of the sound waveform (8- or 16-ms window; e.g., syllable 'h' in Figure 2; n=76 syllables in 22 males). The FF was defined as the distance, in Hz, between the zero-offset peak and the highest peak in the autocorrelation function. To improve the resolution of frequency estimates, we performed a parabolic interpolation of the peak of the autocorrelation function (de Cheveigné and Kawahara, 2002). We computed the coefficient of variation (CV: $\sigma/\mu$) of the FF of such syllables for each experimental condition (e.g., Kao et al., 2005; Kao and Brainard, 2006; Sakata et al., 2008; Stepanek and Doupe, 2010). These syllables were found to have a slight but significant change in FF over the harmonic portion of identified syllables (-0.09±0.02% (mean±SEM) change in FF per ms: $p<0.01$), but this should not affect the analysis of social context effects on syllable structure because we measured the same window across control and experimental days.

The social context in which song is produced also influences the speed at which song is delivered (Cooper and Goller, 2006; Kao and Brainard, 2006; Sakata et al., 2008; Stepanek and Doupe, 2010; Dunning et al., 2014). To analyze the effect of social context on song tempo in Bengalese finches, we calculated the duration of frequently produced sequences of syllables (n=40 sequences in 22 males), using the interval between the onset of the first syllable and the onset of the last syllable in the sequence (e.g., Kao and Brainard, 2006; Sakata and Brainard, 2006; Long and Fee, 2008; Sakata et al., 2008). Onsets were based on amplitude threshold crossings and selected as boundaries because the change in amplitude is sharper and less variable for onsets than for offsets, allowing for more accurate estimates of

sequence duration. The speed at which song is produced varies as a function of song length: sequence durations are shorter at the beginning of a song bout than later in the song bout (e.g., Chi and Margoliash, 2001; Glaze and Troyer, 2006; James and Sakata, 2014). Therefore, we only analyzed the first instance of each sequence within each bout.

Tissue collection and immunocytochemistry for EGR-1 expression:

In a subset of birds from which we collected behavioral data, we also analyzed EGR-1 expression in forebrain areas using established immunocytochemical protocols (e.g., Kimpo and Doupe, 1997; Whitney et al., 2000; Castelino and Ball, 2005; Hara et al., 2007). We compared EGR-1 expression across FD, UD and silent birds (n=8/grp; see above) to analyze the neural circuitry underlying context-dependent changes in song structure. There are a number of other genes including *c-fos* and *dusp1* that can be used to map functional circuits (e.g., Kimpo and Doupe, 1997; Horita et al., 2012), but we selected EGR-1 because EGR-1 is expressed throughout song control nuclei, up-regulated during vocal production, and modulated by social context in songbirds like the zebra finch (e.g., Jarvis et al., 1998; Whitney et al., 2000; Hara et al., 2007; Castelino and Ball, 2005). By analyzing EGR-1 expression in FD, UD and silent Bengalese finches, we could assess the effect of social context on EGR-1 expression (i.e., FD vs. UD birds) as well as general effects of song production on EGR-1 expression (FD and UD birds vs. silent birds). Behavioral differences across UD and FD birds in this subset were comparable to the larger dataset (see Results).

Because we were interested in revealing brain areas that were affected specifically by social context and related to context-dependent variation in song structure, we took precautions to minimize differences in the quantity and rate of singing between FD and UD birds, both of which can influence immediate early gene expression. We ensured that the amount of song produced by FD birds (FD song: 116±12.6 (mean±SEM) seconds) and UD birds (UD song: 124±1.4 seconds) was not significantly different (t-test: $t_{14}$=0.43, p=0.67). In addition, because Bengalese finches can produce UD songs in rapid succession and because of the temporal

dynamics of immediate early gene expression (e.g., Mello and Clayton, 1994), we encouraged UD birds to space out their songs by occasionally tapping on the soundbox such that their pattern of individual song production resembled that for FD birds. As such, the average interval between songs was not significantly different between UD and FD birds (t-test: $t_{14}$=1.7, p=0.11).

Lights were turned off 60-90 min after of the start of data collection (see above). Within 5 minutes after lights off, birds were deeply anaesthetized with isoflurane vapor, then transcardially perfused with heparinized saline (100 IU/mL) followed by 150 mL of 4% paraformaldehyde (pH=7.4). Brains were removed, post-fixed for 4 hours at 4°C in 4% paraformaldehyde and then transferred to a 30% sucrose solution overnight at 4°C. Coronal sections were cut at 40 µm using a sliding microtome (Leica Biosystems, Wetzlar, Germany) and stored in 0.025M phosphate-buffered saline (PBS) containing 0.05% sodium azide at 4°C.

To analyze context-dependent changes to immediate early gene expression in the song system (Figure 1), we processed brains for EGR-1 and other neuronal markers. To facilitate the identification of brain areas, we processed brain sections for tyrosine hydroxylase (TH), a rate-limiting enzyme in catecholamine synthesis, as well as NeuN (Neuronal Nuclei; n=18 birds) or DAPI (n=6 birds; Bottjer, 1993; Soha et al., 1996; Appeltants et al., 2000; Reiner et al. 2004b; Matheson and Sakata, 2015). Briefly, we rinsed sections for 10 min in 0.025M PBS (pH=7.4) 3X then blocked the sections for 1 hr in PBS + 5.0% donkey serum + 0.3% Triton-X. Thereafter, we incubated sections for 48 hrs at 4°C with sheep anti-TH (NB300110, Novus Biologicals, Littleton, CO, USA) and rabbit anti-EGR-1 (SC189; Santa Cruz Biotechnology, Santa Cruz, CA, USA), each diluted 1:1000 in PBS + 2.5% donkey serum + 0.3% Triton-X + 0.05% sodium azide. This was followed by 2X 30 min rinses in PBS and a 2 hr incubation in donkey anti-rabbit secondary conjugated to Alexa Fluor 594 (5 µl/ml; Life Technologies) and donkey anti-sheep conjugated to Alexa Fluor 488 (3 µl/ml; Life Technologies) in PBS + 2.5% donkey serum + 0.3% Triton-X. Sections were then rinsed for 10 min 3X in PBS and transferred to

PBS+0.05% sodium azide before mounting. To stain for NeuN, we included mouse anti-NeuN (1:1000 dilution; EMD Millipore, Billerica, MA, USA) in the primary incubation, and donkey anti-mouse secondary antibody conjugated to Alexa Fluor 350 (5µl/ml) in the secondary incubation. Sections were then rinsed 3X 10 min rinses in PBS and transferred to PBS+0.05% sodium azide before mounting on chrom-alum subbed slides. Brain sections stained with NeuN were mounted and coverslipped with Prolong Gold Antifade without DAPI (n=18) whereas sections not stained with NeuN were coverslipped with Prolong Gold Antifade with DAPI (n=6) to label cell nuclei.

Image acquisition and analysis

Across FD, UD, and silent birds, we analyzed the density of EGR-1 expression in song control nuclei. Specifically, we quantified EGR-1 expression in NIf, HVC, RA, the basal ganglia nucleus Area X, and LMAN (Figure 1). We used TH, NeuN, or DAPI staining as well as alternate series of Nissl stained sections to help identify brain areas for quantification. Thereafter, monochrome photomicrographs (594 nm) of EGR-1 expression were taken in the center of each brain area in each hemisphere at 20X using a Zeiss Axio Imager upright microscope and AxioCam MRm Zeiss camera (Carl Zeiss, Jena, Germany; image frame resolution: 1388 x 1040 pixels). Images were imported into Photoshop (Adobe) and cropped down to a specified window for quantification. We quantified EGR-1 expression in a 0.20x0.20 mm window for HVC, RA, and Area X, a 0.15x0.15 mm window for LMAN, and a 0.10x0.10 mm window for NIf. EGR-1 positive neurons were manually counted using FIJI (Image J, NIH). Thereafter, we computed the number of EGR-1 neurons per unit area (i.e., density of EGR-1 expression). We counted EGR-1 expression in 4.12±0.18 (mean±SEM) sections per bird, and values were averaged across sections and hemispheres within an individual for data analysis. Image acquisition and quantification were done blind to experimental condition by one experimenter (LEM), and the reliability of scoring was very high (correlation between repeated measurements of the same images: r=0.98; n=92).

Statistical analysis

Before analysis, all data were checked for normality using the Shapiro-Wilks test ($\alpha = 0.05$). In cases in which normality was violated (EGR-1 data for RA, LMAN, and NIf), data were cube-root transformed to improve normality. For cube-root transformed data, means and standard errors were back-transformed before plotting (Bland and Altman, 1996).

Generally speaking, to assess the effects of social context on vocal motor control, we analyzed how song features differentially changed from the control day to the experimental day in FD and UD birds. Control days were, modally, 1 day before the experimental day (range: 1-10 days; median: 1 day), and the median number of days between the control and experimental days was not significantly different between FD and UD birds (Kruskal-Wallis test: Z=0.358, $p=0.72$). Because of the procedure to analyze EGR-1 expression (see above), we recorded and analyzed only songs produced immediately after lights on for the experimental day. To control for potential circadian variation in song (e.g., Derégnaucourt et al., 2005), we analyzed only songs produced immediately after lights on for the control day as well. We analyzed the same amount of song on the control and experimental days such that sample sizes were not significantly different ($p>0.50$ for all song features).

We analyzed the effect of social context on song by comparing the degree of change for each song feature across control and experimental days for FD and UD birds. As mentioned above, multiple examples of some song features were analyzed for an individual bird (e.g., the FF of multiple syllables, transition entropy of multiple branch points, duration of multiple sequences per bird). To avoid pseudoreplication in the analysis, we computed the weighted average change ('weighted change') from the control to the experimental day across all examples of a feature for a bird; consequently, only one value was assigned to each bird for statistical analysis. To compute the weighted change, we calculated the magnitude of change from the control to the experimental day for each example of a song feature (e.g., percent

change in the duration of each sequence) and weighted the change by the relative occurrence of that example for that bird. For example, if sequence durations decreased by 2% for a sequence that was produced 100 times and by 10% for another sequence that was produced 200 times, the weighted change would be 7.36% for this bird. After computing the weighted change, we used t-tests to analyze whether the weighted change was significantly different than zero for FD and UD birds ($H_0$: mean=0) as well as whether the weighted change was significantly different between FD and UD birds. For features in which only one measure was taken per bird (i.e., number of introductory notes preceding song), we computed the percent change across control and experimental days for each bird and analyzed this change in FD and UD birds using t-tests.

To examine the effects of social context on EGR-1 expression, we used an ANOVA with Condition (silent, UD, and FD) as the independent variable and the density of EGR-1 expressing neurons as the dependent variable. Because of variation in staining across batches, we also included "Batch" as a variable in the analysis. All experimental groups were represented in equal numbers within each batch. Tukey's HSD was used for all post-hoc analyses.

All statistical analyses were performed using JMP 10.0 (SAS Institute, Cary, NC, USA) for Macintosh, and $\alpha = 0.05$ for all tests.

## RESULTS

*Effects of social context on song organization:*

Social context affects song structure in a number of species, including the Bengalese finch (Sakata et al. 2008; Hampton et al. 2009; Sakata and Brainard 2009; Dunning et al., 2014; Heinig et al., 2014). For example, relative to spontaneous, undirected (UD) songs, songs directed at females during courtship interactions (female-directed (FD) songs) consist of syllables that are more stereotyped in structure and

arranged in more stereotyped sequences. Furthermore, FD songs are faster and preceded by more introductory notes.

To ensure that our manipulations of social context affected the organization of Bengalese finch song, we compared songs produced on a control day before the experimental day to songs produced on the experimental day for FD birds (n=11) and UD birds (n=11; Figure 3a). We hypothesized that the songs of FD birds would be significantly different between the control day in which only UD songs were produced and the experimental day in which only FD songs were produced. We also anticipated no significant differences between songs produced on the control and experimental days for UD birds because they produced UD songs on both days. Furthermore, we predicted that the degree to which song changed from the control to experimental day would be significantly larger for FD birds than UD birds.

Social context affects the stereotypy of syllable structure. In particular, the fundamental frequency (FF) of syllables with relatively flat, harmonic structure is less variable when birds produce FD song than UD song (Sakata et al. 2008; Hampton et al. 2009; Sakata and Brainard 2009). Similar to previous papers, we found that the variability (coefficient of variation, or CV) of FF was lower when FD birds produced FD song on the experimental day than UD song on the control day. In the example provided in Figure 3b, the CV of FF of a syllable from an individual FD bird was lower on the experimental day (CV=0.0139) than on the control day (CV=0.0206). Such decreases in the CV of FF from the control to experimental day were consistent across FD birds; consequently, the weighted percent change (per bird) from the control to experimental day was significantly less than zero for FD birds (-18.9±3.2% change (mean±SEM); $t_{10}$=6.0, $p<0.01$; Figure 3c). This was comparable to the magnitude of context-dependent changes observed in previous studies (e.g., Sakata et al., 2008; Hampton et al., 2009). In contrast, there was no significant change in the CV of FF across control and experimental days for UD birds (-5.4±3.1% change (mean±SEM); $t_{10}$=1.7, $p=0.12$; Figure 3c). The weighted percent

change was also significantly more negative for FD birds than for UD birds ($t_{20}$=3.0, $p<0.01$).

Just as syllable structure is less variable during FD song than UD song, the sequencing of syllables at branch points is less variable when Bengalese finches produce FD song than UD song (Sakata et al., 2008; Hampton et al., 2009; Sakata and Brainard, 2009; Heinig et al., 2014). Consistent with previous data, we found that syllable sequencing was less variable when FD birds produced FD song on the experimental day than UD song on the control day. An example of such an effect is depicted in Figure 3d. In this example, the FD bird produces the syllables 'g' or 'n' after the branch point 'bcdef, and the transitions probabilities to these syllables varied across control and experimental days. Relative to transition probabilities on the control day, the bird biased his transitions to 'g' on the experimental day. As such, the transition entropy, a measure of sequence variability, for this branch point was lower on the experimental day (0.406) than on the control day (0.998). Such changes in transition entropy from control to experimental days were common for FD birds, and the weighted change (per bird) in entropy tended to be less than zero for FD birds (-0.11±0.05 (mean±SEM); $t_{10}$=2.13, p=0.06). In contrast to FD birds, the weighted change in transition entropy was not significantly different than zero for UD birds (0.01±0.03 (mean±SEM); $t_{10}$=0.25, p=0.80). Furthermore, the weighted change in entropy tended to be more negative for FD birds than for UD birds (Figure 3e; $t_{20}$=1.96, p=0.06).

Birds produce faster songs when directing songs to females than when singing UD song in isolation (Sakata et al., 2008; Hampton et al., 2009; Sakata and Brainard, 2009; Dunning et al., 2014). Similarly, we found that FD birds but not UD birds produced faster songs on the experimental day than on the control day. For example, the duration with which an FD bird produced the sequence 'abcde' was 2.3% shorter on the experimental day (mean±SEM: 459.1±6.8 ms) than on the control day (469.9±8.2 ms; Figure 3f). Overall, the weighted percent change (per bird) in sequence duration from the control to experimental day was significantly

less than zero for FD birds (-2.5±0.9% (mean±SEM); $t_{10}$=2.77, p=0.02), and the magnitude of this change was comparable to previous studies (Sakata et al., 2008; Hampton et al., 2009; Sakata and Brainard, 2009). However, the weighted percent change was not significantly different than zero for UD birds (-0.5+0.3% (mean±SEM); $t_{10}$=1.86, p=0.09). Additionally, the weighted change was significantly more negative for FD birds than for UD birds ($t_{20}$=2.17, p=0.04; Figure 3g). The percent change in sequence duration was particularly negative for one individual FD bird (Figure 3g), but results were identical even after the removal of this individual (p<0.05).

FD songs are preceded by more introductory notes than UD songs (e.g., Sakata et al. 2008). In contrast to previous results, neither FD nor UD birds showed significant changes in the number of introductory notes from the control to the experimental day (p>0.25 for both), and the magnitude of change across days was not significantly different between FD and UD birds ($t_{20}$=1.3, p=0.21).

Taken together, these analyses indicate that our manipulations of social context altered the sequencing, structure, and timing of song syllables in adult Bengalese finches in a manner consistent with previous studies. Moreover, these analyses support the use of our experimental paradigm to analyze the effects of social context on EGR-1 expression.

*Effects of social context on EGR-1 expression in the song system:*

The temporal and spectral properties of birdsong are controlled by discrete and specialized circuits ('song system'; Figure 1), and previous studies in the zebra finch demonstrate that social context affects the expression of EGR-1 in a variety of nuclei within these circuits (e.g., Jarvis et al., 1998; Castelino and Ball, 2005; Hara et al., 2007). As such, we analyzed context-dependent differences in EGR-1 expression in the vocal motor pathway (VMP), which includes NIf (nucleus interface of the nidopallium), HVC (used as proper name), and RA (robust nucleus of the

arcopallium), and in the anterior forebrain pathway (AFP), which includes Area X and LMAN (lateral magnocellular nucleus of the anterior nidopallium).

Neurons in NIf generate premotor commands for song and influence the temporal structure of song (McCasland, 1987; Hosino and Okanoya, 2000; Naie and Hahnloser, 2011; Lewandowski et al., 2013). However, little is known about immediate early gene expression in NIf during song production (but see Feenders et al., 2008). We found that singing in both social contexts increased EGR-1 expression in NIf ($F_{2,15}$=9.8, $p<0.01$; Figure 4a): EGR-1 expression was significantly higher in UD and FD birds than in silent birds ($p<0.02$ for both). However, the social context in which song was produced did not affect EGR-1 expression in NIf since there was no significant difference between UD and FD birds ($p=0.51$).

Neurons in HVC are critical for song production and control, modulate the structure, sequencing and timing of song syllables, and receive direct input from NIf (Fee and Scharff, 2010; Brainard and Doupe, 2013; Lewandowski et al., 2013). As in NIf, EGR-1 expression in HVC was significantly different among silent, FD, and UD birds ($F_{2,15}$=28.8, $p<0.01$; Figure 4b,c). EGR-1 expression was significantly higher in UD and FD birds than in silent birds ($p<0.02$ for both). Furthermore, EGR-1 expression in HVC was significantly higher in UD birds than in FD birds ($p<0.01$). Thus, context-dependent differences in the song system first emerge in the HVC of Bengalese finches.

Neurons in HVC project to RA in the VMP as well as to the basal ganglia nucleus Area X in the AFP, and we found that social context and singing affected EGR-1 expression in both downstream areas. In RA, EGR-1 expression was significantly different among silent, FD, and UD birds ($F_{2,15}$=18.4, $p<0.01$; Figure 4d). In particular, EGR-1 expression in RA was significantly higher in UD and FD birds than in silent birds and, moreover, higher in UD birds than in FD birds ($p<0.05$ for all contrasts).

In Area X, EGR-1 expression was significantly different among silent, FD, and UD birds ($F_{2,15}$=49.1, p<0.01; Figure 5a,b), with significantly higher EGR-1 expression in both UD and FD birds than in silent birds (p<0.01 for both). Moreover, EGR-1 expression in Area X was higher in UD birds than in FD birds (p<0.01).

Area X projects indirectly to LMAN, an area important for the social modulation of song (Kao et al., 2005; Kao and Brainard, 2006; Hampton et al., 2009; Stepanek and Doupe, 2010). EGR-1 expression in LMAN was significantly different among silent, FD, and UD birds ($F_{2,15}$=17.8, p<0.01; Figure 5c). EGR-1 expression was significantly higher in UD birds than in silent birds (p<0.01) but not significantly different between FD and silent birds (p=0.14). Additionally, EGR-1 expression was significantly higher in UD birds than in FD birds (p<0.01).

*Relationship between variation in EGR-1 expression in the song system and variation in song features:*

The previous analyses indicate that, broadly speaking, context-dependent variation in song is correlated with context-dependent variation in EGR-1 expression in HVC, RA, Area X and LMAN. However, the degree to which variation in song features is related to variation in EGR-1 expression, independent of social context, remains unknown. To this end, we computed the residuals from the preceding ANOVA models of EGR-1 expression (i.e., Condition and Batch as independent variables), then analyzed the degree to which individual variation in the CV of FF, transition entropy, and song tempo explained this residual variation in EGR-1 expression.

In order to conduct such analyses, we first computed the weighted averages of song features for each bird. We summarized the sequence variability for an individual bird by computing the average transition entropies of his branch points, weighted by the prevalence of each branch point. With regard to the variability of FF, we computed the average CV across all syllables measured within a bird, weighted by the prevalence of each syllable. For song tempo, we computed the average number of syllables per second for each bird. Sequence durations, which were examined in

the analysis of social context effects, were not used here because the number of syllables within such stereotyped sequences varied across birds, and this variation does not reflect variation in song tempo (only variation in the length of a stereotyped sequence). Because previous studies have found that the amount of song is positively related to EGR-1 mRNA expression in song control nuclei (e.g., Jarvis et al., 1998; Hara et al., 2007), we also included the total amount of song produced by each bird (total song duration) as an independent variable in the model. All independent variables were normally distributed and not significantly correlated with each other (i.e., no multicollinearity).

For all brain areas, there was no significant relationship between any song feature and residual EGR-1 expression ($p>0.05$ for all; Figure 6). This suggests that after adjusting for context-dependent differences in the density of EGR-1-expressing neurons, little residual variation in EGR-1 expression was explained by variation in song.

## DISCUSSION

Social context acutely affects the organization of vocal signals in a variety of species, including humans and songbirds, and such changes influence the salience, interpretability, and attractiveness of signals (Catchpole and Slater, 2008; Woolley and Doupe, 2008; Seyfarth et al., 2010; Kuhl, 2010; Sakata and Vehrencamp, 2012; Dunning et al., 2014). For example, male Bengalese and zebra finches produce more stereotyped songs when courting females than when producing songs in isolation (Cooper and Goller, 2006; Kao and Brainard, 2006; Sakata et al. 2008; Hampton et al. 2009; Sakata and Brainard 2009), and female finches prefer the more stereotyped courtship songs (Woolley and Doupe, 2008; Dunning et al., 2014). Despite their importance, little is known about the neural mechanisms underlying social influences on behavior.

To gain insight into the neural circuits that could contribute to social context-dependent changes to song, we analyzed differences in immediate early gene (EGR-1) expression among Bengalese finches that produced female-directed (FD) song during courtship interactions with females, produced undirected (UD) songs in isolation, or remained silent. We found that singing and social context affected the number of neurons expressing EGR-1 protein throughout forebrain and basal ganglia nuclei underlying song control ('song system'). The song system is comprised of two main pathways: the vocal motor pathway (VMP), which includes NIf, HVC, and RA, and the anterior forebrain pathway (AFP), which includes Area X and LMAN (Figure 1). In all song nuclei examined, the production of FD and UD song led to increases in EGR-1 protein expression, supporting their involvement in song control and production. Moreover, social context modulated EGR-1 protein expression in a number of brain areas in the song system: EGR-1 expression was significantly higher in HVC, RA, Area X, and LMAN in Bengalese finches producing UD song than in Bengalese finches producing FD song. Context-dependent differences were not observed in NIf, the primary nucleus in the VMP that sends motor commands to HVC. This suggests that context-dependent variation in EGR-1 expression in the song system first emerges in HVC and that changes to HVC, RA, Area X, and LMAN could contribute to context-dependent changes in syllable structure, timing, and sequencing.

Neurons in HVC, RA, Area X, and LMAN regulate various aspects of song control and have been implicated in the social modulation of song (Sakata and Vehrencamp, 2012; Brainard and Doupe, 2013; Bertram et al., 2014; Woolley and Kao, 2014). For example, neurophysiological activity in Area X, LMAN, and RA is significantly different when male songbirds perform UD and FD song (Hessler and Doupe, 1999; Kao et al., 2005; Kao and Brainard, 2006; Kao et al., 2008; Sober et al., 2008; Woolley et al., 2014), and manipulations of Area X, LMAN, and RA activity significantly affect the control and social modulation of syllable structure (Vu et al., 1994; Ashmore et al., 2005; Ölveczky et al., 2005; Kao and Brainard, 2006; Hampton et al., 2009; Leblois et al., 2010; Stepanek and Doupe, 2010; Warren et al., 2011; Charlesworth et

al., 2012). Neural activity in HVC encodes information about syllable sequencing and timing, and perturbations of HVC activity acutely affect syllable sequencing and timing (Vu et al., 1994; Yu and Margoliash, 1997; Schmidt, 2003; Ashmore et al., 2005; Thompson and Johnson, 2007; Long and Fee, 2008; Sakata and Brainard, 2008; Wang et al., 2008; reviewed in Jin, 2009; Aronov et al., 2011; Fujimoto et al., 2011; Rajan and Doupe, 2013; Basista et al., 2014).

We propose that context-dependent changes in Area X, LMAN, and RA differentially contribute to context-dependent changes to syllable structure, whereas context-dependent changes in HVC differentially contribute to context-dependent changes to syllable sequencing. Support for this notion comes from studies documenting that manipulations of activity in Area X and LMAN affect the social modulation of spectral but not temporal features of song. For example, LMAN lesions eliminate the social modulation of fundamental frequency but do not affect the modulation of syllable sequencing in Bengalese finches (Hampton et al., 2009). Similarly, inactivation and lesions of LMAN as well as antagonism of D1 receptors in Area X eliminate the social modulation of syllable structure but do not affect context-dependent changes to the number of introductory notes preceding song or the number of motifs per bout (Kao and Brainard, 2006; Leblois et al., 2010; Stepanek and Doupe, 2010; Leblois and Perkel, 2012). Consistent with these data, EGR-1 protein and mRNA expression are affected by social context in Area X, LMAN, and RA in the same manner across Bengalese and zebra finches, two species in which social context affects the variability of syllable structure (Jarvis et al., 1998; Castelino and Ball, 2005; Kao and Brainard, 2006; Teramitsu and White, 2006; Hara et al., 2007; Sakata et al., 2008).

Because of the conserved role of HVC activity in the control of syllable sequencing, we propose that context-dependent changes to syllable sequencing are mediated by context-dependent changes to HVC activity. For example, neural activity in HVC encodes information about syllable sequencing in Bengalese and zebra finches (Yu and Margoliash, 1997; Schmidt, 2003; reviewed in Jin, 2009; Fujimoto et al., 2011),

and manipulations of HVC activity acutely affect syllable sequencing (Vu et al., 1994; Ashmore et al., 2005; Thompson and Johnson, 2007; Long and Fee, 2008; Wang et al., 2008; Aronov et al., 2011; Basista et al., 2014). Species differences in the effects of social context on syllable sequencing and EGR-1 expression in HVC similarly support this notion. In particular, social context affects the variability of syllable sequencing in Bengalese finches but not zebra finches (Kao and Brainard, 2006; Sakata et al., 2008; Hampton et al., 2009; Sakata and Brainard, 2009) and has greater effects on HVC EGR-1 expression in Bengalese finches than in zebra finches (Jarvis et al., 1998; Castelino and Ball, 2005; Hara et al., 2007). However, it remains possible that neurons in the AFP could contribute to context-dependent changes to syllable sequencing because some manipulations of Area X and LMAN activity have been found to affect syllable sequencing (e.g., Kobayashi et al., 2000; Hamaguchi and Mooney, 2012; Kubikova et al., 2014).

The degree to which nuclei in the VMP and AFP differentially contribute to the social modulation of song tempo remains unknown, but there is evidence that activity in HVC, RA, Area X, and LMAN can influence the speed of song. For example, manipulations of HVC activity and temperature significantly affect the timing of syllables (e.g., Vu et al., 1994; Long and Fee, 2008; Aronov et al., 2011). Of particular interest here are the findings that HVC temperature is elevated during the production of the faster FD song and that experimental increases in HVC temperature similarly increase song tempo (Long and Fee, 2008; Aronov et al., 2011; Andalman et al., 2011; Aronov and Fee, 2012). Additionally, LMAN lesions have been found to affect song tempo and the magnitude of context-dependent changes to sequence durations (e.g., Williams and Mehta, 1999; Brainard and Doupe, 2001; Kao and Brainard, 2006; Thompson et al., 2011). However, a number of studies have found that manipulations of Area X and LMAN activity do not affect the social modulation of song tempo (e.g., Hampton et al., 2009; Stepanek and Doupe, 2010; Leblois and Perkel, 2012). Of particular relevance here is the finding that lesions of LMAN, the primary projection from the AFP to the VMP, do not affect the magnitude of context-dependent changes to song tempo in Bengalese finches

(Hampton et al., 2009). Therefore, we propose that context-dependent changes in song tempo could be differentially influenced by neurons in the VMP in Bengalese finches.

Precisely how differences in immediate early gene expression relate to differences in song structure is unclear but we propose that context-dependent changes to EGR-1 expression reflect changes to neurophysiological activity that regulate song on a moment-by-moment basis. While the relationship between elevated EGR-1 expression and increased song variability is merely correlational in our study, the expression of immediate early genes like EGR-1 and FOS in song control nuclei are related more to vocal motor production and less to the sensory processing of song (e.g., Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997; Feenders et al., 2008). Consequently, we hypothesize that context-dependent differences in EGR-1 expression in song nuclei reflect differences in vocal motor commands. Elevated levels of EGR-1 protein are correlated with increased and more variable neural activity in a number of brain areas, including Area X, LMAN, and RA (e.g., Hessler and Doupe, 1999; Kao et al., 2005, 2008; Sober et al., 2008; Woolley et al., 2014). Further, greater variability of neural activity in Area X, LMAN, and RA correlate with greater variability in syllable structure (Kao et al., 2005; Ölveczky et al., 2005, 2011; Woolley et al., 2014). Consequently, our data suggest that the firing patterns of neurons in Area X, LMAN, and RA could be more variable when Bengalese finches produce the more variable UD song than the more stereotyped FD song (e.g., Sober et al., 2008). Furthermore, our data also suggest the possibility that neurophysiological activity in HVC will be greater and more variable during the production of UD song than FD song in Bengalese finches, and, given the key role of HVC to the control of syllable sequencing, this difference in HVC activity could underlie context-dependent differences in the variability of syllable sequencing.

Taken together, our data support the notion that social context-dependent changes to Bengalese finch song are mediated by context-dependent changes to activity in the song system. Our data underscore that context-dependent changes in the song

system first emerge at the level of HVC in Bengalese finches. The degree to which context-dependent changes in HVC drive context-dependent changes in EGR-1 expression in downstream areas in the Bengalese finch is unknown, but results in the zebra finch suggest that context-dependent changes to EGR-1 expression in Area X, LMAN, and RA can arise without context-dependent changes to HVC (Jarvis et al., 1998; Castelino and Ball, 2005; Hara et al., 2007). These data further highlight EGR-1 as an immediate early gene that is sensitive to the social context in which song is produced and suggest the possibility that the expression of other immediate early genes like *c-fos* or *dusp1* could be similarly modulated by social context. Furthermore, because of species differences in the effects of social context on song structure and EGR-1 expression in the brain, these data suggest that HVC could be an important brain area for understanding species diversity in song control and social behavior.

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## FIGURE LEGENDS

**Figure 1.** The 'song system' of songbirds. The song system consists of two main pathways, the vocal motor pathway (VMP; grey) and the anterior forebrain pathway (AFP; white). The VMP includes the forebrain nuclei NIf (nucleus interface of the nidopallium), HVC (used as proper name), and RA (robust nucleus of the arcopallium). The AFP consists of the basal ganglia nucleus Area X, DLM (dorsal lateral nucleus of the medial thalamus) and LMAN (lateral magnocellular nucleus of the anterior nidopallium).

**Figure 2.** Organization of Bengalese finch song. Plotted is a spectrogram (frequency on the y-axis, time on the x-axis, intensity as lightness) of a single rendition of Bengalese finch song. Above the spectrogram are letters used to label individual syllables in Bengalese finch song for offline analysis. Syllables are arranged in stereotyped sequences (e.g., 'bcde' and 'nof') as well as variable sequences called 'branch points'. An example of a branch point is 'bcdef', which can be followed by 'g' or 'n'. The syllable 'h' is an example of a syllable with relatively flat, harmonic structure that was measured in the analysis of fundamental frequency. Scale bar= 200 ms.

**Figure 3.** Effects of social context on syllable structure, sequencing, and timing. (a) Experimental design. Songs were collected and analyzed on control and experimental days. On control days, all Bengalese finches produced only undirected (UD) songs. On the experimental day, FD birds (n=11) produced only female-directed (FD) song whereas UD birds (n=11) produced only UD song. We analyzed how song changed from the control to the experimental day for FD and UD birds. (b) An example of the effect of social context on the variability of fundamental frequency (FF) for an individual FD bird. We measured the variability (coefficient of variation (CV)) with which birds produced syllables with relatively flat, harmonic structure on control and experimental (expt) days. In this example, the CV of the FF of a syllable was 0.0206 on the control day (n=32 renditions) and 0.0139 on the

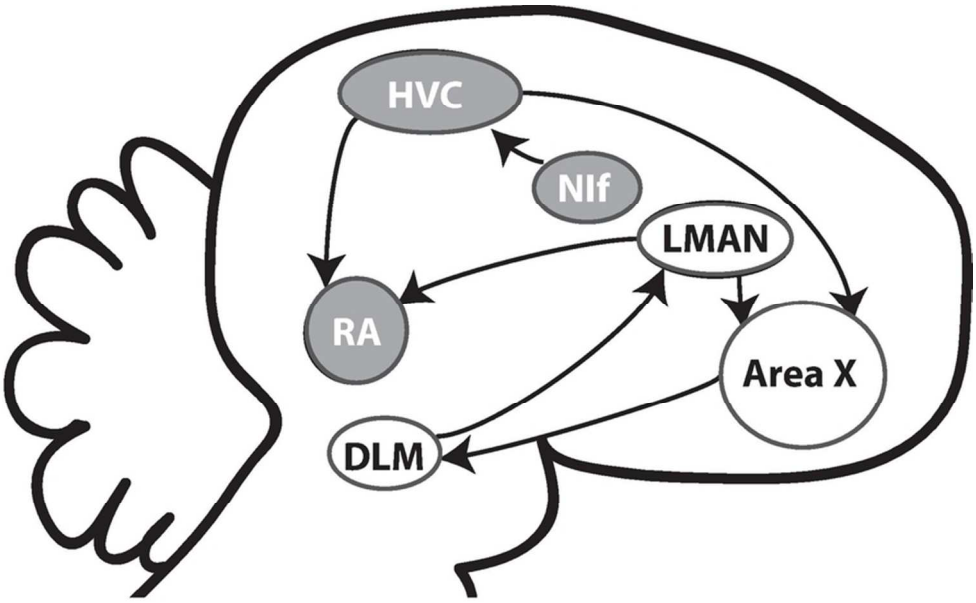
experimental day (n=30 renditions). (c) The weighted percent change in the CV of FF was significantly less than zero for FD birds (top; -18.9±3.2% (mean±SEM)) but not for UD birds (bottom; -5.4±3.1% (mean±SEM)). Furthermore, the weighted change was significantly more negative for FD birds than for UD birds. (d) An example of the effect of social context on syllable sequencing for an individual FD bird. Depicted are transition probabilities following the branch point 'bcdef' (Figure 2) on the control and experimental ('expt') days. On the control day in which he produced only UD song, the bird produced the 'g' and 'n' transitions at roughly equal probabilities, whereas on the experimental ('expt') day in which he produced only FD song, he predominantly produced the 'g' transition following 'bcdef'. Consequently, the transition entropy, which captures the variability of syllable sequencing, is higher on the control day than on the experimental day for this bird. (e) The weighted change in transition entropy (from control to expt day) was less than zero for FD birds (top; -0.11±0.05 (mean±SEM); p=0.06) but not for UD birds (bottom; 0.01±0.03 (mean±SEM); p=0.80). Furthermore, the weighted change was more negative for FD birds than for UD birds (p=0.06). (f) An example of the effect of social context on song tempo for an individual FD bird. In this example, we measured the duration of the sequence 'abcde' (from the onset of 'a' to the onset of 'e'). Plotted below the spectrogram are histograms of the durations of 'abcde' on the control (white bars) and experimental ('expt'; black bars) days. Scale bar= 100 ms. (g) The weighted percent change in sequence durations (from the control to expt day) was significantly less than zero for FD birds (top; -2.5±0.9% (mean±SEM); p=0.02) but not UD birds (bottom; -0.5+0.3% (mean±SEM); p=0.09) and significantly more negative for FD birds than UD birds. All contrasts remained significant even after the removal of a single FD bird. For (c), (e), and (g), data are plotted with some random vertical jitter to allow for better visualization of data points.

**Figure 4.** Effect of social context on EGR-1 expression in the vocal motor pathway. (a) The density of EGR-1 expressing (EGR-1-ir) neurons in NIf was significantly higher in birds producing UD and FD song than in silent birds. However, EGR-1

expression was not significantly different between UD and FD birds. (b) Examples of EGR-1 expression (white) in the HVC of silent, FD and UD birds. Scale bar = 50 μm. (c) The density of EGR-1 expressing neurons in HVC was significantly higher in birds producing UD and FD song than in silent birds. Furthermore, EGR-1 expression was also significantly higher in UD birds than in FD birds. (d) The density of EGR-1 expressing neurons in RA was significantly higher in UD and FD birds than in silent birds and higher in UD birds than in FD birds. For (a), (c), and (d), different letters indicate groups that are significantly different from each other (Tukey's HSD; $p<0.05$).

**Figure 5.** Effect of social context on EGR-1 expression in the anterior forebrain pathway. (a) Examples of EGR-1 expression (white) in Area X of silent, FD and UD birds. Scale bar = 50 μm. (b) The density of EGR-1 expressing (EGR-1-ir) neurons in Area X was significantly higher in birds producing UD and FD song than in silent birds. Furthermore, EGR-1 expression was significantly higher in UD birds than in FD birds. (c) The density of EGR-1 expressing neurons in LMAN was significantly higher in birds producing UD song than in silent birds and marginally higher in birds producing FD song than in silent birds. Furthermore, EGR-1 expression was significantly higher in UD birds than in FD birds. For (b) and (c), different letters indicate groups that are significantly different from each other (Tukey's HSD; $p<0.05$).

**Figure 6.** Relationship between song features and EGR-1 expression in song control nuclei, independent of social context. We computed the residuals from the previous analyses of context-dependent differences in EGR-1 expression in NIf, HVC, RA, Area X, and LMAN. Thereafter, we analyzed the degree to which individual variation in mean CV of FF, transition entropy, number of syllables per second (i.e., speed of song), and total song duration covaried with individual variation in residual EGR-1 expression (multiple regression). For all brain areas examined, there was no significant relationship between any feature of song and residual variation in EGR-1 expression. Open squares = FD birds; filled circles = UD birds.




# Figure 1

The 'song system' of songbirds. The song system consists of two main pathways, the vocal motor pathway (VMP; grey) and the anterior forebrain pathway (AFP; white). The VMP includes the forebrain nuclei NIf (nucleus interface of the nidopallium), HVC (used as proper name), and RA (robust nucleus of the arcopallium). The AFP consists of the basal ganglia nucleus Area X, DLM (dorsal lateral nucleus of the medial thalamus) and LMAN (lateral magnocellular nucleus of the anterior nidopallium).
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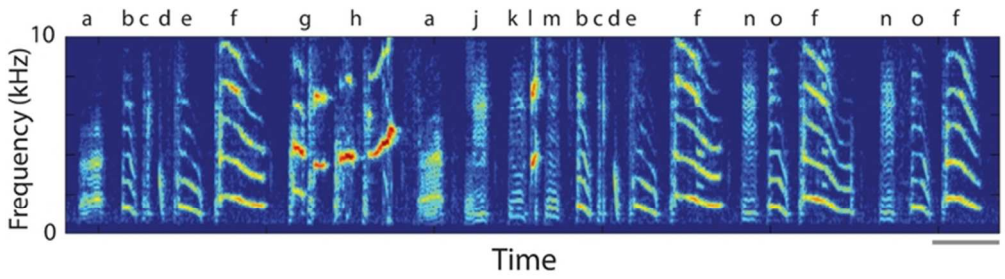


# Figure 2

Organization of Bengalese finch song. Plotted is a spectrogram (frequency on the y-axis, time on the x-axis, intensity as lightness) of a single rendition of Bengalese finch song. Above the spectrogram are letters used to label individual syllables in Bengalese finch song for offline analysis. Syllables are arranged in stereotyped sequences (e.g., 'bcde' and 'nof') as well as variable sequences called 'branch points'. An example of a branch point is 'bcdef', which can be followed by 'g' or 'n'. The syllable 'h' is an example of a syllable with relatively flat, harmonic structure that was measured in the analysis of fundamental frequency. Scale bar= 200 ms.
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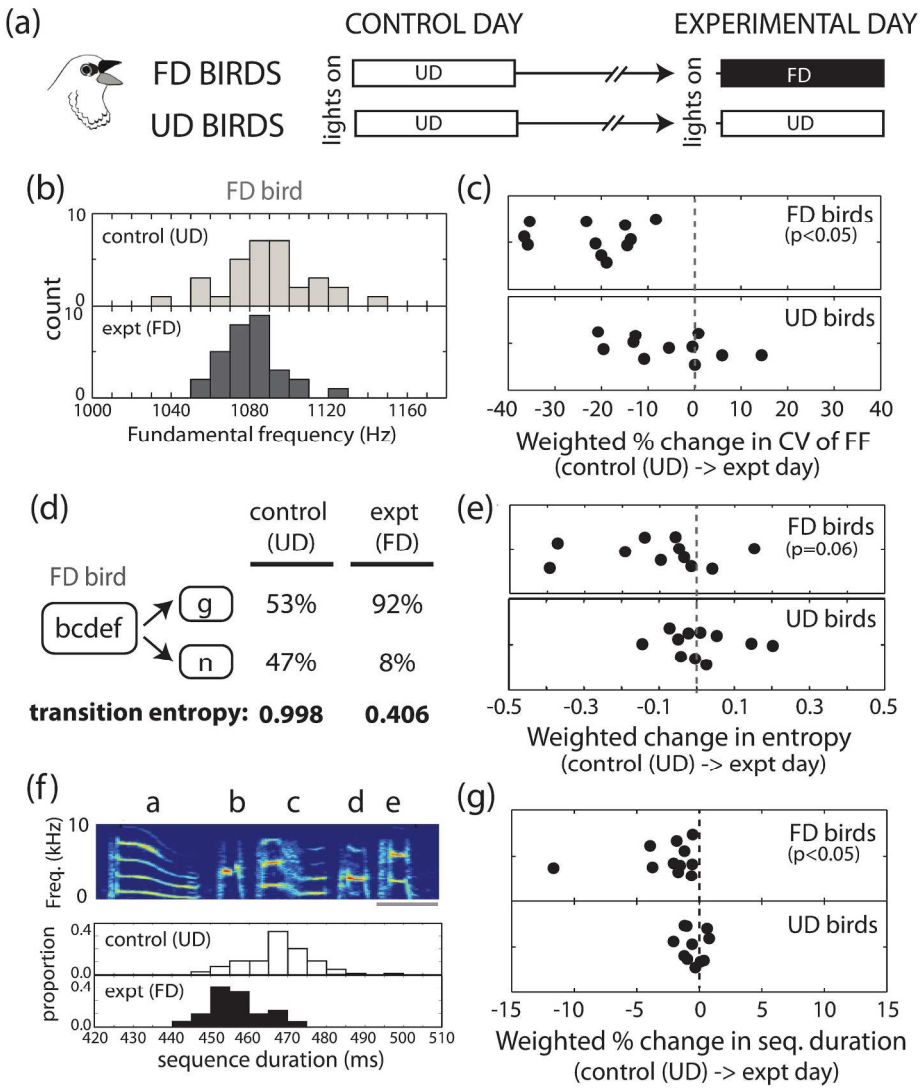


# Figure 3

Effects of social context on syllable structure, sequencing, and timing. (a) Experimental design. Songs were collected and analyzed on control and experimental days. On control days, all Bengalese finches produced only undirected (UD) songs. On the experimental day, FD birds (n=11) produced only female-directed (FD) song whereas UD birds (n=11) produced only UD song. We analyzed how song changed from the control to the experimental day for FD and UD birds. (b) An example of the effect of social context on the variability of fundamental frequency (FF) for an individual FD bird. We measured the variability (coefficient of variation (CV)) with which birds produced syllables with relatively flat, harmonic structure on control and experimental (expt) days. In this example, the CV of the FF of a syllable was 0.0206 on the control day (n=32 renditions) and 0.0139 on the experimental day (n=30 renditions). (c) The weighted percent change in the CV of FF was significantly less than zero for FD birds (top; -18.9+3.2% (mean+SEM)) but not for UD birds (bottom; -5.4+3.1% (mean+SEM)). Furthermore, the weighted change was significantly more negative for FD birds than for UD birds. (d) An example of the effect of social context on syllable sequencing for an individual FD bird. Depicted are transition probabilities following the branch point 'bcdef'

(Figure 2) on the control and experimental ('expt') days. On the control day in which he produced only UD song, the bird produced the 'g' and 'n' transitions at roughly equal probabilities, whereas on the experimental ('expt') day in which he produced only FD song, he predominantly produced the 'g' transition following 'bcdef'. Consequently, the transition entropy, which captures the variability of syllable sequencing, is higher on the control day than on the experimental day for this bird. (e) The weighted change in transition entropy (from control to expt day) was less than zero for FD birds (top; -0.11+0.05 (mean+SEM); p=0.06) but not for UD birds (bottom; 0.01+0.03 (mean+SEM); p=0.80). Furthermore, the weighted change was more negative for FD birds than for UD birds (p=0.06). (f) An example of the effect of social context on song tempo for an individual FD bird. In this example, we measured the duration of the sequence 'abcde' (from the onset of 'a' to the onset of 'e'). Plotted below the spectrogram are histograms of the durations of 'abcde' on the control (white bars) and experimental ('expt'; black bars) days. Scale bar= 100 ms. (g) The weighted percent change in sequence durations (from the control to expt day) was significantly less than zero for FD birds (top; -2.5+0.9% (mean+SEM); p=0.02) but not UD birds (bottom; -0.5+0.3% (mean+SEM); p=0.09) and significantly more negative for FD birds than UD birds. All contrasts remained significant even after the removal of a single FD bird. For (c), (e), and (g), data are plotted with some random vertical jitter to allow for better visualization of data points.
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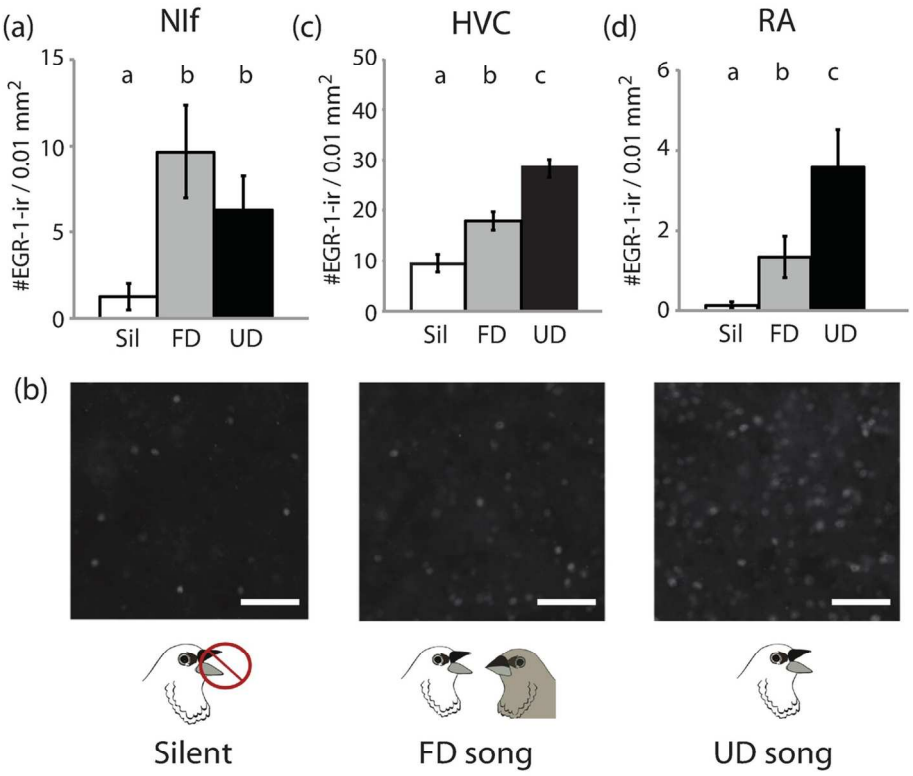


# Figure 4

. Effect of social context on EGR-1 expression in the vocal motor pathway. (a) The density of EGR-1 expressing (EGR-1-ir) neurons in NIf was significantly higher in birds producing UD and FD song than in silent birds. However, EGR-1 expression was not significantly different between UD and FD birds. (b) Examples of EGR-1 expression (white) in the HVC of silent, FD and UD birds. Scale bar = 50 μm. (c) The density of EGR-1 expressing neurons in HVC was significantly higher in birds producing UD and FD song than in silent birds. Furthermore, EGR-1 expression was also significantly higher in UD birds than in FD birds. (d) The density of EGR-1 expressing neurons in RA was significantly higher in UD and FD birds than in silent birds and higher in UD birds than in FD birds. For (a), (c), and (d), different letters indicate groups that are significantly different from each other (Tukey's HSD; $p<0.05$).
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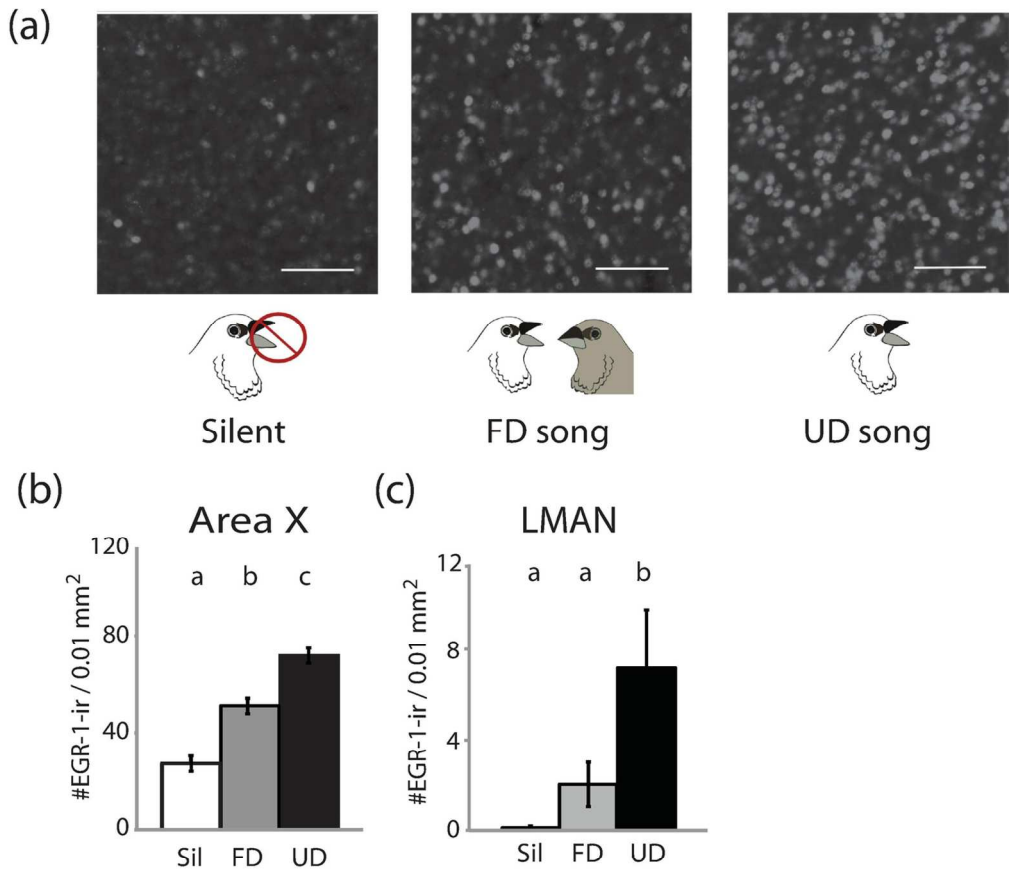


# Figure 5

Effect of social context on EGR-1 expression in the anterior forebrain pathway. (a) Examples of EGR-1 expression (white) in Area X of silent, FD and UD birds. Scale bar = 50 µm. (b) The density of EGR-1 expressing (EGR-1-ir) neurons in Area X was significantly higher in birds producing UD and FD song than in silent birds. Furthermore, EGR-1 expression was significantly higher in UD birds than in FD birds. (c) The density of EGR-1 expressing neurons in LMAN was significantly higher in birds producing UD song than in silent birds and marginally higher in birds producing FD song than in silent birds. Furthermore, EGR-1 expression was significantly higher in UD birds than in FD birds. For (b) and (c), different letters indicate groups that are significantly different from each other (Tukey's HSD; $p<0.05$).
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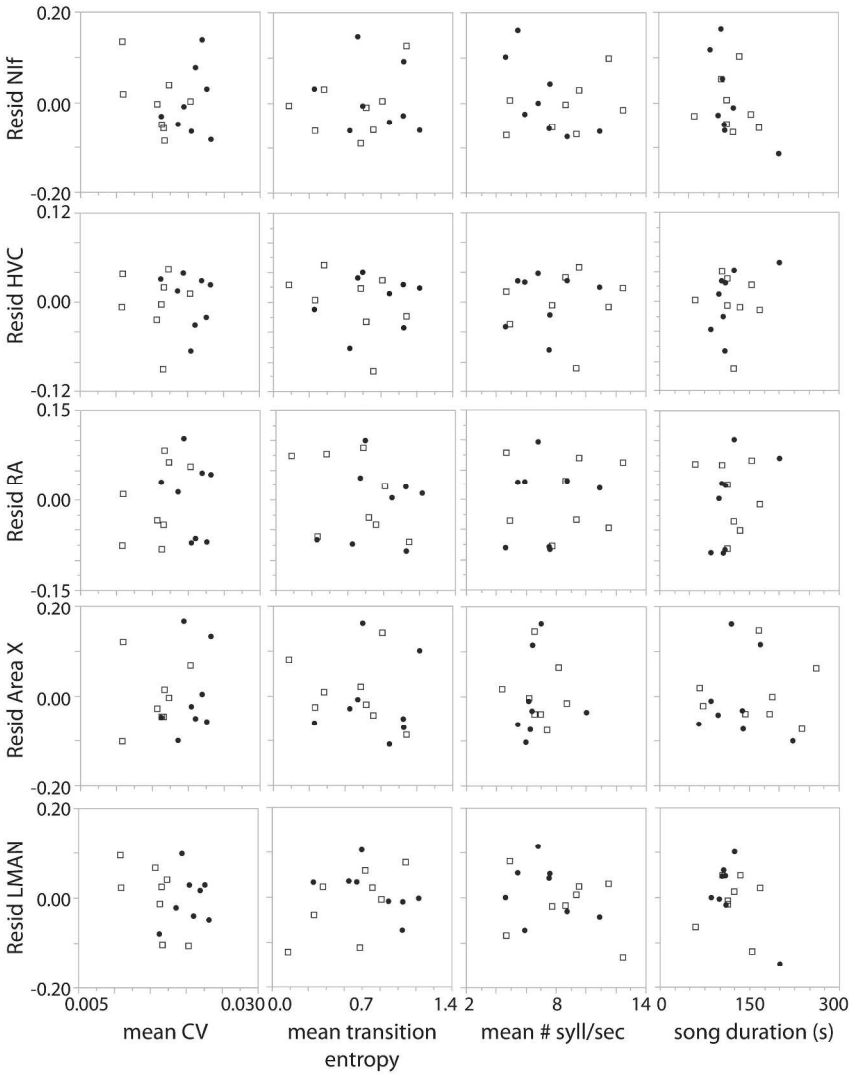


Figure 6

Relationship between song features and EGR-1 expression in song control nuclei, independent of social context. We computed the residuals from the previous analyses of context-dependent differences in EGR-1 expression in NIf, HVC, RA, Area X, and LMAN. Thereafter, we analyzed the degree to which individual variation in mean CV of FF, transition entropy, number of syllables per second (i.e., speed of song), and total song duration covaried with individual variation in residual EGR-1 expression (multiple regression). For all brain areas examined, there was no significant relationship between any feature of song and residual variation in EGR-1 expression. Open squares = FD birds; filled circles = UD birds.
234x332mm (300 x 300 DPI)