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Auditory cortical feedback signals are modulated during songbird courtship

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eLife Assessment

This **important** study reports that neural activity in the auditory cortex (field L) of singing male zebra finches can be modulated by the presence of a female conspecific. These findings extend recent work showing that the activity of dopaminergic neurons in songbirds is also affected by an audience. **Solid** evidence is presented for the importance of singing context in modulating auditory processing during vocal production, but the study does not yet fully establish that these effects arise specifically from audience-dependent modulation of auditory feedback, as opposed to possible acoustic, temporal, or recording-related confounds. This work should be of interest to researchers studying the context dependence of sensory processing, the role of auditory feedback, and vocal communication during courtship behavior.

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Abstract

Auditory feedback is important for vocal learning and control, but it remains unclear how the presence of an audience affects neural representations of self-produced sounds. Here we recorded neural activity from the auditory pallium in zebra finches practicing singing alone and directing courtship songs to females. We first discovered that many auditory neurons changed their singing-related discharge patterns during courtship singing. We used syllable-targeted distorted auditory feedback (DAF) to test how signals related to song feedback distortion depend on courtship context. Though past work showed that dopamine neurons uniformly reduce DAF responses during courtship, pallial auditory neurons exhibited heterogeneous DAF signal re-tuning in the presence of the female. Thus, single neurons in the songbird auditory pallium process feedback from self-produced actions differently when singing alone compared to when singing female-directed courtship song.

Introduction

Motivational drive affects attention and associated sensory responses to external events (Allen et al., 2019; Aton, 2013; Hindmarsh et al., 2021). Hungry or thirsty animals are more alert to food or water cues, which evoke larger brainwide neural responses (Allen et al., 2019; Burgess et al., 2018). Courtship also influences behavioral and neural responses to sensory events (Sakata and Brainard, 2009, 2008; Skals et al., 2005; Zhang et al., 2018), but it remains unclear how representations of self-produced behaviors change with the presence of an audience during courtship. Here we recorded auditory cortical neurons in birds singing alone and to females to test how auditory feedback signals depend on courtship-associated changes in motivational state.

Adult male zebra finches practice undirected song alone and direct courtship song to females (Kao et al., 2008; Zann, 1996). Both undirected and directed songs combine introductory notes, calls, and a stereotyped sequence of phonologically distinct syllables called motifs (Hyland and Tchernichovski, 2019; Rajan and Doupe, 2013). The acoustic structure of undirected and

directed motifs is highly similar in adult finches, enabling singing-related neural activity to be precisely aligned and compared across contexts (Kao et al., 2008; Woolley et al., 2014; Zann, 1996). The first goal of this study was to examine motif-aligned discharge in auditory cortical neurons to test if neural representations depend on courtship state.

A second goal was to test how song distortions are represented across performance modes. In past work, distorted auditory feedback (DAF) targeted to specific syllables has been used to drive syllable-specifc vocal plasticity (Andalman and Fee, 2009; Tumer and Brainard, 2007). Though DAF does not capture all aspects of natural song learning, it brings song evaluation under experimental control (Ali et al., 2013; Canopoli et al., 2014; Hoffmann et al., 2016). Birds modify their song to avoid DAF, and several studies support a reinforcement learning framework. Dopaminergic (DA) projections from the ventral tegmental area to Area X, the striatal nucleus of the song system, are necessary for both natural and DAF-based learning (Duffy et al., 2022; Gadagkar et al., 2016; Hisey et al., 2018; Hoffmann et al., 2016; Xiao et al., 2018a; Ali et al., 2013; Harding, 2004; Scharff and Nottebohm, 1991).

Recently, we discovered that DA signals retune during courtship. Specifically, DAF-associated DA signals known to evaluate undirected song are uniformly reduced during female-directed singing - as if a bird does not attend to his own mistakes (Roeser et al., 2023). This discovery raises two possibilities. First, DAF-related DA signals might be retuned at the level of VTA, for example by behavioral state-dependent modulation of intrinsic or synaptic excitability of VTA DA neurons (Xiao et al., 2018b). If this is the case, then DAF responses in upstream inputs to VTA could exhibit similar tuning to auditory feedback during directed and undirected song, but their influence on DA spiking is altered. Another possibility is that auditory processing differs when alone versus when courting a female, analogous to how motivational drives such as thirst or hunger retune widespread neural responses to water or food cues (Allen et al., 2019; Burgess et al., 2018), or how auditory cortical signaling is acutely affected by attention (Ahissar et al., 1992; Hubel et al., 1959) and context (Fritz et al., 2003; Jaramillo et al., 2014). This idea predicts that auditory representations even in a primary pallial area would change with possible attentional and motivational changes associated with a transition from singing alone to singing to a potential mate.

To test these possibilities, we recorded single neuronal activity in the auditory pallium of birds singing alone and to females. We targeted our recording electrodes to Field L, a primary auditory pallial area that projects into multiple higher auditory areas that, in turn, project to VTA (Bottjer et al., 2000; Foster and Bottjer, 1998; Mandelblat-Cerf et al., 2014; Moore and Woolley, 2019). Field L is composed of multiple subdivisions and surrounds the interfacial nucleus and because the implanted wire bundles spread in a small radius of up to ~0.5 mm, our recordings likely included large territories of the auditory pallium (Figure S1). Although Field L is classically described as a primary auditory thalamorecipient region, its activity may also be shaped by contextual signals related to the courtship context, potentially via recurrent interactions with higher-order auditory forebrain regions such as the caudal mesopallium (CM) and caudomedial nidopallium (NCM) (Bauer et al., 2008; Figure S1C).

Here we report that auditory responses to both bird's own song and DAF changed in heterogeneous ways at the transition from undirected to courtship directed singing. Thus, a bird's auditory representations of its own song vary across practice and courtship performance modes. Together with past work showing a re-tuning of premotor signals during courtship (Kao et al., 2008; Singh Alvarado et al., 2021; Woolley et al., 2014; Hessler and Doupe, 1999), our discovery that a sensory area retunes is consistent with distributed changes in sensorimotor processing during courtship.

Results

Singing-related auditory discharge is altered during courtship

Because we were interested in how auditory activity changed during female directed song, we recorded at least 20 song motifs during undirected singing and then presented females to elicit courtship song (n=147 neurons, n=11 birds). Consistent with past work (Keller and Hahnloser, 2009), we observed heterogenous singing-related firing in pallial auditory neurons, ranging from highly temporally precise to variable discharge across motif renditions (Figure 1A-C). To compare neural responses between undirected and directed singing, we focused on motif-aligned activity to compare acoustically similar vocalizations in the undirected and directed states (Figure 1A-F). For each neuron, we analyzed how mean firing rates, burst fraction, and the temporal precision of motif-locked discharge depended on courtship state, using analyses previously described (Kao et al., 2008; Sakata and Brainard, 2009; Woolley et al., 2014). Burst fraction was computed as the fraction of spikes occurring in burst events, defined as three (or more) spikes with two (or more) consecutive interspike intervals less than the 25th percentile of the ISI distribution. The temporal precision of neuronal song-locked firing was computed as the intermotif correlation coefficient (IMCC) using a 20 ms Gaussian kernel for smoothing (Goldberg and Fee, 2010; Olveczky et al., 2005; Kao et al., 2008; Sakata and Brainard, 2009; Woolley et al., 2014).

At the population level, neither the temporal precision nor mean firing rate exhibited significant shifts in one direction (Figure 1H-I; Paired t-test, FR: p=0.14, IMCC: p=0.99, n=138 neurons). A small but significant increase in burst fraction was observed (paired t-test, p=9.3x10-6, n=138 neurons, mean ± SEM: 0.11 ± 0.006 vs 0.15 ± 0.007, Figure 1J). Thus auditory neurons did not uniformly change their singing-related firing patterns at the transition to courtship singing, but individual neurons could exhibit significant changes in discharge with changes in courtship state, including increases or decreases in their average motif-locked firing rate (n=19 increase; n=16 decrease, p<0.01), burst fraction (n=20 increase; n=2 decrease, p<0.01), or precision of timing within the motif (n=9 increase; n=5 decrease, p<0.01) (Figure 1D-F, p values derived from Monte Carlo shuffles by condition).

Singing-related feedback signals can retune during courtship

Past work showed that Field L neurons can exhibit responses to distorted auditory feedback (DAF) during undirected singing (Keller and Hahnloser, 2009). To test if DAF responses exist and/or retune during courtship, we recorded pallial auditory neurons during lone and directed singing as we delivered syllable-targeted DAF (Andalman and Fee, 2009; Tumer and Brainard, 2007). For each bird, a specific syllable was probabilistically targeted with a 50 ms broadband sound played through speakers surrounding the bird (Chen et al., 2020; Gadagkar et al., 2016; Hamaguchi et al., 2014). To quantify DAF responses across the population of neurons and across conditions, we compared neural activity between randomly interleaved renditions of distorted and undistorted songs. DAF responsiveness within each behavioral condition was assessed using circular-shift permutation tests with max-statistic correction across bins. To test whether the DAF response differed between behavioral contexts, we separately permuted context labels (undirected vs directed) within undistorted and distorted trials, recomputed the bin-wise difference-of-differences contrast, and used the maximum absolute shuffled value across bins to obtain family-wise-error-corrected p-values.

Overall, responses to DAF were highly heterogeneous and variable in magnitude. While, as expected, some Field L neurons robustly responded to DAF with activations following song distortion (Figure 2A,D), some neurons' responses were more subtle (Figure 2C), suggesting a continuum in DAF responsiveness. Interestingly, neural responses to DAF could be modulated by the courtship context in heterogeneous ways. The neuron in Figure 2.2A was DAF responsive during directed singing, but the magnitude of the response was significantly decreased relative to the DAF response while singing alone.

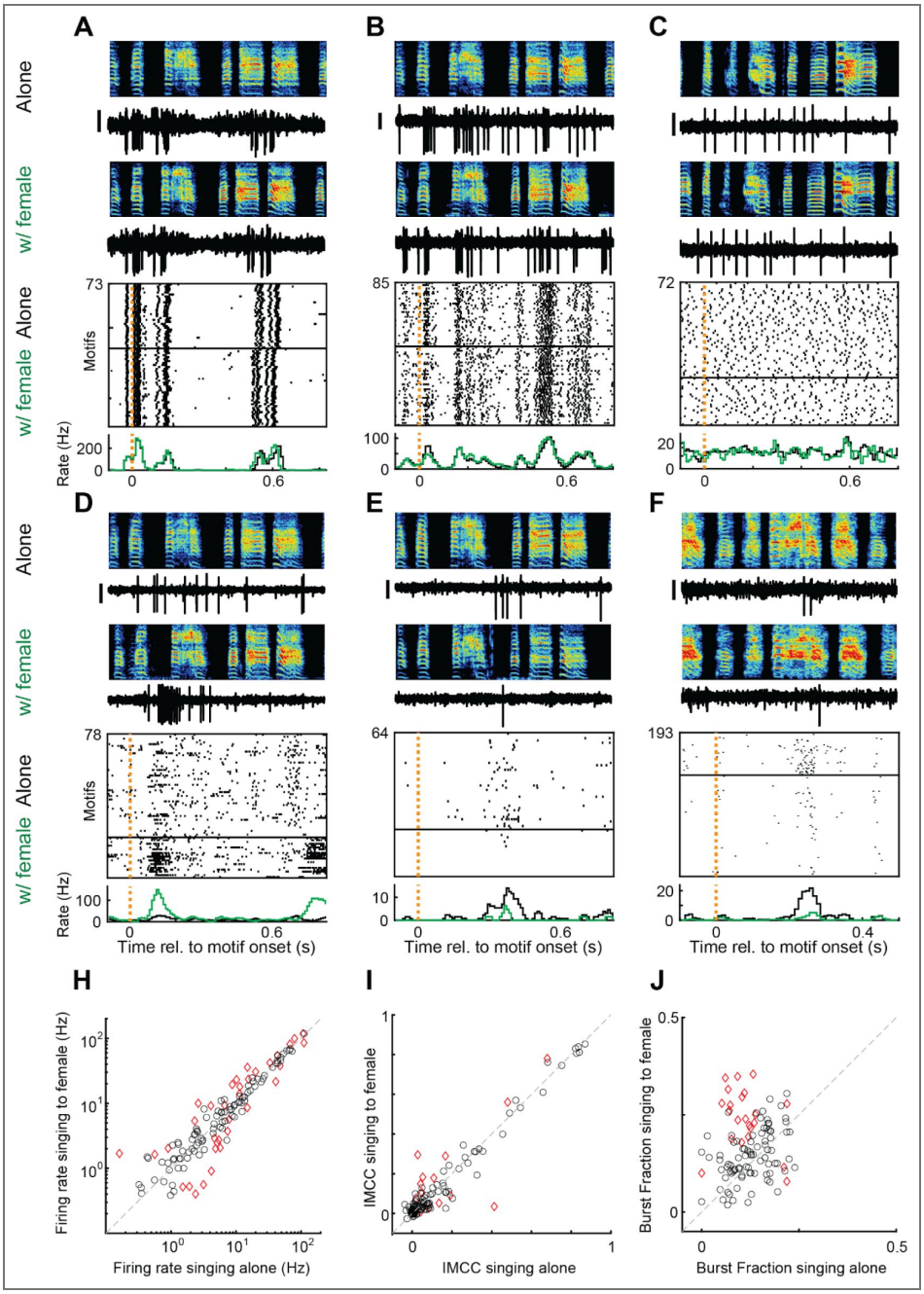

Figure 1. Singing related discharge can retune during courtship.

(A-C) Three example neurons with stable firing properties across alone and female-directed song conditions. Top to bottom: single trial example spectrograms, spike discharge, corresponding spike raster plots, and rate histograms (aligned to motif onset) from motif renditions singing alone (black) and to the female (green). Black vertical scale bar for spiking activity is 0.3 mV, y-axis limits of spectrograms are 0.2 to 8 kHz. (D-F) Data plotted as in A-C for three example neurons with significant context-dependent changes in firing. (H-J) Scatter plots of mean firing rates (H), IMCC values (I) and neuronal burst fraction (J) for 138 neurons when singing alone and to the female. Red diamonds: neurons with significant change across conditions ($p<0.01$, shuffled permutations).

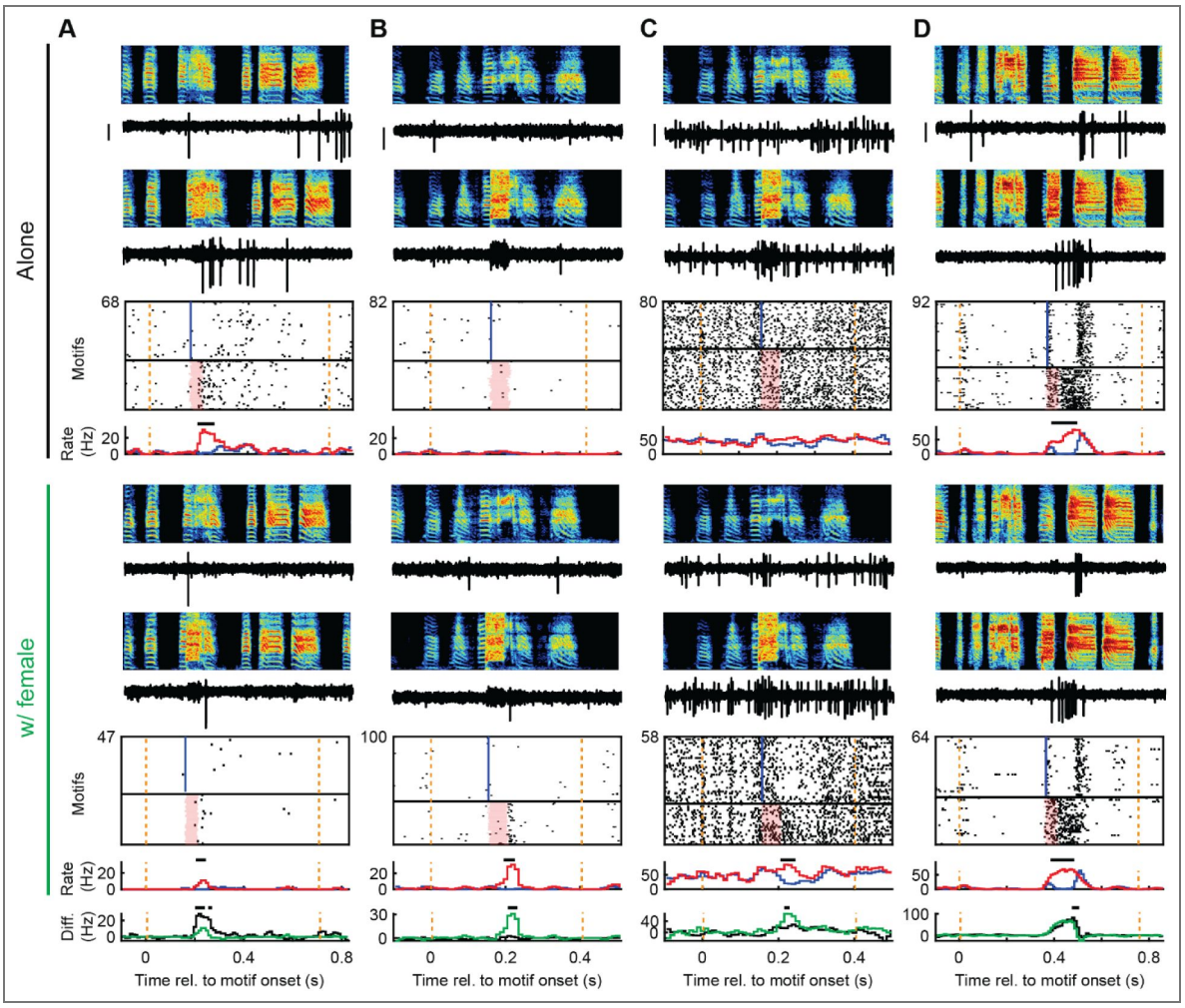

Figure 2. DAF-activated neurons can retune during courtship.

(A-D) Four example neurons with DAF responses that vary in the degree to which they are affected by the courtship context. Top to bottom: single trial example spectrograms and spiking activity for undistorted and distorted trials, corresponding raster plots (blue vertical bar denotes feedback target time in undistorted renditions; orange vertical dotted lines denotes onset and offset of song motif). Corresponding rate histograms for undistorted renditions (blue trace) and distorted renditions (red trace). Below are the same plots, but for songs directed to the female. Bottom: difference between undistorted and distorted rate histograms for singing alone (black) and singing to the female (green). All data are time-aligned to motif onset. Black lines above rate histograms and rate difference traces denote bins for significant DAF responses and significant changes in DAF responses, respectively. Scale bar for spiking activity is 0.3 mV. Vertical axis limits for spectrograms are 0.2 to 8 kHz.

Unexpectedly, some neurons (as in Figure 2B) were not DAF responsive during undirected singing, but became DAF responsive during directed singing. The neuron in Figure 2C was moderately DAF responsive during undirected singing, but more so during directed singing. Some neurons, however, as in Figure 2D, exhibited largely unchanged DAF responsiveness across the two social contexts.

Unexpectedly, some neurons were not activated by the song distortion but rather by the lack of target syllable distortion. These activations following undistorted renditions could also depend on the courtship context, as was the case for the neurons in Figure 3A-C, while some neurons were relatively insensitive to the courtship context (Figure 3D).

Together, these neural responses exemplify the heterogeneity of DAF-related signaling in the auditory cortex, and that DAF-related signaling can be dependent on the social context in which the bird is performing the song. Importantly, because these neural recordings were performed over long time courses (~2-8 hours), a strict threshold for stability was imposed. A Pearson's correlation coefficient of at least 0.99 between the average neural waveform during undirected and directed singing was required for a unit to be considered stable (Dickey et al., 2009; Figure S2). Statistical tests defining auditory neurons as DAF-responsive or not in a binary fashion may not be suitable if the underlying population of DAF-related responses exist on a continuum from responsive to non-responsive. However, to provide a summary of the observed responses, we performed three different analyses. First, we replicated the same analysis as in a previous Field L recording study (Keller and Hahnloser, 2009). This analysis identified 71/147 neurons as DAF responsive in at least one behavioral condition, whereas 76/147 were not responsive in either condition. Next, to visualize changes in DAF responses as a population, we scored each neuron's DAF response by computing the maximum z-scored difference between undistorted and distorted firing rates in a time window 0-100 ms after distortion target time onset (Figure S3). We found explaining a neuron's DAF response and change in DAF response across contexts difficult to describe with a single number that was highly sensitive to parameter choices. Because the previously used tests were highly sensitive and did not explicitly test for a change in DAF responsiveness, we adopted the previously mentioned permutation-based test. This more conservative approach identified 48/147 neurons as DAF responsive in at least one condition, with 13 of those neurons exhibiting a significant modulation in their DAF response between undirected and female-directed singing. Together, these data show that auditory responses to distorted auditory feedback during singing can retune during courtship.

A previous study recording from Field L in zebra finches reported neural activations prior to the onset of singing, consistent with premotor signaling (Keller and Hahnloser, 2009). We tested for context dependent changes in premotor activity by examining peaks in neural activity aligned to motif onsets. Across the population neurons did not exhibit significant changes in the timing of motif onset-aligned activity (Figure S4).

Action potential width of individual neurons has previously been used to classify putative interneurons or putative principal cells in the zebra finch auditory pallium (Calabrese and Woolley, 2015). We tested if DAF-response scores, mean firing rates during singing, burst fraction, IMCC, and the change in all of these between undirected and directed singing was correlated with spike half width (spike half-width measured as peak to trough time; Figure S5). DAF-response in either condition, the change in DAF response across conditions, and the change in firing rate, burst fraction, and IMCC were not significantly correlated with spike width (Figure S5A-B). Consistent with previous literature, firing rate was significantly correlated with spike width (Pearson's correlation, p=0.003; Figure S5C). Interestingly, burst fraction (p=8.3x10-4) and IMCC (p=5.4x10-5) were also significantly correlated with spike width (Figure S5C).

Discussion

By recording from the auditory pallium in zebra finches singing alone and to females, we discovered, for the first time to our knowledge, that auditory representations of an animal's own vocalizations change with an audience. These findings extend past work in finches showing that social context affects auditory responses to the calls of conspecifics (Angeloni and Geffen, 2018;

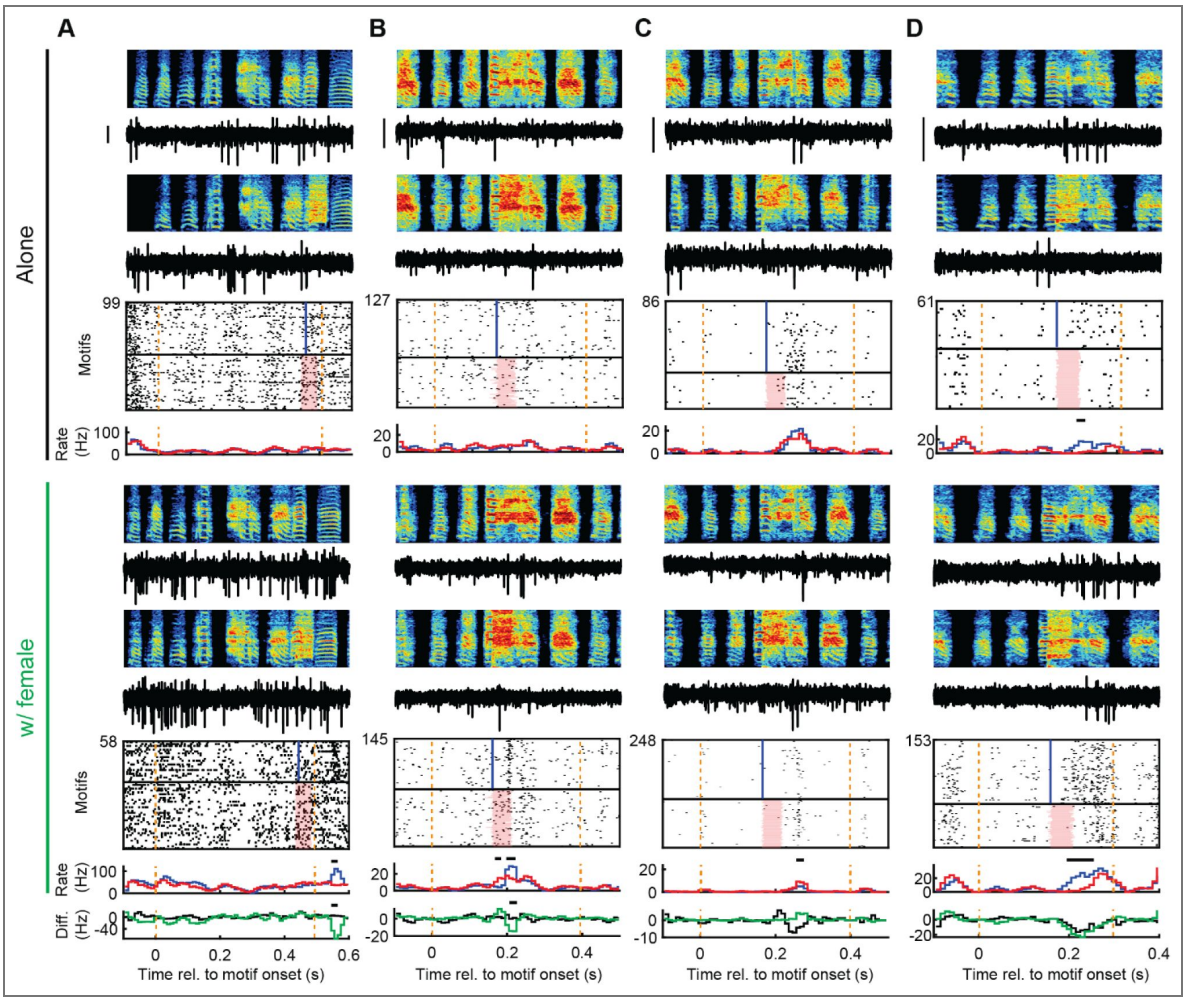

Figure 3. Neurons activated by undistorted singing could depend on courtship context.

(A-D) Four example neurons with activations following undistorted renditions that vary in the degree to which they are affected by the courtship context. Top to bottom: single trial example spectrograms and spiking activity for undistorted and distorted trials, corresponding raster plots (blue vertical bar denotes feedback target time in undistorted renditions; orange vertical dotted lines denotes onset and offset of song motif). Corresponding rate histograms for undistorted renditions (blue trace) and distorted renditions (red trace). Below are the same plots, but for songs directed to the female. Bottom: difference between undistorted and distorted rate histograms for singing alone (black) and singing to the female (green). All data are time-aligned to motif onset. Black lines above rate histograms and rate difference traces denote bins for significant DAF responses and significant changes in DAF responses, respectively. Scale bar for spiking activity is 0.3 mV. Vertical axis limits for spectrograms are 0.2 to 8 kHz.

Menardy et al., 2014; Remage-Healey et al., 2010). More broadly, these findings support the idea that auditory cortical activity is modulated not just by acoustic features (Gervain and Geffen, 2019; King et al., 2018) but also diverse phenomena such as attention (Hubel et al., 1959), primary rewards (David et al., 2012), task parameters (Downer et al., 2015; Fritz et al., 2003); (Angeloni and Geffen, 2018; Menardy et al., 2014, 2012; Remage-Healey et al., 2010), and even hormone levels (Angeloni and Geffen, 2018; Menardy et al., 2014, 2012; Remage-Healey et al., 2010).

Detecting differences between predicted and actual sensory feedback is a crucial aspect of motor learning (Keller and Mrsic-Flogel, 2018), and auditory cortical neurons in diverse species can signal mismatch errors (Eliades and Wang, 2008; Keller and Hahnloser 2009; Parras et al., 2021; Ulanovsky et al., 2003). However, it is important to note that DAF-related changes in firing in auditory neurons do not necessarily imply that neurons compute sensory prediction errors. DAF-related responses could arise from ordinary auditory tuning to the broadband distortion stimulus. In past recordings from VTA we observed a bimodal distribution of error responses, and the error-responding neurons were the ones that projected to Area X (Gadagkar et al., 2016). Yet in cortex there is uncertainty about the suitability of classifying single neurons by response profile, as task-relevant parameters that can be decoded from neuronal populations may not be apparent when examining single neuronal representations (Kaufman et al., 2014; Mante et al., 2013; Rigotti et al., 2013; Williams et al., 2018). We observed a continuum of DAF responses during both directed and undirected singing which made it difficult to unambiguously define a neuron as being DAF responsive or not.

Because the main goal of this study was to test if courtship-associated reduction in DAF signaling, recently observed in VTA DA neurons (Roeser et al., 2023), resulted from a local process in VTA or reflected a retuning of auditory responsiveness, we explicitly tested for changes in DAF responsiveness between alone and female-directed singing. Surprisingly, we discovered that Field L neurons could retune at the transition from lone to courtship singing in diverse ways, consistent with distributed modulation of auditory processing that does not fully explain the uniform DAF-signal attenuation observed in VTA. Interestingly, Area X and LMAN neurons uniformly increase their temporal precision and reduce their burstiness during courtship singing (Kao et al., 2008; Roeser et al., 2023; Sakata and Brainard, 2008; Singh et al., 2021; Woolley et al., 2014). In contrast, auditory pallium neurons could exhibit increases or decreases in burstiness, temporal precision, or mean rate with courtship.

An open question is how auditory pallium receives information about whether a female is present, and how this information influences neural activity. Several neuromodulatory systems with possible information about courtship state project to songbird auditory forebrain, including acetylcholine (Shea and Margoliash, 2010), serotonin (Yip et al., 2020), and norepinephrine (Cardin and Schmidt, 2004). Estrogens can also rapidly modulate auditory firing (Remage-Healey et al., 2010; Scarpa et al., 2022). Yet how the courtship state may be orchestrated across multiple brain regions remains unclear. One possibility is that hypothalamic nuclei such as the medial preoptic nucleus (MPOA) initiate the courtship state. The MPOA projects to multiple brainstem neuromodulatory areas which in turn project broadly throughout the forebrain, including the song system and auditory system (Riters and Alger, 2004; Ben-Tov et al., 2023; Castelino and Ball, 2005; Singh Alvarado et al., 2021). It will be interesting in future studies to examine the neural signals propagating through these pathways at transitions into and out of the courtship state.

Materials and Methods

Subjects

Sixteen adult male zebra finches (>90 days post hatch) were the subjects of this study. Animal care and experiments were carried out in accordance with NIH guidelines and were approved by the Cornell Institutional Animal Care and Use Committee.

Surgery and awake-behaving electrophysiology

For chronic neural recordings, subjects were anesthetized with isoflurane inhalation and mounted on a stereotaxic instrument for probe implantation. All probes were 16-channel moveable electrode bundles (Innovative Neurophysiology). Probes were implanted 1.5-2.0 mm anterior and 1.5-2.0 mm lateral of the bifurcation of the mid-sagittal sinus to target Field L (Keller and Hahnloser 2009) at a head angle of 80 degrees (measured as the angle from the tip of the beak to the center of the ear bars relative to the horizontal plane). The end of the cannula was implanted 1.5 mm ventral to the surface of the brain. Birds were then placed alone in a sound isolation chamber (12 hour light/dark cycle) with ad libitum food and water and allowed 1 day to recover post op before being subjected to the distorted auditory feedback (DAF) protocol. 2-3 days were allotted for habituation to DAF and to ensure the bird began to spontaneously sing enough motifs in social isolation before neural recordings. Electrode bundles were extended from the end of the cannula on a daily basis by ~50 μm. DAF was implemented with a custom LabView acquisition program that analyzed song syllables in real-time and delivered syllable-targeted feedback. DAF (50 ms broadband noise bandpass filtered at 1.5-8 kHz to match frequency range of zebra finch song) was played over speakers in the recording chamber on top of a specific target syllable randomly on 50% of motif renditions. Experiments were carried out in the male's home cage, which was inside a sound isolation chamber. When the homecage lights came on each day, recording began and the male was left alone to sing at least 40 undirected song motifs. Female directed motifs were then recorded by presenting a female in the chamber in ~10 minute intervals throughout the day until at least 40 directed song motifs were elicited (Roeser et al., 2023). Electrode placement was verified at the end of the experiment with small electrolytic lesions, histology, and dark field imaging. 11 of the 16 implanted birds yielded single unit recordings and sang sufficient motifs for the experiment. Many channels on the probes recorded multi-unit activity, which were taken note of but not analyzed in this study.

Neural recording and analysis

Neural signals were acquired with the Intan RHD recording controller and 16-channel Intan headstages that directly interfaced with the moveable bundles. Sampling rate was set to 20kHz and recording was manually controlled with the Intan recording software, where a 60 Hz notch filter was applied and spiking activity of single units could be visualized in real time. Audio data and a real-time digital copy of the DAF signal were simultaneously recorded with neural data in the recording controller such that all data could be easily time aligned. A custom MATLAB GUI was used for visualizing song and neural data, and for spike sorting. Neural recordings were bandpass filtered between 0.4 kHz and 6-8 kHz and single unit spiking activity was manually sorted as previously described (Goldberg and Fee, 2010). Initially, 161 neurons were recorded, but only highly stable neurons with a correlation coefficient of at least 0.99 between the average waveform during undirected and directed singing were kept (Dickey et al., 2009). Spike half width was measured as peak to trough time on the average waveform.

Motif aligned spiking activity was time-warped to the median duration of undirected or directed motifs. Any motifs that had overlapping time with a female call in directed motifs was excluded from analysis. Firing rate (FR) histograms were computed by binning spiking events in 10 ms windows and smoothing with a 3-bin moving average. To calculate the significance of the FR changes across behavioral contexts, we randomly assigned spike trains from each song motif trial as undirected or directed groups while conserving trial numbers, then calculated FR values for the randomized dataset, as previously described (Goldberg and Fee, 2010). This shuffling without replacement was repeated 10,000 times for each neuron, yielding 10,000 new changes in FR values. Measured changes in firing rate that were greater than the 99th percentile of the shuffled distribution were considered significant, as in previous studies (Sakata and Brainard, 2008). This procedure was repeated for IMCC and burst fraction. A p value less than 0.01 was considered significant to account for multiple comparisons. To quantify the degree to which neuronal firing was time-locked to song, the intermotif correlation coefficient (IMCC) was calculated as described previously (Chen et al., 2019; Kao et al., 2008; Olveczky et al., 2005). To compute IMCC,

motif-aligned FR was mean-subtracted and smoothed with a Gaussian kernel of 20 ms SD, resulting in a rate vector, $\boldsymbol{r_i}$, for each motif. IMCC was defined as the mean of all pairwise correlation coefficients between $\boldsymbol{r_i}$ as follows:

$$IMCC = \frac{1}{N_{pairs}} \sum_{j>i}^{N_{pairs}} CC_{ij}$$

$$CC_{ij} = \frac{\boldsymbol{r}_i \cdot \boldsymbol{r}_j}{\sqrt{\boldsymbol{r}_i^2 \boldsymbol{r}_j^2}}$$

We defined bursts as events containing three or more spikes with consecutive interspike intervals less than the 25th percentile of the 25th percentile of the interspike interval distributions during singing. Burst fraction was calculated as the fraction of spikes during song motifs that occurred during bursts.

We quantified the DAF responsiveness for each neuron in two ways, following approaches previously described (Chen et al., 2019; Gadagkar et al., 2016; Keller and Hahnloser, 2009). Spike counts in 30 ms time windows shifted in 5 ms steps up to 50 ms after DAF offset were generated for undistorted and distorted trials. A WRS test assessed the significance of each 30 ms window, and only neurons with 2 or more consecutively significant ($p<0.05$) windows were considered a significant DAF response (Keller and Hahnloser, 2009).

To more stringently test for DAF responsiveness and explicitly test for changes in DAF responsiveness between undirected and directed singing, we used the motif-aligned, time-warped spike trains counted in 10ms bins, smoothed over 3 bins, and defined bin-wise DAF-response vectors as distorted minus undistorted activity within the undirected and directed conditions. A bin-wise difference-of-differences vector was used to quantify context-dependent changes in the DAF response. Within-condition DAF responsiveness was tested using circular-shift permutation tests in which each trial's spike-count vector was independently circularly shifted by a random number of bins, preserving within-trial temporal structure while disrupting stimulus alignment (Harrison and Geman, 2011; Stella et al., 2022; Amarasingham et al., 2011). For each shuffle, shuffled DAF-response vectors were recomputed and the maximum absolute value across bins was stored to generate max-statistic null distributions. To test whether DAF responsiveness differed between contexts, context labels were randomly permuted separately within undistorted and distorted trials, shuffled DAF-response vectors were recomputed for each context, and the shuffled difference-of-differences vector was calculated; again, the maximum absolute value across bins was stored on each shuffle. Family-wise-error-corrected bin-wise p-values were obtained by comparing the absolute observed statistic at each bin to the corresponding null distribution of shuffled maxima.

To investigate premotor related activity, we aligned spikes to motif onsets that had at least 30 ms of preceding silence. Spikes were binned in 3ms bins and smoothed over 3 bins. Trial-averaged firing rates were computed and peaks were detected using MATLAB's findpeaks function. The significance of a peak was determined by circularly shuffling the spike trains and recomputing the maxima, yielding a null distribution to compare the measured value against. All peaks with $p<0.05$ were considered significant, and the earliest peak in firing rate was plotted in Figure S4A.

Figure supplements

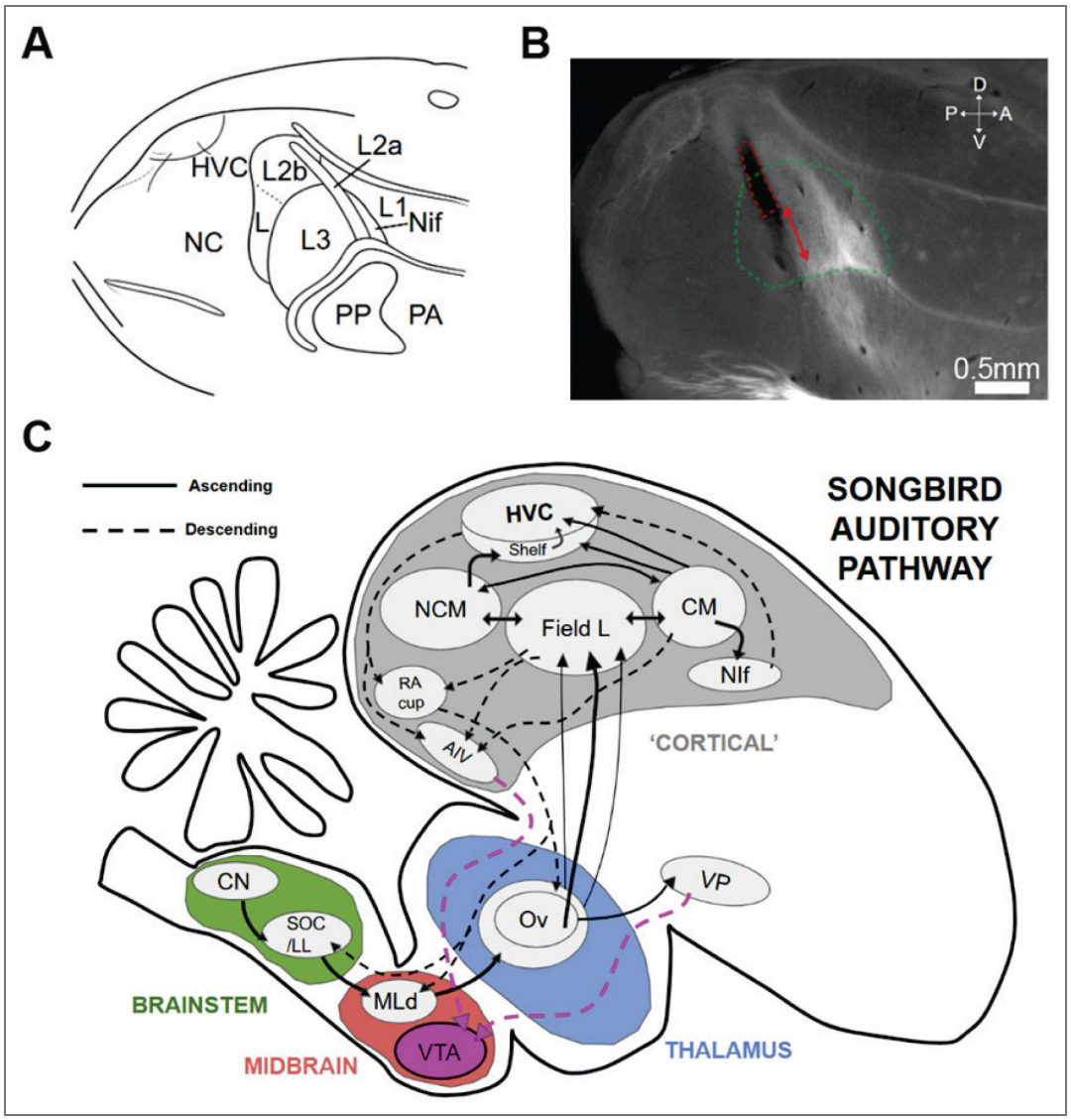

Figure S1. Auditory system of the zebra finch and example histology (**A**) Example sagittal view of the anatomical structure of auditory areas in the zebra finch brain, adopted from a previous study (Fortune and Margoliash, 1992). (**B**) Example brain slice approximately 1.5mm medial from the midsagittal sinus showing the wire bundle implant location (dotted red line) and the travel path of the moveable bundle (red arrows). Green dotted line denotes the approximate region recorded across all birds, estimated from histology of each bird (**C**) Connectivity diagram showing the ascending and descending connections between auditory areas in the zebra finch brain. Pink lines show connections to the VTA. Acronyms: CN; cochlear nucleus, SOC; superior olivary complex, MLd; dorsal mesencephalic nucleus, VTA; ventral tegmental area, Ov; nucleus ovoidalis, VP; ventral pallidum, CM; caudal mesopallium, NIf; interfacial nucleus, NCM; caudomedial nidopallium, RA; robust nucleus of the arcopallium, AIV; ventral portion of the intermediate arcopallium. Connections between areas adopted from connectivity diagrams in previous studies (Shaevitz and Theunissen, 2007; Vates et al., 1996; Bauer et al., 2008).

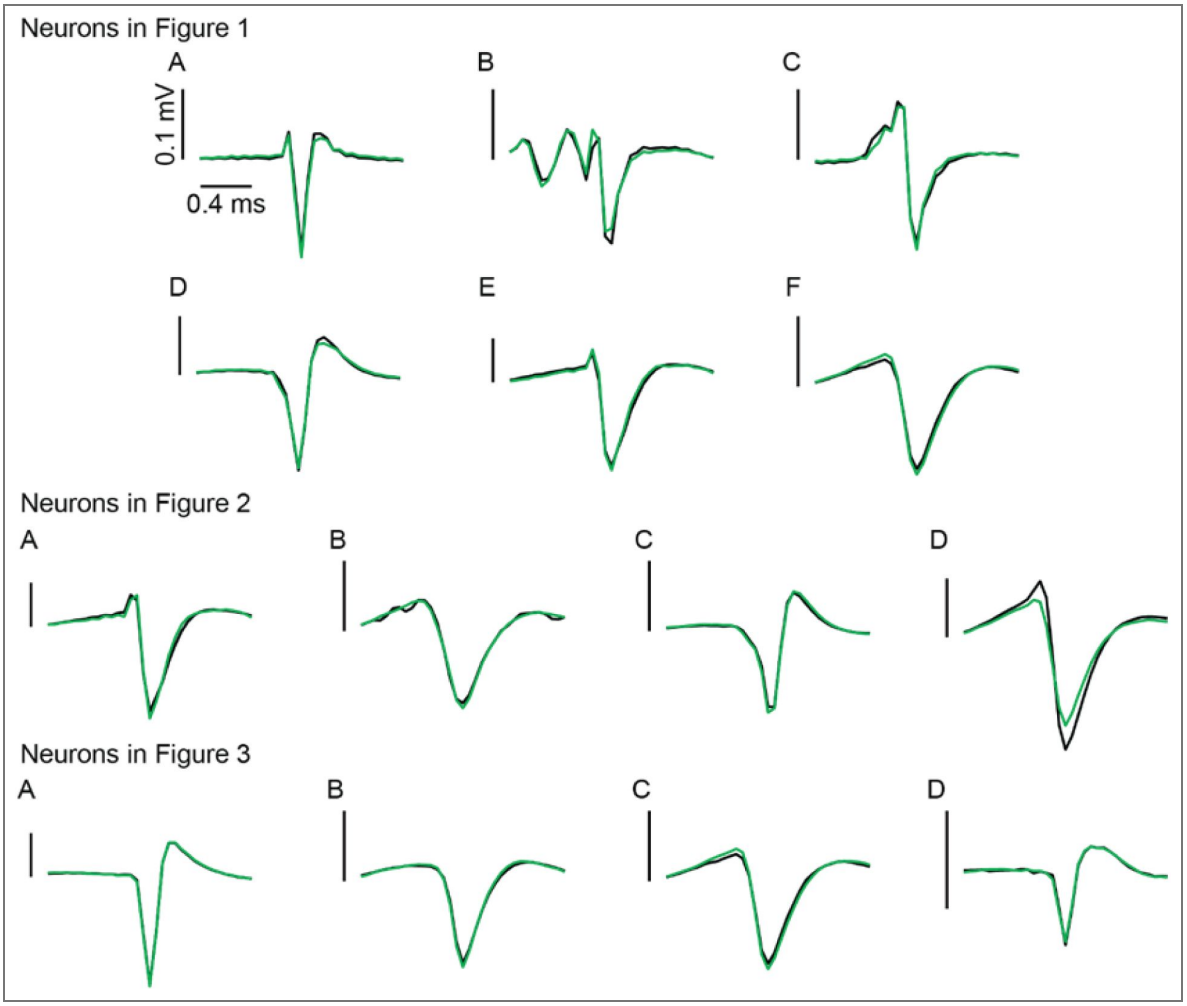

Figure S2. Spike waveforms are stable across undirected and directed singing.

Average waveforms for single units from Figures 2.1-2.3 during undirected singing (black) and directed singing (green). All units included in this study had a Pearson's correlation coefficient between the undirected and directed average waveforms of >0.99.

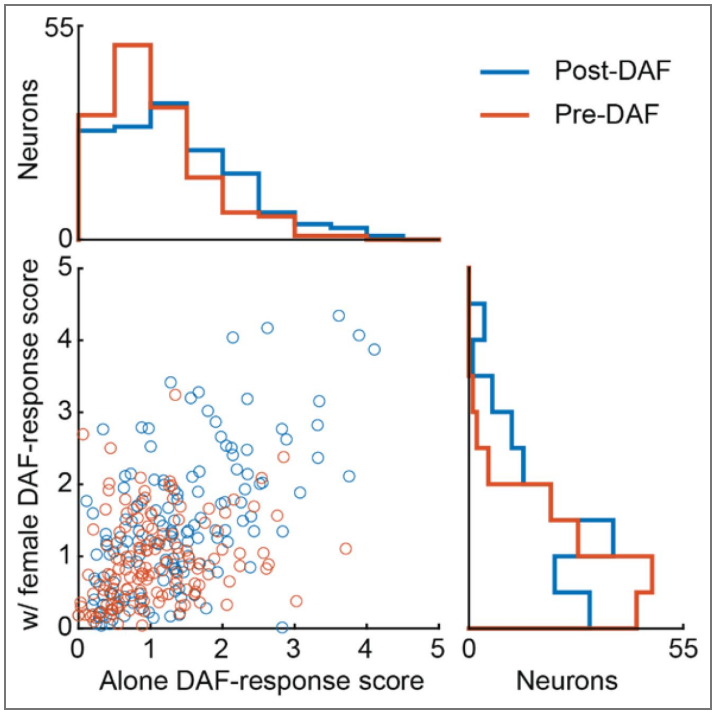

Figure S3. Distributions of absolute DAF-response scores in pallial auditory neurons.

Scatter plot where each dot represents the absolute z-scored DAF-response of a single neuron during the 100 ms interval after the onset of DAF (blue) and 100 ms before DAF onset (orange). Corresponding histograms for each condition are projected along the x and y axes.

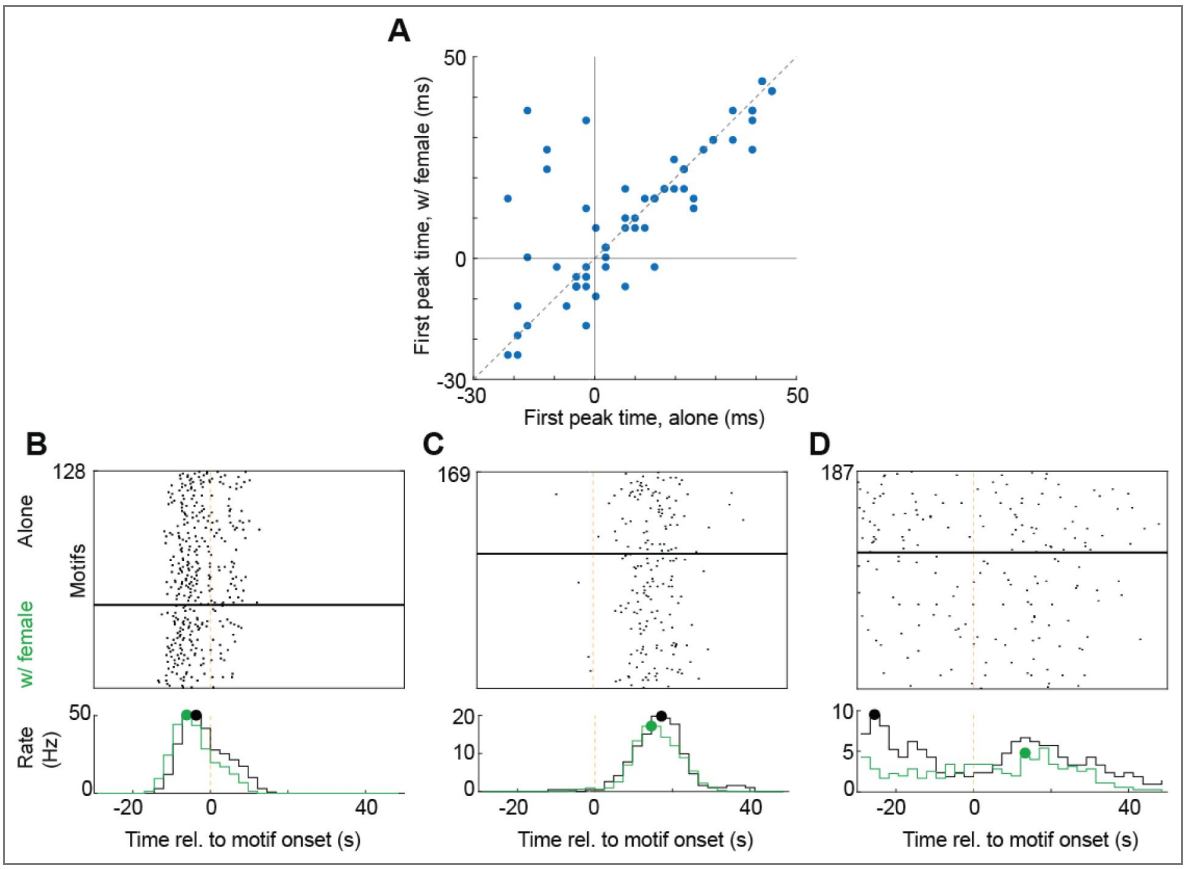

Figure S4. Latencies of neural response vary from pre- to post-motif onset and are largely stable across conditions.

(**A**) Scatter plot of latencies of first firing rate peak between undirected and directed singing. (**B**) Example neuron with activity before motif onset; raster of spike times and average firing rates for undirected (black) and directed (green) aligned to motif onset. Green and black dots indicate the earliest significant peak. (**C**) Example neurons plotted as in (B) but for a neuron responding post motif onset. (**D**) Example neuron plotted as in (B-C) but for a neuron with a relatively larger change in peak response time.

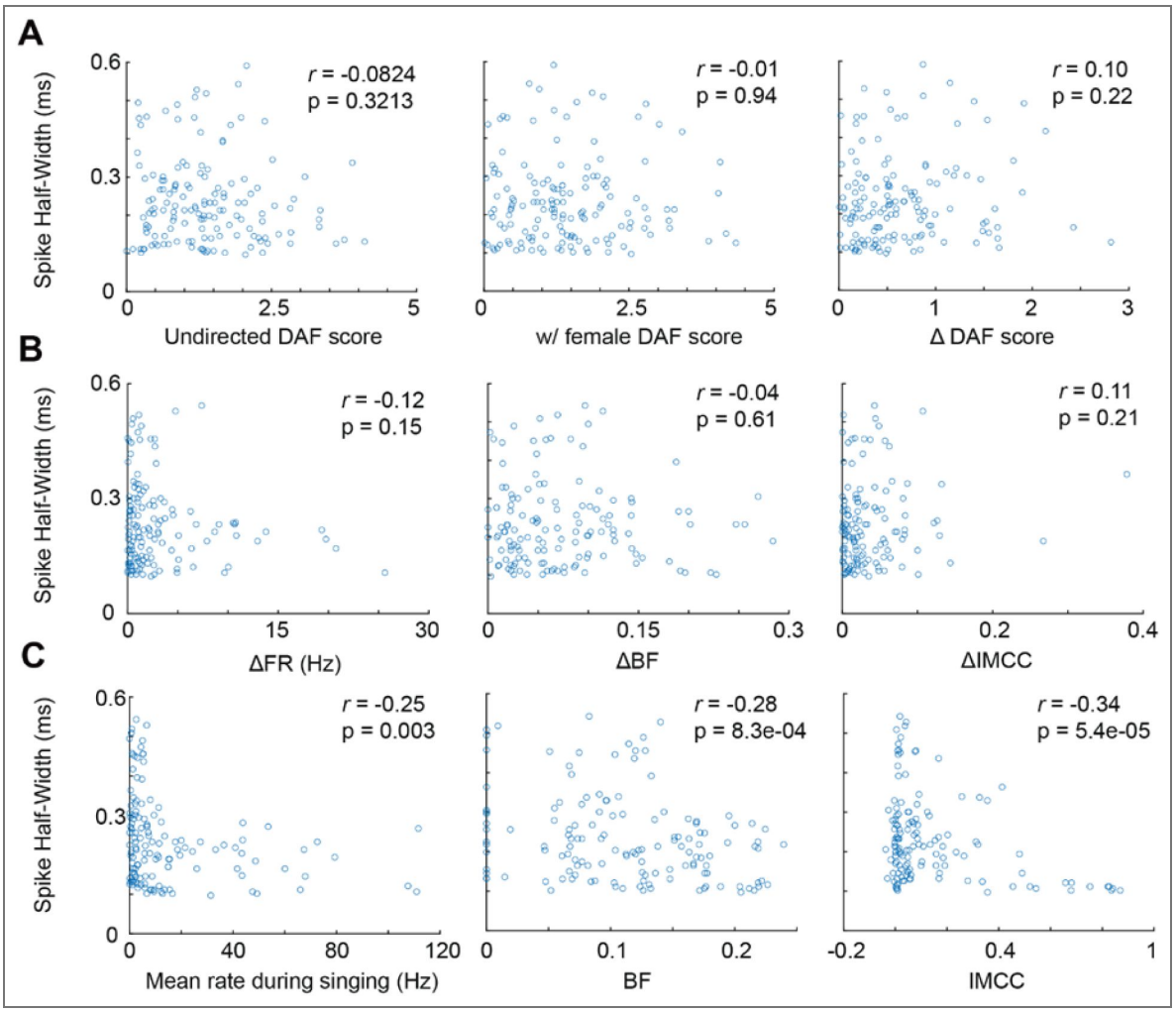

Figure S5. Spike half-width correlations with neural discharge properties.

(**A**) Scatter plots of DAF-response scores in undirected and directed, and the change between them (undirected-directed) vs spike half-width with no significant correlations (**B**) Scatter plots of the change in firing rate, burst fraction (BF) and IMCC (undirected-directed) vs spike half-width with no significant correlations. (**C**) Scatter plots of mean firing rate, burst fraction, and IMCC, all during undirected singing, vs spike half-width. All correlations in (C) are significant with correction for multiple comparisons (uncorrected p-values shown). Pearson's linear correlation coefficients and associated p values are shown in each plot.

Data availability

Processed data files (stored as MATLAB .mat files) containing sorted electrophysiology spike times and acoustic segment timing, as well as MATLAB scripts used to analyze those data files for this paper have been deposited: Direct link: *https://osf.io/ftg84* DOI link: *https://doi.org/10.17605/OSF.IO/FTG84*. Raw data files are too large to practically store online, but can be made available on request.

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Peer reviews

Reviewer #2 (Public review):

This study asks whether auditory responses in the songbird auditory pallium/field L during singing are modulated by social context. Specifically, the authors examine neural responses to delayed auditory feedback during male zebra finch song produced either alone or in the presence of a female. This is an interesting and important question because the evaluation of self-generated vocal output may differ when the song has a dedicated social function.

The main strength of the work is that it addresses auditory feedback processing during natural vocal behavior and does so across two naturalistic contexts. The revised manuscript is strengthened by additional analyses of spike waveform similarity, response significance, response latency stability, and exclusion of motifs overlapping with female calls. These additions make the reported context-dependent response differences more credible and help address some concerns about recording stability and contamination by female vocalizations.

The results show that some auditory pallium neurons respond differently to feedback perturbations during directed and undirected song. This finding is potentially significant because it suggests that auditory processing during vocal production is not rigid but could subserve a social function that depends on the listener. If robust, this would add an important dimension to models of song monitoring and sensorimotor control.

However, the strength of evidence remains moderate rather than conclusive. Several alternative explanations are not fully ruled out. Directed and undirected songs may differ

acoustically in ways that could influence neural responses, and it is not yet clear that relevant song features were directly compared or controlled across contexts. The experimental sequence also appears to be ordered, with undirected song recorded before directed song, which makes it difficult to fully separate social-context effects from time-dependent changes in recording quality or neural responsiveness. The added waveform analysis is useful, but does not completely establish continuous unit stability across long recording sessions. In addition, possible song changes around the delayed-feedback target point, including compensatory modifications before or after feedback, remain an important potential confound. Finally, clarification of the time-warping and spike-alignment procedures is important because condition-specific alignment could affect comparisons between directed and undirected song.

Overall, the data support context-dependent differences in neural responses in some neurons, but do not yet fully establish that these differences arise specifically from audience-dependent modulation of auditory feedback processing rather than from acoustic, temporal, or recording-related confounds. The work is likely to be useful to researchers interested in vocal communication, auditory feedback, and social modulation of sensorimotor processing, particularly as a foundation for future experiments using counterbalanced designs and more direct controls of song structure across contexts.

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Reviewer #3 (Public review):

In this study, Jones et al. examine how neural activity in auditory regions (the auditory pallium) of singing male songbirds is modulated by the presence or absence of an audience (a female conspecific). They test whether activity in auditory pallium differs between conditions in which the male is singing to a female (directed song) or alone (undirected song) and whether response to distortions of auditory feedback (DAF) differ between these conditions. Previous work has shown that in other parts of the songbird brain, sensory-motor activity can differ between directed and undirected song, and that responses to DAF are attenuated when males sing directed song versus undirected song. These prior results raise the interesting question of the extent to which such modulations of activity by the presence of an audience are already present in primarily auditory areas within the pallium. This possibility is also motivated by prior work that has shown that activity in the auditory pallium is not exclusively explained by auditory input, but can also be modulated by the bird's state - whether it is singing or not.

Against this background, the questions asked here are of interest for two inter-related reasons:

(1) The authors address whether the presence of an audience (a female conspecific) alters activity in an auditory region during singing. Primary songbird auditory areas such as Field L, and analogous mammalian thalamo-recipient cortical regions such as A1, are often thought of as responding very specifically to the features of sensory stimuli, but are also understood to be modulated by a variety of factors including the attentional and behavioral state of the animal. For audition, such modulation includes whether or not animals are vocalizing and listening to themselves or listening to playback of their own vocalizations. Cited works from Keller (2009) as well as Eliades and Wang (2008) have indicated that the act of vocalizing can modulate auditory responses to self-generated feedback in primary auditory areas relative to those arising from playback of the same sounds. Here, the question is whether responses to self-generated feedback differ between conditions of singing alone versus singing to a female audience. A demonstration that the presence of an audience matters to responses in auditory pallium would add to a general understanding of how it is that non-auditory factors can modulate activity within regions that are considered primarily sensory.

(2) The authors address the possible source of an audience-dependent modulation of responses to feedback perturbation in the VTA previously reported by Goldberg and colleagues (2023). In the VTA, responses to perturbations during singing are consistently attenuated when males are singing to females versus when they are singing alone, but the underlying mechanisms of this modulation are unknown. Here, the authors test the possibility that such modulation by an audience is already present at the level of auditory pallium. The previously reported attenuation in VTA is a nice example of how neural processing can differ with varying behavioral priorities. Understanding whether this modulation of responses to DAF arises already in auditory areas would further a mechanistic understanding of an intriguing example of state-dependent modulation of sensory processing and behavior and lend broad insight into related phenomena.

The authors report 1) that activity in the auditory pallium differs between directed and undirected singing at many individual recording sites, but that these changes are heterogeneous, with both increases and decreases in activity, so that there is no consistent change across the population and 2) that modulation of activity by DAF can differ between directed and undirected song, but that there is no consistent attenuation of response (as observed in the VTA) and instead heterogeneous increases and decreases in response to DAF so that there is no net change at the population level.

These findings are important and of general interest; while they do not readily explain the source of the audience-dependent attenuation of auditory responses to DAF in the VTA, the demonstration of audience-dependent modulation of self-generated feedback and its disruption in the auditory pallium provides an opportunity for further investigation of how changes in social context influence brain and behavior.

Additional comments and suggestions:

The authors have done a good job of addressing many of the issues that were raised in the initial round of reviews. There is additional analysis that strengthens the study, including 1) applying a stability criterion to assess the quality of unit isolation, 2) shifting away from a categorical identification of units as "retuning" or not, to an analysis that presents a continuum of changes to neural firing between conditions, 3) use of non-parametric statistics for the assessment of significance of differences in response measures between conditions and 4) exclusion from analysis data from motifs during which females were observed to be vocalizing.

The authors also have added to the text several important clarifications, and modified language in several ways that improve the presentation and interpretation of results. This includes 1) noting that differences between neural activity during singing with and without DAF does not necessarily reflect "error detection" but could instead reflect how neurons with fixed auditory receptive fields might respond differently to the distinct auditory inputs present between these conditions, 2) clarifying that the recordings were not specifically restricted to Field L, but were distributed more broadly across the auditory pallium, and 3) discussing some of the mechanisms whereby tuning might change due to various sensory, motor and internal factors associated with differences between singing alone and singing to a female.

I only have a couple of areas of remaining concern that I think could be addressed with further analysis, or some additional discussion, according to the authors' preferences.

(1) Stationarity of neural response

My main residual concern relates to the issue raised in the previous round of review of how much of the observed difference in activity between morning sessions when the male is alone and later sessions when the male is singing to a female could reflect changes in neural

response properties (non-stationarity) due to the passage of time (sometimes at least several hours) rather than specifically due to the presence or absence of an audience.

The authors restriction of data to recordings that passed a stability criterion for unit waveforms is helpful in addressing whether the same units are 'held' over the course of the experiment. However, even with well isolated units, the response properties or tuning of units can change over time due to a variety of factors that include changes in internal state, circuit excitability, up and down states, neural plasticity, etc.

The previous review noted several examples of data from the manuscript that illustrated this concern - instances where response properties of neurons appeared to change over time within a given condition. Any such changes in response properties that occur in the absence of a change in audience would tend to contribute to the reported "retuning" of responses.

One thing that the authors could do to address this issue would be to discuss potential contributions of non-stationarity of responses over time as a potential confounding variable and then editorialize about why they think this seems unlikely to explain many cases in which response properties change between conditions. See comments to authors for one specific suggestions along these lines.

Alternatively, the authors could carry out additional analyses to evaluate this issue more quantitatively. For example, by measuring the magnitude of "spontaneous" changes in responsiveness observed within conditions (such as by comparing the motif aligned activity for the first n examples within a condition against the activity during the last n examples) and comparing that with the magnitude of changes observed across conditions.

Another approach would be to carry out some sort of "change point analysis" on the motif-related activity for each experiment in order to establish how often the most abrupt changes in activity occur at the transition between conditions versus spontaneously at other times.

Lastly, while the experimental design didn't specifically include interleaved blocks of undirected (alone) singing and female directed singing, the methods indicate that the female directed singing data were collected by repeatedly introducing females for 10 minutes at a time. If there are even a couple of cases where the males produced song alone in the periods between female presentation, it would be worth testing whether modulation of neural firing tracked these interleaved conditions.

Previously published work such as the interleaved recordings of Hessler and Doupe indicate a close and reversible tracking between modulation of neural activity in sensorimotor song system nuclei and switches between singing alone and singing to a female. With respect to the possibility raised in the rebuttal of whether males continue to sing 'female directed song' even after the removal of a female, these and other published data suggest that this is not likely to be the case. But if this were a concern in interpreting any data, the authors could directly assess the male's song for previously described changes in acoustic variability that also track changes in the presence of an audience.

(2) Further discussion of how an audience might influence responses.

With respect to the mechanisms whereby an audience might modulate neural responses, a somewhat expanded discussion of possibilities with reference to relevant literature would be helpful. This could include reference to evidence for various neuromodulatory systems participating in modulating singing related activity in song system nuclei based on presence or absence of a female - do these neuromodulatory systems project to the relevant regions of the auditory pallium or its lower-level inputs within the ascending auditory pathway such that they could concurrently act on auditory circuitry?

In addition to possibility that the presence or absence of an audience affects auditory circuitry via a change in attention, alertness, or motivation, might efference signals associated with singing or locomotion/dancing reach and influence auditory pathways? Given that premotor activity and acoustic structure of song differ between conditions, might any singing-related efference copy activity that reached auditory regions also differ between conditions? A related interesting possibility that could be worth noting is that the presence of a female generally elicits increased locomotion and dancing on the part of the male that accompanies female directed song. Several studies have noted that general forms of locomotion can also result in efference copy signals reaching and influencing auditory regions (e.g. see Schneider and Mooney, Annual Review, 2018; Han et al. "Locomotion-induced neural activity independent of auditory feedback in the mouse inferior colliculus" iScience 2026 - the latter reference is interesting in that it appears to indicate bi-directional modulation of neural activity as observed across units in the current study).

Minor:

(1) The authors describe some units as showing "Activation by the absence of DAF." Because the birds in the study have extensive experience with DAF on a subset of trials, it is possible that the increased responses in the absence of DAF reflect a positive deviation from expectation of distortion (as seems to be the case for VTA neurons in previous work). But it is also possible that the broadband DAF stimulus drives inhibition of auditory responses in some cases, and the greater responses in the interleaved trials with normal feedback simply reflect the absence of that inhibition (rather than a positive deviation from a learned expectation). In keeping with the authors shift away from the use of "error detection" elsewhere in the manuscript, it might also be good to use less interpretive language here instead of "activation by absence of DAF".

(2) At the authors discretion, it would be interesting to know if there is any relationship between the way in which changes in audience affect activity with normal auditory feedback versus with DAF. For example, if normal singing responses are attenuated in the female directed condition, are the responses to DAF also attenuated?

(3) In figure 2, the vertical dashed lines associated with the rasters indicate the onset and offset of motifs. For several of the figure panels, the spectrograms show motifs that are not aligned with these onsets and offsets. Please clarify or modify (are the rasters from time-warped data but the spectrograms are not -time warped?).

(4) The authors equate peaks in activity before the onsets of motifs with premotor activity: ["A previous study recording from Field L in zebra finches reported neural activations prior to the onset of singing, consistent with premotor signaling (Keller and Hahnloser, 2009). We tested for context dependent changes in premotor activity by examining peaks in neural activity aligned to motif onsets. Across the population neurons did not exhibit significant changes in the timing of motif onset-aligned activity (Figure S4)."]

However, the spectrograms as shown in Figures 1 and 2 indicate that each motif is often preceded immediately by other song syllables such as introductory notes or syllables from the end of the preceding motif. Further analysis would be required in the current study to demonstrate that the activity present before motif onsets reflects premotor activity rather than auditory responses to the proceeding syllables. Please soften the claim that this reflects premotor activity or provide additional analysis or argument.

https://doi.org/10.7554/eLife.91769.2.sa1

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

This study examines the context-dependent modulation of auditory cortical neurons in response to expected sensory input, either self-generated sounds or expected perturbations of self-generated sounds. Specifically, using songbirds, the authors ask whether social context (the presence of a female conspecific) affects 1) the response of auditory cortical neurons to the bird's own song when he is singing; and 2) the response of neurons to perturbations of auditory feedback that the bird has been trained to expect.

Strengths:

First, the authors report that across the population, the responses of the neurons does not differ when a male bird sings alone or if he sings to a female. A fraction of auditory cortical neurons, however, do show significant differences in the firing rate, precision, and/or degree of burst firing when males sing alone vs. when they sing to females. This finding is broadly consistent with the literature showing that sensory neurons (visual, auditory, somatosensory, etc.) can be rapidly reconfigured into different "information processing modes" depending on behavioral state (e.g., quiescence vs. vigilance).

For the perturbation experiments, the authors trained birds to expect distorted auditory feedback during a particular syllable. They found that some neurons showed greater responses during perturbation when a female was present (compared to when males were alone) while other neurons had smaller responses during perturbation when a female was present. In addition, the response of a small number of auditory cortical neurons were not affected by behavioral state. These results contrast with their prior report that the responses of midbrain dopaminergic neurons that project to the basal ganglia are "uniformly reduced" in the presence of a female, raising a question of how an evaluation signal is transformed in the circuit from the primary sensory region to the midbrain.

Weaknesses:

While the experiments and analysis are solid, the finding that social context can alter responses of auditory cortical neurons in a multitude of ways (increase, decrease or no change) raises several questions that can be examined with additional analysis. For example, do context-dependent differences in auditory responses derive from context-dependent differences in the songs? Are context-dependent differences present in all classes of neurons and throughout the auditory system?

The observed heterogeneity in the firing properties of auditory cortical neurons, both in response to self-generated sounds and during perturbations of auditory feedback, raises the question of which neurons are sensitive to social context (which likely can be addressed by the authors in a revision). The authors should provide additional details about the recordings:

(a) What are the locations of the recording sites? Prior work has shown that there is an organized map of spectrotemporal features of sounds in the auditory cortex of songbirds; spectral tuning widths change along the medial-lateral axis and temporal tuning widths differ between the input and output layers of Field L. Were the recordings primarily in Field L2 (thalamo-recipient region), L1 or L3? Were some recordings lateral to Field L in secondary auditory regions? Were the neurons that showed context-

> *dependent changes in firing properties localized or distributed throughout Field L (i.e., were the context-dependent differences in neural responses truly brain-wide)? At a minimum, the authors should include a schematic showing the different regions of Field L and a summary of the location of the recording sites. Images of the processed tissue with electrolytic lesions would also be helpful.*

We agree that the anatomical targeting and limits of localization should be made explicit. In the original manuscript, we referred broadly to recordings from "Field L" and described targeting coordinates in the Methods. In the revised manuscript, we have softened the anatomical claim from "Field L" to "auditory pallium" where appropriate, while explicitly stating that electrodes were aimed at Field L. We also added anatomical caveats and a new supplemental figure.

The revised title and abstract now reflect this more conservative anatomical framing. For example, the abstract now states: "Here we recorded neural activity from the auditory pallium in zebra finches practicing singing alone and directing courtship songs to females." In the Introduction, we now explicitly state both the intended target and the limitation: "We targeted our recording electrodes to Field L, a primary auditory pallial area that projects into multiple higher auditory areas that, in turn, project to VTA."

We then added the caveat: "Field L is composed of multiple subdivisions and surrounds the interfacial nucleus and because the implanted wire bundles spread in a small radius of up to ~0.5 mm, our recordings likely included large territories of the auditory pallium (Figure S1)."

We also added mechanistic/anatomical context for why these recordings may reflect activity shaped by broader auditory forebrain circuitry: "Although Field L is classically described as a primary auditory thalamorecipient region, its activity may also be shaped by contextual signals related to the courtship context, potentially via recurrent interactions with higher-order auditory forebrain regions such as the caudal mesopallium (CM) and caudomedial nidopallium (NCM) (Bauer et al., 2008; Figure S1C)."

Changes made in revision: We added Figure S1, which includes anatomical subdivisions, an example histological slice showing the area where the cannula was implanted, and auditory pathway connectivity. We also revised the wording throughout the manuscript from "Field L neurons" to more conservative phrasing such as "auditory pallium neurons" or "pallial auditory neurons" when appropriate. We did not claim layer-specific localization, because the revised manuscript explicitly states that we cannot make such claims.

> *(b) Was the context-dependent modulation limited to a particular class of neurons (distinguished by spike waveform shape, spontaneous firing rate, or other feature)?*

We agree that identifying whether context-dependent modulation is associated with specific neuronal classes is important. In the revision, we added analyses examining relationships between spike width and various firing characteristics. We also looked for potential relationships between mean rate and DAF response, IMCC and DAF response, and found no clear trend. Overall, we did not observe any clear relationship between DAF response, DAF response modulation, and metrics like spike width or mean firing rate.

The revised manuscript states: "Action potential width of individual neurons has previously been used to classify putative interneurons or putative principal cells in the zebra finch auditory pallium (Calabrese and Woolley, 2015)."

We then describe the new analysis: "We tested if DAF-response scores, mean firing rates during singing, burst fraction, IMCC, and the change in all of these between undirected and directed singing was correlated with spike half width (spike half-width measured as peak-to-trough time; Figure S5)."

The revised result is: "DAF response in either condition, the change in DAF response across conditions, and the change in firing rate, burst fraction, and IMCC were not significantly correlated with spike width (Figure S5A-B)."

We also report that some general firing properties did correlate with spike width: "Consistent with previous literature, firing rate was significantly correlated with spike width (Pearson's correlation, p=0.003; Figure S5C). Interestingly, burst fraction (p=8.3x10-4) and IMCC (p=5.4x10-5) were also significantly correlated with spike width (Figure S5C)."

Changes made in revision: We added Figure S5 and associated text analyzing whether context-dependent changes in DAF response, firing rate, burst fraction, and IMCC were correlated with spike half-width. These analyses did not support the conclusion that context-dependent DAF modulation was restricted to a waveform-defined neuronal class.

> *(a) Prior work has shown that songs of zebra finches differ slightly when males sing alone compared to when they sing to females: songs are faster; pitch is less variable; and the number of introductory elements is greater when males sing to females. Do some of the observed social context-dependent differences in the responses of auditory neurons reflect differences in the songs in the two conditions? Did the authors of this study also find premotor activity in Field L, and if so, did it differ between the two social contexts? Might differences in Field L responses reflect motor/song differences?*

The revised manuscript now addresses the issues of context-dependent changes in song in several ways. First, we explain why motif-aligned comparisons are meaningful: "The acoustic structure of undirected and directed motifs is highly similar in adult finches, enabling singing-related neural activity to be precisely aligned and compared across contexts."

Second, we added analysis and discussion of premotor-related activity. Changes made in revision: New results paragraph and new Fig S4. "A previous study recording from Field L in zebra finches reported neural activations prior to the onset of singing, consistent with premotor signaling (Keller and Hahnloser, 2009). We tested for context-dependent changes in premotor activity by examining peaks in neural activity aligned to motif onsets. Across the population neurons did not exhibit significant changes in the timing of motif onset-aligned activity (Figure S4)."

> *(b) For the perturbation experiments, this raises a question of whether perturbation amplitude is different when a male is alone and when a female is present. It would be useful to know if (and how much) perturbation amplitude varied depending on the location inside the cage as well as whether the sound pressure level of the underlying song was higher (e.g., Lombard effect).*

We previously calibrated the perturbation amplitude in Roeser et al., 2023, in an identical recording setup. Two speakers deliver the feedback on either side of the bird's home cage. We acknowledge the possibility that the position and orientation of the bird can affect the way the sound hits either of the bird's eardrums and thus potentially affect a neural response. However, neural activations following the absence of distortion playbacks were a major feature of the dataset and were context-dependent in some cases.

The Methods state: "DAF was implemented with a custom LabVIEW acquisition program that analyzed song syllables in real-time and delivered syllable-targeted feedback." and "DAF (50 ms broadband noise bandpass filtered at 1.5-8 kHz to match frequency range of zebra finch song) was played over speakers in the recording chamber on top of a specific target syllable randomly on 50% of motif renditions."

The revised manuscript also makes clear that experiments occurred in the bird's home cage: "Experiments were carried out in the male's home cage, which was inside a sound isolation

chamber."

Importantly, the revised Results show that not all DAF-related responses were simple activations to additional sound. Some neurons were activated by the absence of distortion: "Unexpectedly, some neurons were not activated by the song distortion but rather by the lack of target syllable distortion." and "These activations following undistorted renditions could also depend on the courtship context."

Changes made in revision: We clarified the DAF stimulus and recording setup in Methods and added Figure 3 showing neurons activated following undistorted renditions. These data argue that context-dependent responses are not simply explained by DAF sound amplitude, although we do not claim that position-dependent acoustic variation was fully eliminated.

> *Finally, it would be helpful if the authors could include a model and/or more discussion of how the uniform attenuation in midbrain dopaminergic neurons may arise given the heterogeneous responses in Field L.*

The revised manuscript provides evidence for context-dependent retuning upstream of VTA, but does not offer a direct mechanistic explanation for the uniform attenuation seen in dopaminergic neurons. The revised Discussion states: "Because the main goal of this study was to test if courtship-associated reduction in DAF signaling, recently observed in VTA DA neurons (Roeser et al., 2023), resulted from a local process in VTA or reflected a retuning of auditory responsiveness, we explicitly tested for changes in DAF responsiveness between alone and female-directed singing."

It then explicitly contrasts auditory pallium and VTA: "Surprisingly, we discovered that Field L neurons could retune at the transition from lone to courtship singing in diverse ways, consistent with a more widespread process in the brain that does not fully explain the uniform DAF-signal attenuation observed in VTA."

Changes made in revision: We expanded the Discussion to explicitly state that auditory pallium retuning is heterogeneous and therefore does not fully explain the uniform attenuation observed in VTA. We do not present a formal circuit model, but we now more clearly frame the result as evidence for broader sensory retuning that is likely transformed downstream.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *In the manuscript, Jones and Goldberg study auditory cortex in male zebra finches. They explore song-related responses in two different contexts, when the male is either alone or in the presence of a female. They find a heterogeneity of responses, in line with auditory cortical neurons computing the social modulation of responses found in VTA.*
>
> *Weaknesses:*
>
> *Stability of responses has not been studied: some neurons seem to have responses that slowly drift in time, which could lead to observed differences between alone and with-female conditions. Also, possible motor confounds and sound-of-audience confounds should be addressed. The language is often imprecise.*
>
> *Stability and Reversal: It is a bit unfortunate that stability of effects seemingly has not been studied by reversing experimental conditions. The work would be much stronger if authors could show that audience-dependent tuning is robust in individual cells. Did they record from some neurons during reversal back to the alone condition?*

We agree that recording stability is essential. A reversal experiment was not feasible for this dataset, as it is difficult to confirm whether song motifs produced immediately following female presence represent undirected singing or are directed to an unseen but recently present female. Instead, the revised manuscript adds a strict unit-stability criterion based on waveform similarity across conditions.

The revised Results state: "Importantly, because these neural recordings were performed over long time courses (~2-8 hours), a strict threshold for stability was imposed." The exact criterion is: "A Pearson's correlation coefficient of at least 0.99 between the average neural waveform during undirected and directed singing was required for a unit to be considered stable (Dickey et al., 2009; Figure S2)."

Changes made in revision: The strict waveform-stability inclusion criterion and new Figure S2 directly showcase unit stability across the time course of the experiments.

> *Motor responses: Does DAF playback change song? If so, especially if it applies only in one of the two conditions (audience/no audience), then the observed response differences could be motor-related rather than auditory responses.*

We agree that motor confounds must be minimized. We previously found that DAF did not affect the acoustics of the subsequent syllable (Gadagkar et al., 2016). The revised manuscript clarifies that DAF and undistorted trials were randomly interleaved and analyzed by comparing matched renditions within conditions. Importantly, we only analyzed motif-aligned activity, ensuring that all syllables within the song motif are the same.

Changes made in revision: We clarified the DAF analysis framework and added a more conservative permutation-based analysis comparing distorted and undistorted trials within each context, then comparing those DAF-response vectors across contexts. We do not claim that all possible motor consequences of DAF are eliminated, but the analysis directly tests neural responses to randomly interleaved distorted versus undistorted renditions.

> *Similarly, motif-aligned spiking activity was time warped to the median duration of undirected or directed motifs. Could the shorter motifs during directed song lead to alignment differences that would account for the different error responses in alone/with-female conditions?*

We agree this is an important technical point. The time-warping we conducted, standard in the field, compensates for the tempo differences between directed and undirected song. Importantly, our main analysis of change in error response no longer uses a 100 ms response window, but rather includes all windows in the motif.

Changes made in revision: We clarified that the revised DAF response analysis uses motif-aligned, time-warped spike trains. Importantly, the revised analysis moves away from relying on a single scalar response window and uses bin-wise permutation tests with family-wise error correction.

> *Audience versus sound of audience: Is it truly the audience that causes the difference in error responses or is it the sounds the audience makes?*

We agree that the sensory cues defining "audience" cannot be fully separated in this experiment. The reviewer raises an important point that female zebra finches occasionally call at the male. We have excluded all song motifs from analyses that include an overlapping female call.

The revised Methods now explicitly state that motifs overlapping with female calls were excluded: "Any motifs that had overlapping time with a female call in directed motifs was excluded from analysis."

We also revised the Discussion to treat the mechanism by which auditory pallium receives information about the female as an open question: "An open question is how auditory pallium receives information about whether a female is present, and how this information influences neural activity."

Changes made in revision: We excluded motifs overlapping with female calls and added discussion explicitly acknowledging that how female presence is represented in auditory pallium remains unresolved. We do not claim to distinguish visual, auditory, social, or motivational components of the female-present condition.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *In this study, Jones et al. examine how neural activity in a primary auditory area (field L) of singing male songbirds is modulated by the presence or absence of an audience (a female conspecific). Prior work has demonstrated that the presence of an audience attenuates the responses of dopaminergic neurons to distortions of auditory feedback (DAF). Here the authors report that even in a region that is primarily considered sensory, responses to DAF are also modulated by the audience, although in a heterogeneous manner. However, to be fully persuasive, additional analyses will be required to address how much of the apparent modulation by audience may be explained by other factors such as changes in recorded neurons or their properties over time.*
>
> *(1) A central concern relates to whether the main reported effects associated with differences in singing directed versus undirected song reflect only those changes in conditions, versus contributions from changes in unit isolation or response properties over time.*

We completely agree that unit stability is critically important in this study. To address this concern, we now quantify stability and apply strict inclusion criteria adopted from a study that assessed unit stability over days (Dickey et al., 2009). Additionally, we now include average waveform overlays for all example units across conditions as supplemental Figure S2.

Changes made in revision: We added: "Importantly, because these neural recordings were performed over long time courses (~2-8 hours), a strict threshold for stability was imposed." and "A Pearson's correlation coefficient of at least 0.99 between the average neural waveform during undirected and directed singing was required for a unit to be considered stable (Dickey et al., 2009; Figure S2)."

> *(2) A second concern has to do with the categorical definition of 'error neurons'. The authors define a subset of neurons as error responsive only if their responses to DAF exceed a specific threshold (2.5 standard deviations). The problem is that for some neurons categorically defined as being responsive to DAF in only one condition, there is almost certainly not a significant difference in the actual responses to DAF between conditions.*

We overhauled our analyses characterizing DAF responses. Rather than relying only on a 2.5 z-score threshold, we now use a more conservative permutation-based approach that directly tests DAF responsiveness and context-dependent changes in DAF responsiveness.

The revised Results state: "Statistical tests defining auditory neurons as DAF-responsive or not in a binary fashion may not be suitable if the underlying population of DAF-related responses exist on a continuum from responsive to non-responsive."

The updated result is: "This more conservative approach identified 48/147 neurons as DAF-responsive in at least one condition, with 13 of those neurons exhibiting a significant modulation in their DAF response between undirected and female-directed singing."

> *(3a) Some discussion of what is already known about the auditory tuning of Field L, and the extent to which responses associated with distortion of feedback may reflect the frequency tuning of Field L neurons versus something that might be construed as more specifically as detecting an error in perceived feedback.*

We agree that DAF-related changes in firing do not necessarily imply that neurons are explicitly detecting an "error" between predicted and actual feedback. Field L neurons can have spectrotemporal receptive fields and frequency tuning such that a broadband DAF stimulus could drive excitation or inhibition simply because the stimulus overlaps with excitatory or inhibitory regions of a neuron's receptive field. We therefore revised the manuscript to use more cautious language and to describe these responses as DAF-related or feedback-related signals rather than categorically as "error responses".

Changes made in revision: The title was changed from "Auditory cortical error signals retune during songbird courtship" to "Auditory cortical feedback signals are modulated during songbird courtship". We also added a sentence to the Discussion: "However, it is important to note that DAF-related changes in firing in auditory neurons do not necessarily imply that neurons compute sensory prediction errors. DAF-related responses could arise from ordinary auditory tuning to the broadband distortion stimulus."

> *(3b) It would also be useful to discuss further previous work on differences in auditory tuning or responses between conditions when subjects are vocalizing, versus when vocalizations are played back, and to what extent efference copy signals might contribute to the processing of feedback distortions.*

We agree these are important points. Our experimental design did not include sufficient passive bird-own-song (BOS) playback trials to permit quantitative comparisons with vocalizing conditions, and we therefore cannot draw firm conclusions about the contribution of efference copy signals to the DAF responses described here. We did observe robust motif onset-associated neural activations, including some activity preceding motif onset, which were present across both social contexts (see new Figure S4). These observations are consistent with prior reports of premotor-related signals in Field L (Keller and Hahnloser, 2009), but whether such signals contribute differentially to DAF processing across contexts remains an open question that we now acknowledge in the Discussion.

> *(3c) To what extent did the current study control for any vocalizations or other sounds produced by females during the directed singing, and could this have contributed to differences in Field L activity between conditions?*

Please see response R2.4 above, in which we describe the exclusion of all song motifs that overlapped in time with a female call. This exclusion criterion was applied throughout all analyses of directed singing.

> *Figure 1D: In the directed condition there are no spikes at all following the first handful of motif renditions. Were the directed and undirected recordings interleaved here?*

Undirected and directed trials were not interleaved. The raster plots are presented in chronological order; however, for each behavioral condition, rows are sorted with the

earliest renditions at the bottom and the most recent at the top. We have clarified this in the figure legend.

> *A minor issue: the raw example trace with male alone does not seem to have a corresponding set of points in the raster plot. For panel E, I also cannot find rasters that correspond to the example recordings shown at top.*

In the original version, we randomly downsampled the condition with more trials to equalize trial counts across conditions in the example rasters, while performing all analyses on the full set of recorded trials. As a result, the example spike shown in the raw trace was drawn from one of the downsampled trials not displayed in the raster.

Changes made in revision: For greater transparency, we now include all trials from both conditions for each example neuron in Figure 1.

> *Figure 2A also shows a neuron that looks like it has non-stationarity; for the alone condition without altered feedback, the main peak has no spikes for the bottom half of the rasters.*

In the original version, example neurons were selected to illustrate the DAF-response scoring method, which in some cases highlighted neurons with less stable response profiles. In the revised manuscript, we have replaced this example with neurons that exhibit more robust and stable DAF-related responses, and we now provide a broader set of example neurons illustrating both increases and decreases in DAF responsiveness across conditions.

> *Other figures show firing rate distributions that appear to be very non-Gaussian, with some motifs during which there is a lot of activity, and others in which there is little activity. Please consider applying non-parametric tests as appropriate.*

We agree. In general, some neurons exhibited non-uniform firing rate distributions across trials. All of our main analyses are now conducted using non-parametric permutation tests, which do not assume a Gaussian distribution of trial-by-trial firing rates.

> *Approaches to addressing the non-stationarity issue could include more specifically indicating examples in which recordings from the alone condition and directed condition are interleaved and exhibit reversible changes in the pattern of responses.*

Unfortunately, nearly all of our undirected and directed recording periods were not interleaved, as the experimental design required a block of undirected singing followed by directed singing with female presence. We find it informative, however, that DAF-response modulation was observed in both directions, with some neurons losing DAF responsiveness during directed song and others gaining it, a pattern that is difficult to attribute to a simple unidirectional drift in recording quality. We now provide additional examples illustrating both directions of modulation in Figures 2 and 3.

> *The methods and/or raster plots should include some further explanation of the time periods over which recordings were made in the alone versus directed conditions, and the extent to which they are interleaved or not.*

We have clarified this in the revised Methods. In brief, recording began when the home cage lights came on each day, with the male left to sing alone until at least 40 undirected song motifs were collected. A female was then introduced in approximately 10-minute intervals until at least 40 directed song motifs were collected. The total recording duration on a given day ranged from 0.56 to 10.27 hours, reflecting variability across birds in the time required to elicit sufficient singing in each context. We have added this information to both the Methods and relevant figure legends.

> *It would be most helpful to assess the stability of waveforms and unit isolation across time.*

We now apply strict inclusion criteria based on waveform stability, as described in R3.1 above. SNR was quantified as Vpp/(2*sigma_noise), where Vpp was the peak-to-peak amplitude of each filtered spike waveform and sigma_noise was estimated from the median absolute deviation of the filtered voltage trace. This combines the peak-to-peak normalization used by Nordhausen et al. (1996) with the robust noise estimator described by Rey et al. (2015). Waveform overlays for all included example units are provided in Figure S2.

> *It would be reassuring to see that significant differences between conditions are equally or more prevalent under the conditions of greatest unit isolation and recording stability.*

The average SNR of neurons ultimately included in the analysis was 9.47 +/- 3.57, with a minimum of 4.69. Neurons that exhibited significant DAF-response modulation did not have a significantly different SNR than neurons that did not exhibit significant modulation (Wilcoxon rank-sum test, p=0.38). The mean SNR for significantly modulated neurons was 8.70, compared to 9.5 for non-modulated neurons, indicating that the detection of context-dependent modulation was not systematically biased toward neurons with lower recording quality.

> *One other way that the authors might be able to address the main concern would be to look at the stability of firing patterns within conditions.*

We agree that stability of firing patterns within conditions is an important consideration, and this concern directly motivated the adoption of the permutation-based analysis described above. In this framework, the observed DAF-response difference between conditions is compared to a null distribution generated by shuffling condition labels across trials. This approach inherently accounts for within-condition trial-by-trial variability and does not assume stationarity of firing rates.

> *It would be helpful to have additional explanations of the criteria used for counting spikes, and assessing stability of recordings.*

Spike waveforms were visually inspected for consistency using our custom MATLAB GUI on a 12-second file basis. Interspike interval violations below 1 ms were explicitly checked as an indicator of multi-unit contamination. Detection thresholds were manually set, and each recording file included in the analysis was independently inspected. We have added a more explicit description of these procedures to the Methods section.

> *For the specific examples shown in figures, it would be useful to indicate by small tick marks or otherwise which spikes were counted as single units.*

We appreciate this suggestion. In the revised figures, we have improved the clarity of the example raw voltage traces by annotating the detection threshold and, where multiple units were present on a channel, indicating the waveform amplitude range corresponding to the isolated single unit. We believe this provides sufficient transparency regarding spike identity without requiring tick marks on every individual spike, which would substantially reduce legibility of the example traces.

> *What were the criteria for determining multi-unit versus single-unit activity?*

In the context of this manuscript, "multi-unit activity" refers to channels on which no single neuron could be reliably distinguished from others based on waveform shape and amplitude. Units ultimately included in the study were those for which a single, consistent waveform cluster could be identified and isolated in the custom GUI. In cases where a second

distinguishable unit was present on the same channel, it was manually excluded from the sorted single-unit record. We have clarified this distinction in the Methods.

> *Categorical scores: This definition results in cases where responses of 2.45 vs 2.55 are described as 'retuned', even if these responses are not significantly different. Retuning would be more persuasively demonstrated if the authors could provide a test of whether or not the responses for individual neurons differ significantly between conditions.*

We completely agree, and thank the reviewer for motivating us to develop a more rigorous statistical approach. Our revised analysis uses a non-parametric permutation test that explicitly tests for significantly different DAF responses between undirected and directed singing conditions, with correction for multiple comparisons. This replaces the previous threshold-based categorical classification and directly addresses the concern that neurons near the threshold boundary were being treated as categorically different.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Minor comments:*
>
> *(1) Please include a schematic of the brain, including the different subregions of Field L and the connections between auditory regions and the midbrain.*

Done. Figure S1 has been added, including a schematic of Field L subdivisions and auditory pathway connectivity.

> *(2) The authors should include some additional information about the recordings, such as the proportion of Field L neurons that exhibited singing-related changes in firing rate. It would be helpful to include some examples of spontaneous activity when the bird is quiescent in Figs. 1-2, especially for cells that do not show firing locked to song.*

We appreciate this suggestion. Given the scope of the current revision and the primary focus on DAF-response modulation, we have elected not to add spontaneous activity examples to Figures 1-2 at this time. We agree this would be a valuable addition in future work and have noted it as a limitation in the Discussion.

> *(3) Methods, p. 10: Surgery and awake-behaving electrophysiology: "The of the cannula" - this is the only mention of a cannula. Do the authors mean the ends of the probes?*

Cannula placement and wire bundle extension from the end of the cannula has been clarified in the Methods.

> *(4) Bottom of p. 10: Fix reference for biorxiv paper: "ref andreas paper"*

Fixed.

> *(5) Methods, p. 12: Redundant sentences regarding significant error response criteria.*

Fixed. The redundant sentences have been removed.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *(1) The abstract is too vaguely formulated. Authors should try to quantify the statements already in the abstract.*

We have reworded the abstract to align with the revision's more conservative claims regarding social context modulation of auditory feedback, and have added specific quantitative statements where possible.

> *(2) Authors repeatedly refer to 'perceived song errors' without performing experiments or reporting on behavioral readouts of how birds perceive the jamming sounds. The wording should be changed to something more neutral, e.g. 'DAF responses'.*

We revised the manuscript throughout to use more neutral language centred on "DAF-related" or "feedback-related" responses rather than "perceived errors" or "mistakes". The title was changed from "Auditory cortical error signals retune during songbird courtship" to "Auditory cortical feedback signals are modulated during songbird courtship". We similarly revised the abstract and all relevant passages in the Results and Discussion.

> *(3) Authors write that 33 neurons were DAF responsive in both conditions. How should we interpret this overlap relative to independence and identity assumptions?*

We agree that the original presentation made the interpretation of overlap across conditions unclear. The observed overlap is greater than expected under a strict independence assumption but smaller than expected if responsiveness were identical across conditions, consistent with partial but incomplete sharing of DAF responsiveness across social contexts. In the revised manuscript, however, we have moved away from this binary classification framework because DAF responsiveness appears to vary continuously across neurons. The permutation-based analysis now directly tests for changes in DAF responsiveness across contexts without requiring categorical assignment.

> *(4) Only 10 neurons were not affected by courtship state or only 10 error responsive neurons were not affected? I suggest authors do a multivariate analysis or use a mixed effect model and summarize the result as a table.*

We agree that the categorical accounting of neurons across conditions was difficult to follow in the original manuscript. In the revised manuscript, we clarified the distinction between neurons responsive to DAF within a condition and neurons exhibiting significant modulation of DAF responsiveness across conditions. We now explicitly report: "This analysis identified 71/147 neurons as DAF responsive in at least one behavioral condition, whereas 76/147 were not responsive in either condition." and "This more conservative approach identified 48/147 neurons as DAF responsive in at least one condition, with 13 of those neurons exhibiting a significant modulation in their DAF response between undirected and female-directed singing."

> *(5) It would help if authors could define 'z-scored difference'. Better known is d prime, is this the same?*

For each neuron, the z-scored DAF response was computed as the z-scored firing rate difference between distorted and undistorted trials. Importantly, our revised main analysis avoids any normalization such as z-scoring, and instead uses a permutation-based approach applied directly to spike counts.

> *(6) Is the 'retuning' assessment a bit conservative? Neurons could also retune by showing error scores greater than 2.5 in both conditions but a shifted response time.*

We agree that neurons could retune by shifting the latency of DAF responses. Although potential latency shifts are beyond the scope of the current study, we did observe suggestive evidence of possible latency changes in some example neurons across conditions. We have noted this as an interesting direction for future analysis.

> *(7) Could the stability of DAF response across trials be described? E.g. as the ratio between intra versus inter condition variability?*

We agree that stability of DAF responses across trials is an important concern. In addition to imposing strict waveform stability requirements, our permutation-based statistical test

explicitly accounts for trial-by-trial variability by constructing null distributions from within-condition trial shuffles. We have also replaced the previously shown unstable example neuron with neurons that exhibit more consistent DAF-related responses across trials, and provide additional examples in Figures 2 and 3.

> *Minor:*
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> *(8) 'significant increase in burst fraction': specify effect size of t test in results section.*

We now specify in the main text: "A small but significant increase in burst fraction was observed (paired t-test, p=9.3x10-6, n=138 neurons, mean +/- SEM: 0.11 +/- 0.006 vs 0.15 +/- 0.007, Figure 1J)."

> *(9) The IMCC parameter should be specified in the main text.*

The Gaussian smoothing parameter (20 ms) has now been specified in the main text.

> *(10) Fig. 2: indicate the windows within which error scores are computed.*

This is no longer applicable, as the revised permutation-based analysis does not rely on scoring error responses within a fixed window.

> *(11) In Fig. 2A, the neuron has an error score of -2.54 (significant), but the red and blue curves look almost the same.*

We agree that the previous error score quantification did not always capture firing rate differences in an intuitive way. This example neuron has been replaced in the revised manuscript, and the new analysis avoids scalar error scores in favor of the permutation-based approach.

> ***Reviewer #3 (Recommendations For The Authors):***
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> *Minor points:*
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> *(1) "(ref andreas paper)." Add reference here?*

Fixed.

> *(2) Hessler and Doupe 1999 is a good reference for premotor signal re-tuning during courtship.*

We agree. The reference has been included in the revised manuscript.

> *(3) Page 5: "discharge depended on courtship state, using" - should this be "depending"?*

The original wording was intentional: "we tested how discharge depended on courtship state." We have verified this reads correctly in context and made no change.

> *(4) Page 9: "consistent with a brainwide process" - what is meant here?*

We have revised this wording. The revised manuscript replaces "brainwide process" with clearer language describing a distributed modulation of auditory responsiveness that is not confined to a single nucleus.

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