Using temperature to analyze temporal dynamics in the songbird motor pathway

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Summary

Many complex behaviors, like speech or music, have a hierarchical organization with structure on many timescales. How does the brain control the timing of behavioral sequences? Do different circuits control different timescales of the behavior? To address these questions, we use temperature to manipulate the biophysical dynamics in different regions of the songbird forebrain involved in song production. We found that cooling premotor nucleus HVC (proper name) slows song speed across all timescales by up to 45% while only slightly altering the acoustic structure, whereas cooling downstream motor nucleus RA (robust nucleus of the arcopallium) has no observable effect on song timing. Our observations suggest that dynamics within HVC are involved in the control of song timing, perhaps through a chain-like organization. Local manipulation of brain temperature should be broadly applicable to identify neural circuitry that controls the timing of behavioral sequences and, more generally, to study the origin and role of oscillatory and other forms of brain dynamics in neural systems.

Motor behaviors are built out of a sequence of movements that evolve through time. From the most basic, such as locomotion, to the most complex, such as playing the piano, the timing of movements is crucial. For some simple oscillatory behaviors, in which the movement evolves on a single timescale, it has been possible to identify the particular neurons and biophysics that control the temporal dynamics of the behavior — for example, pacemaker neurons in the stomatogastric ganglion 1 or the oscillator network that controls swimming in the leech 2.But what mechanisms underlie more complex learned behaviors that have structure on many timescales?

Birdsong exhibits a remarkably precise and hierarchically organized temporal structure 3, 4 mediated by a number of distinct, well-studied motor nuclei 5, 6 (Fig. 1a), allowing for an unprecedented view into the central control of motor timing. Adult zebra finches generate a 0.5–1.0s long song motif, repeated a number of times during a bout of singing 7. The motif itself is made up of song syllables —individual bursts of sound approximately 100ms in length occurring in a precise order. Syllables are highly stereotyped and often contain complex acoustic structure that can evolve rapidly (10 ms timescale). The duration of song

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elements at all timescales is stereotyped; trial-to-trial fractional variations in song timing are roughly 1% 8–10.

Are different brain regions responsible for the timing of motifs, syllables, and subsyllabic structure? Two forebrain nuclei in particular have been implicated in the control of the temporal structure of song: HVC and RA. HVC projects to RA, which in turn projects to the vocal motorneurons 11 as well as midbrain vocal control and brainstem respiratory areas 12. Previous electrophysiological studies have found evidence that these brain regions contribute to song structure in a hierarchical manner 13, 14 and have suggested that dynamics underlying the generation of different song timescales may reside in different brain regions. For instance, syllable-timescale dynamics have been suggested to occur in HVC 15 n while subsyllable-timescale dynamics may arise in RA 14, 16, 17. Additionally, portions of the midbrain and respiratory areas project back to HVC through thalamic nucleus Uvaeformis (Uva) 18, 19, raising the further possibility that syllables, which are tightly linked to respiratory patterning 20, may be timed by respiratory oscillator circuits9, 21. With current techniques however, it has been difficult to test ideas about the origin of dynamics that underlie the temporal control of song.

Localizing temporal dynamics with temperature

We set out to localize temporal dynamics within song control system, taking advantage of the fact that the speed of brain processes is strongly temperature dependent 22–24. The aim was to produce localized mild heating or cooling 25, 26, rather than inactivation for which cooling has also been used 27. The basic logic of our experiments is as follows: If the circuitry in a particular brain area is involved in controlling song timing, then cooling that area should slow the song. Furthermore, if the neural control of song is organized with a dynamical hierarchy (i.e. different song timescales controlled by biophysical dynamics in different brain areas), it should be possible to differentially alter the behavioral timescales by individually manipulating the temperature in these areas.

Dynamics in HVC

We started by bilaterally manipulating the temperature of nucleus HVC. We designed a device based on the Peltier effect, capable of rapidly heating or cooling HVC in a spatially restricted manner (Fig 1a–c, Supplementary Fig 1). Song timing was strongly affected by changes in HVC temperature. At colder temperatures, song motifs were produced more slowly than control songs (Fig 1d, Supplementary Fig 2). All birds (n = 10 birds) showed a significant increase in motif duration during cooling (range 16.9 to 44.9%, Fig 1e). Fractional change in motif duration (dilation) was found to change fairly linearly with temperature changes in the range 0 to −6.5°C (0A to 1A current, Fig 1f). The slope of this relation was used as a simple metric of temperature-dependent song dilation, which we refer to as stretch (%/°C, see Supplementary Methods). The stretch metric in different birds ranged from −1.89 to −3.97 %/°C, with a mean of −2.83±0.22 %/°C. Changes in song speed during cooling were immediate and persisted for an hour or more (Supplementary Fig 1d). Strikingly, temperature changes in HVC had only a small effect on the acoustic structure of the song (Supplementary Fig 4).

Does HVC cooling slow the song even at the shortest timescale of subsyllabic structure? We quantified this using a standard dynamic time warping algorithm based on the correlation of the control song to the cooled song (Supplementary Fig 5), and also by direct measurement of the duration of subsyllabic elements. The average dilation of subsyllabic structure for each song syllable was computed at each temperature condition (0 to −6.5 °C, Fig 2a), and the slope (in %/°C) of the dilation as a function of temperature was computed for each syllable (Fig 2d). The mean stretch for subsyllabic structure was found to be −2.88±0.12 %/°C, significantly different from zero (t-test, $p < 10^{-6}$). This observation suggests that biophysical dynamics in HVC may be involved in controlling song timing on a fine timescale.

How about the control of syllable onsets? Do respiratory circuits projecting to HVC (e.g. through Uva) act as a 'clock' that autonomously controls the initiation of syllables? If so, cooling HVC should have little effect on syllable onsets. Alternatively, syllable onsets may be linked to the completion of the previous syllable, in which case the interval between syllable onsets should increase during cooling as the duration of each syllable increases. In fact, the intervals between syllable onsets were significantly dilated by an average of −3.05 ±0.11 %/°C (t-test, $p < 10^{-6}$, Fig 2b,d). This is not consistent with a model in which syllable timing (or the timing of singing-related respiration) is autonomously controlled by circuit dynamics in respiratory circuits or any other area upstream or downstream of HVC.

The stretch of syllable-onset intervals was significantly correlated with stretch of the syllable contained within that interval (Fig 2e, $r^2 = 0.607$, slope =0.92±0.16), and more weakly correlated with the stretch of other syllables ($r^2 = 0.47$, see Supplementary Materials). In other words, for syllables that exhibited a larger stretch than average, the onset interval to the following syllable also exhibited a larger stretch than average. This is consistent with a model in which the onset of each syllable may be causally linked to, or triggered by, the end of the previous syllable.

The silent gaps between syllables were also significantly dilated by HVC cooling (−3.70±0.32 %/°C, t-test, $p<10^{-12}$, median=−3.29%/°C), suggesting that biophysical dynamics in HVC are involved in the timing of gaps. The cooling-related stretch of gaps was slightly larger than that observed for syllables (p<0.05, paired t-test, median gap stretch was 12% larger than median syllable stretch). Similarly, the average stretch of a syllable-onset interval was slightly larger than the stretch of the syllable contained within that interval (mean paired difference 0.26±0.088 %/°C, p<0.001, paired t-test, median interval stretch was 3.4% larger than median syllable stretch, Supplementary Fig 6). These observations imply that the circuit mechanisms involved in initiating song syllables may be different from those involved in generating structure within song syllables, as has been suggested by measurements of the variability in timing of gaps and syllables in natural singing [8, 9].

An analogous argument can be made for the timing of motif onsets; if there is a 'motif clock' outside of HVC that independently controls the interval between motif onsets, then cooling HVC should have little effect on motif onset intervals. In fact, motif onset intervals were significantly dilated by an average of −3.19±0.24 %/°C (Fig 2c,d,f, n=7 birds, t-test,

p<10^{-5}), which is not consistent with a model in which motif onsets are timed autonomously by circuit dynamics outside of HVC.

Dynamics in RA

While the HVC cooling experiments strongly suggest the involvement of HVC in generating the fine temporal structure within syllables, they do not rule out some involvement of other brain areas. In particular, circuit dynamics 14, 16, 17, 21 and connectivity 28, 29 within RA, as well as reciprocal connections from RA to HVC 30, have been implicated in the generation of these short timescales. In general, these models would predict that the song timing can be affected by manipulating circuit dynamics in RA. Here we directly test this prediction by bilaterally cooling RA during singing. We use a Peltier device similar to that used for HVC, but with attached gold probes (330 μm dia) that were implanted into RA to facilitate thermal conduction (Fig 3a, Supplementary Fig 7,8). At a distance of 200 μm from the probe, estimated to be the furthest extent of RA neurons, we observed a temperature drop of 10°C at the maximum current used. We also found that the RA cooling device produced a slight temperature change in HVC of roughly 30% of the temperature change in RA at each current level (Fig 3b).

As expected, because of the residual effect of the RA cooling probe on HVC temperature, we observed a slight increase in motif duration at higher cooling currents (n = 4 birds, Fig 3c, Supplementary Fig 9a). The effect of the residual HVC cooling on motif duration can be accurately predicted by the results of the HVC cooling experiments (Fig 1f) and fully accounts for changes in motif duration produced by the RA probe (Fig 3d). Thus, after incorporating a correction for HVC temperature changes, we find no evidence that changes in RA temperature affect song motif duration (p > 0.20, Fig 3e) or the timing of subsyllabic structure (Fig 3f), suggesting that dynamics in RA may not contribute significantly to song timing, at least by any mechanism that is sensitive to temperature changes in the range we were able to achieve here.

The fact that RA cooling had so little effect on song structure led us to ask whether our temperature manipulation had any effect on the neuronal properties in RA. In non-singing birds, RA neurons spontaneously generate tonic regular spiking 14, possibly associated with an intrinsic subthreshold membrane potential oscillation 31. We measured the spiking frequency of single units in RA in an anesthetized preparation while changing the temperature using the RA cooling probe. Cooling produced a rapid, roughly linear decrease in RA neuron tonic spiking rate (Fig 4, 19 cells from 7 birds, slope = 0.85Hz/°C) that resulted in a near cessation of spontaneous spiking at the coldest temperatures (Temp = −10°C). Our observation that a 10°C cooling of RA produces a 2.5-fold reduction in the intrinsic oscillation frequency of RA neurons, while the same manipulation had no detectable effect on song structure or timing, implies that these oscillations are not likely to be a source of dynamics underlying song production.

In contrast, the incidence of high-frequency spontaneous bursts in RA (Fig 4d, top), known to be driven by synaptic input from HVC under anesthesia and during sleep 32, 33, does not show a significant trend with temperature (p>0.6, Fig 4d). The bursts exhibited only a slight

cooling-related decrease in firing rate (5.6 Hz/°C; Supplementary Fig 9c). RA is thus capable of a robust response to bursting input from HVC, even at temperatures low enough to substantially suppress tonic spiking.

The control of song timing by HVC: Lateralization

The HVC and RA cooling experiments highlight the centrality of HVC in controlling song timing. An interesting question is raised by the fact that HVC is a bilateral structure: How are the two HVCs coordinated during singing? Bilateral multiunit recordings in HVC have revealed brief episodes of correlated activity across hemispheres that occur prior to the onset of each syllable (and at some acoustic transitions within long complex syllables) 34, 35, likely mediated by feedback pathways from RA to midbrain areas and bilaterally back to HVC 18, 19. Do these episodes reflect actual bilateral synchronization of the HVCs? If the two HVCs were, hypothetically, synchronized only at the beginning of the motif, after which they operated independently, cooling HVC in only one hemisphere should cause the two HVCs to become misaligned in time by more than a whole syllable by the end of the motif (compare control to bilaterally cooled song, Fig 5c), causing song degradation. However, we found that unilateral cooling of HVC did not produce song degradation, but resulted in slowed songs of normal acoustic structure (Fig 5b,c, Supplementary Fig 10, n=4 birds), ruling out any model in which hemispheric synchronization occurs only at motif onsets.

Do both HVCs contribute to song timing? Given previous observations of hemispheric dominance in songbirds 5, 36, it is conceivable that one HVC acts as a 'master clock' for song timing while the other follows as a slave. In fact, we observed that in all birds (n=4) cooling either HVC alone caused song slowing intermediate to that seen for bilateral cooling (Fig 5b, Supplementary Fig 10), thus ruling out the possibility that song timing is controlled by dynamics in a single hemisphere.

One possible explanation for the observation of intermediate slowing is that the left HVC might control the timing of some parts of the song, while the right HVC might control the timing of other parts of the song. In fact, we found that cooling right HVC or left HVC produced less uniform stretching of the song compared to bilateral cooling, as predicted by this model (Fig 5d–j, bilateral nonuniformity SD = 0.67%/°C; unilateral nonuniformity SD = 1.33%/°C,). This inhomogeneity in stretch during unilateral cooling is comparable to the mean stretch (~1.2%/°C), suggesting that a large fraction of song elements were not stretched by unilateral cooling (bilateral cooling: 14% not significantly stretched, left-only: 71%, right-only: 43%). Furthermore, some elements that were not stretched by right HVC cooling were strongly stretched by left HVC cooling, and vice versa (Fig 5d–h, Supplementary Fig 10). Consistent with this, stretch during left cooling was significantly anti-correlated with stretch during right cooling (Fig 5k, p=.026, see Supplementary Methods). Thus, it appears that there may be some switching of the control of song timing between the two HVCs on the timescale of song syllables or long subsyllabic elements.

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areas. What model of dynamics in the song control system could be consistent with all of
our observations? One possibility is strongly anticipated by the singing-related firing
patterns of HVC neurons that project to RA. These neurons burst extremely sparsely during
singing, each generating a single brief (~6 ms) burst of spikes at a particular moment in
every repetition of the song motif [37]. In addition, different HVC neurons burst at different
times throughout the song. We have proposed that, as a population, these HVC neurons form
chains of activity — like dominoes — that control the timing of the song [38]. The HVC
cooling experiments suggest that the dynamics underlying the sparse sequential activation of
HVC neurons reside at least partially within HVC; if the sparse HVC bursts were driven by
an independent upstream sequence generating circuit, cooling HVC should not have affected
the speed of the song. An interesting possibility is that such sequential chains of activity
arise, in part, from a chain-like synaptic organization within HVC [39–42]. In this case,
cooling HVC might simply increase the time it takes each neuron to burst following activity
in a preceding neuron, thus introducing an accumulating delay that slows down the chain.

While the cooling results rule out models in which brain areas outside HVC independently
or autonomously control song timing at any timescale, they do not exclude the involvement
of other brain areas important for song production, in particular feedback projections from
RA, through the brainstem/midbrain and Uva back to HVC [19, 21]. These projections could
form an integral part of the connectivity that generates sequential bursts in HVC. What is the
timescale on which this feedback might operate? In principle, every burst in HVC could be
driven through fast, rapidly cycling feedback through this loop, rather than intrinsic
connections within HVC. In this view, cooling anywhere in this feedback loop, including
RA, should introduce accumulating delays as well. The fact that we do not observe song
slowing from cooling in RA suggests that the feedback circuitry may not operate at this
rapidly cycling timescale, but less frequently during the song.

Thus, one interesting possibility is that HVC may contain multiple independent chains [8] (or
modules), which may be associated with syllables or long subsyllabic elements [34]. These
modules, each of which could run autonomously by virtue of circuit dynamics within HVC,
may then be linked together in time by the feedback connections from RA through the
thalamus and back to HVC (Supplementary Fig 11). The feedback circuitry may act to
detect the end of one syllable and rapidly and bilaterally initiate the next gap and syllable,
thus simultaneously continuing the song sequence and re-synchronizing the two HVCs. In
this case, cooling HVC would slow the production of a song syllable, because it is generated
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to the termination of the previous syllable by feedback circuitry.

How might a module of chain-like of activity in HVC produce a song syllable? During
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patterns simply follow the timing set by HVC, we would expect RA cooling to have a minimal effect on the timing of RA activity, whereas slowing the chain in HVC would necessarily slow the sequence of bursts in RA. Furthermore, if structures downstream of RA (brainstem motor neurons and syringeal muscles) respond rapidly to descending drive from RA, perhaps on a timescale the fastest song modulations (10–20ms) [38], we would expect that slowing the sequence of bursts in RA might slow the song while having a minimal effect on song acoustic structure. Thus, a simple model of chain-like dynamics in HVC that drives a fast response in RA and downstream structures is consistent with the electrophysiological data and the HVC and RA cooling experiments.

Here we have used local manipulation of brain temperature to identify components within the avian song system that control the timing of a complex behavioral sequence. This approach may be broadly useful to localize specialized brain circuits that control the timing of other behaviors. We have also used temperature changes to test ideas about the contribution of oscillatory dynamics in one brain region to song production, an approach that should be applicable for localizing the biophysical origin of oscillatory and other forms of brain dynamics, and for studying their role in brain function.

METHODS SUMMARY

Subjects

Subjects were adult zebra finches (>120 days posthatch) obtained either from our colony or from an outside distributor (Preferred Birds). All animal procedures were approved by the committee on animal care at MIT.

Cooling Devices

We used a small (0.7g) custom-built thermoelectric device based on the Peltier effect to cool HVC and RA. The HVC cooling device was constructed with two 1mm by 2mm gold cooling elements that contacted bilaterally the surface of the dura overlying left and right HVC. The temperature change in HVC was spatially restricted (Fig 1c), producing a maximal temperature change of only 0.5 °C in RA, the nearest brain region known to be involved in song production in the zebra finch [5, 44]. The current could be switched to flow bilaterally, or unilaterally through only the left or only the right cooling element. The RA cooling devices were equipped with a gold spike implanted into RA to facilitate heat transfer.

RA electrophysiology

A craniotomy was made over RA under isoflurane anesthesia (1.5%). The borders of RA were identified electrophysiologically and the cooling device implanted in the center. Single neurons were isolated using carbon fiber electrodes (Carbostar-1, Kation Scientific), with a signal to noise of greater than 10:1.

Temperature measurements

Temperatures were measured using small thermocouples (Omega, 5SRTC-TT-K-40-36). For HVC calibration, three thermocouples were inserted in one hemisphere, under

anesthesia, at depths of 0.5, 2.0, and 4.0 mm beneath the gold pad. In some birds, an additional probe was placed in contralateral HVC (at a depth of 0.5 mm). Once inserted, the thermocouples were secured with dental acrylic and the bird was placed in a cage and allowed to awaken. At each Peltier current level, three minutes were allowed for the brain to reach a steady-state temperature before measurements were taken from all thermocouple locations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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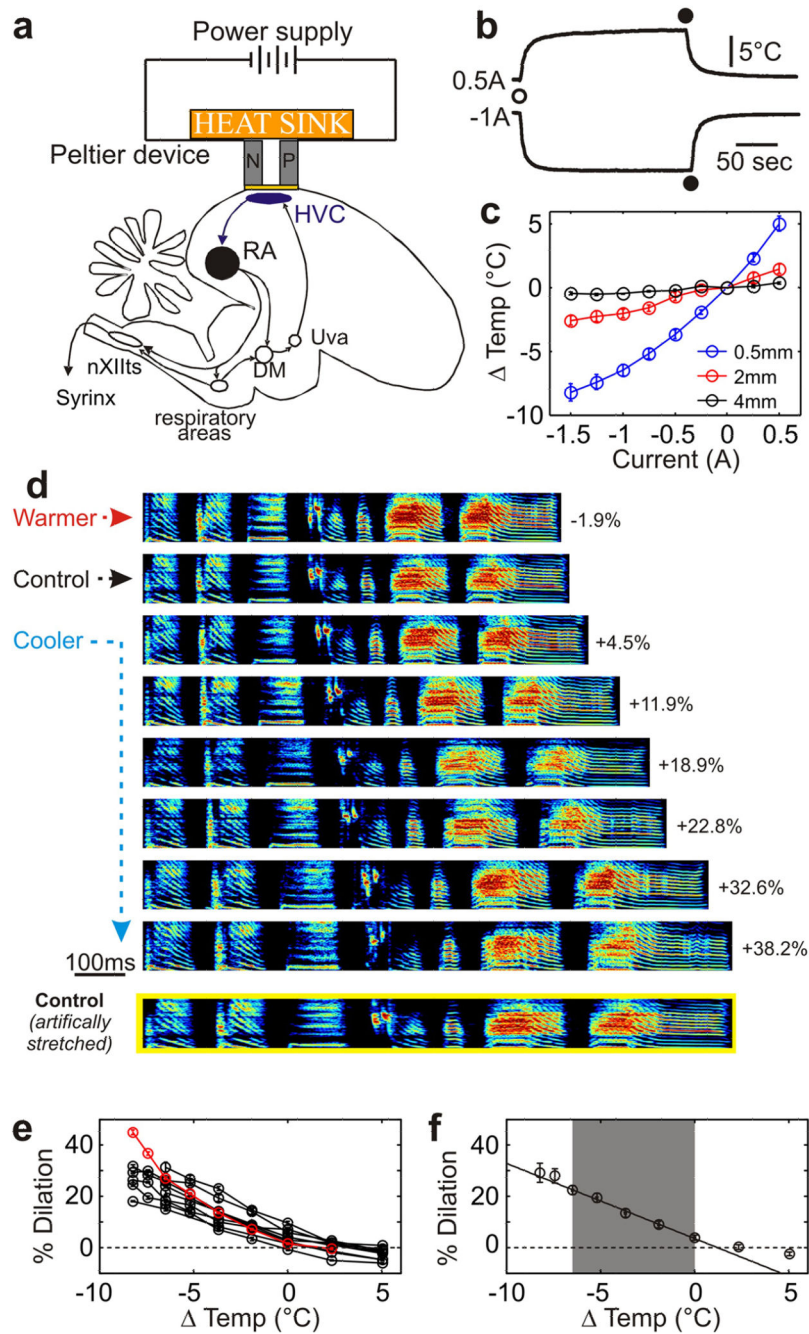
Figure 1. Changes in HVC temperature affect song duration

a, Schematic showing the Peltier device and relevant parts of the song production pathway. **b**, Temperature measurement in HVC as a function of time after onset (open circle) of the indicated current through the Peltier device (heating, top; cooling, bottom); current offset is at closed circles. **c**, Calibration curves for brain temperature changes at various depths under the Peltier device (n = 4 birds). **d**, Representative sonograms (freq: 1kHz to 9kHz) recorded from Bird #3 with HVC heated (0.25A) and cooled (0.25A to 1.5A, 0.25A steps). At bottom, spectrogram of the control motif artificially stretched. **e**, Fractional change in duration

(dilation) of song motif with temperature, relative to the pre-implantation song (n = 10 birds). The red symbols are from bird #3. **f**, Motif dilation averaged over all 10 birds. The shaded area represents the range over which the song stretch metric (%/°C) was calculated.
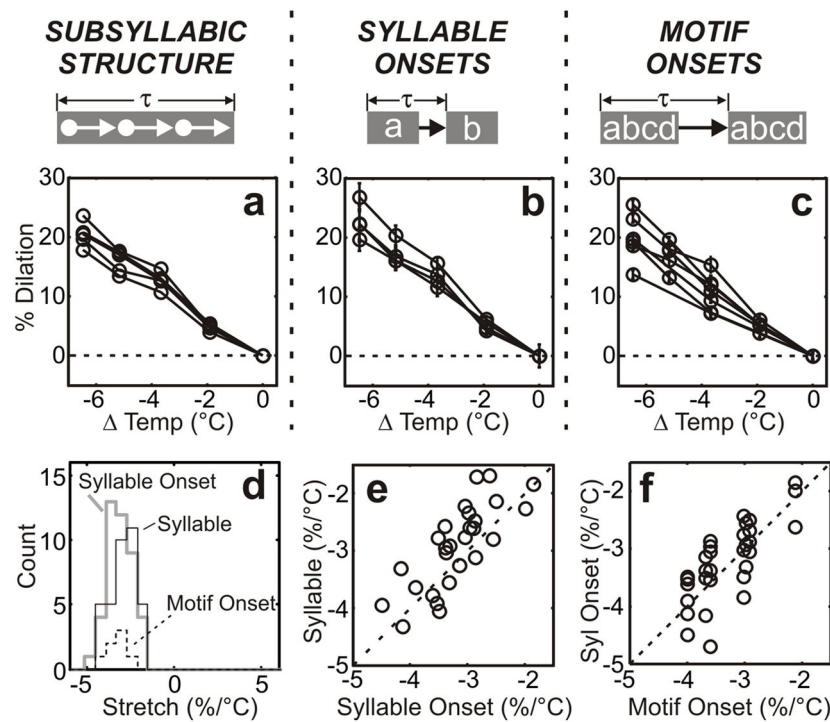
Figure 2. HVC cooling slows the song at all timescales

a, Dilation of subsyllabic structure versus HVC temperature change for all five syllables of bird #8. **b**, Dilation of syllable-onset intervals for the same bird. **c**, Dilation of motif-onset intervals for all 7 birds that produced concatenated motifs at all temperatures. **d**, Distribution of stretch metrics for the entire data set, including syllables (36 syllables, 8 birds,), syllable onsets (43 syllables, 9 birds,), and motif onsets (7 birds). Stretch of syllable-onset interval was strongly correlated with subsyllabic stretch (**e**) and motif-onset stretch (**f**) (for further details, see Supplementary Information).
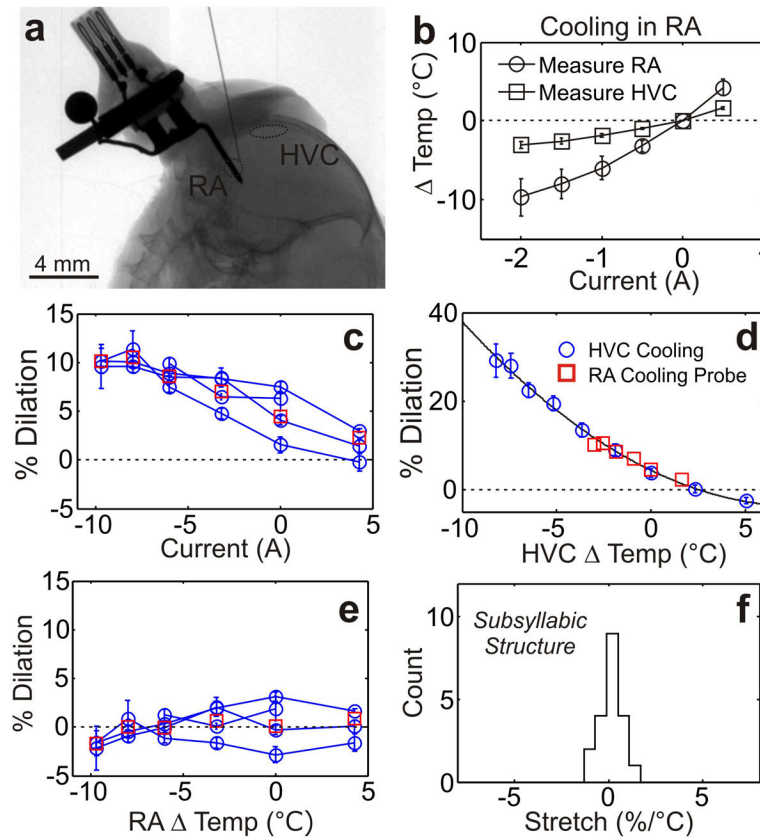
Figure 3. Effects of RA temperature change on song timing

a, X-ray image of the implanted RA cooling device and approximate locations of HVC and RA. **b**, Temperature in RA (200 μm from cooling probe) and HVC as a function of RA probe current. Note that the RA probe produces some cooling in HVC. **c**, Change in motif duration as a function of RA probe current (n=4 birds, red squares show mean). **d**, Average change in motif duration (red squares) during RA probe cooling or heating, plotted as a function of HVC temperature. Also plotted is the average change in motif duration (blue circles) as a function of HVC temperature measured in the HVC cooling experiment (Fig 1f). **e**, Change in motif duration as a function of RA temperature, corrected for the effect of HVC temperature change. **f,** Stretchof subsyllabic elements for the population of RA cooled birds (n = 4 birds, 20 syllables), corrected for HVC temperature change.
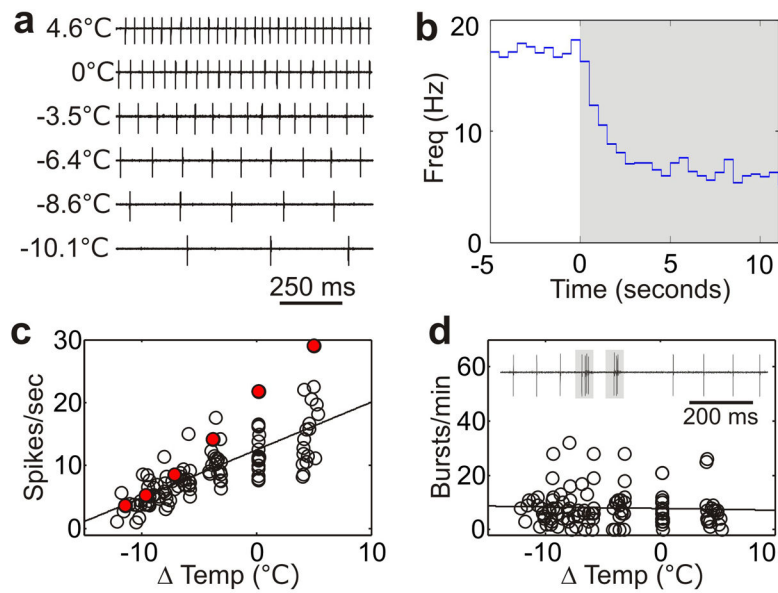
Figure 4. Effects of RA temperature change on RA spiking activity
a, An example of the tonic spiking activity of an RA neuron in an anesthetized bird at various temperature changes. **b**, Average firing rate response (25 trials) to the application of 1A cooling current to the RA probe. **c**, Average tonic spiking rate versus temperature for all recorded neurons (19 cells, 7 birds). Filled red circles are from the example shown in (a). **d**, Spike train showing tonic spiking and spontaneous bursts (top). Incidence of bursts (defined as an instantaneous firing rate greater than 100 Hz) for all neurons (bottom).
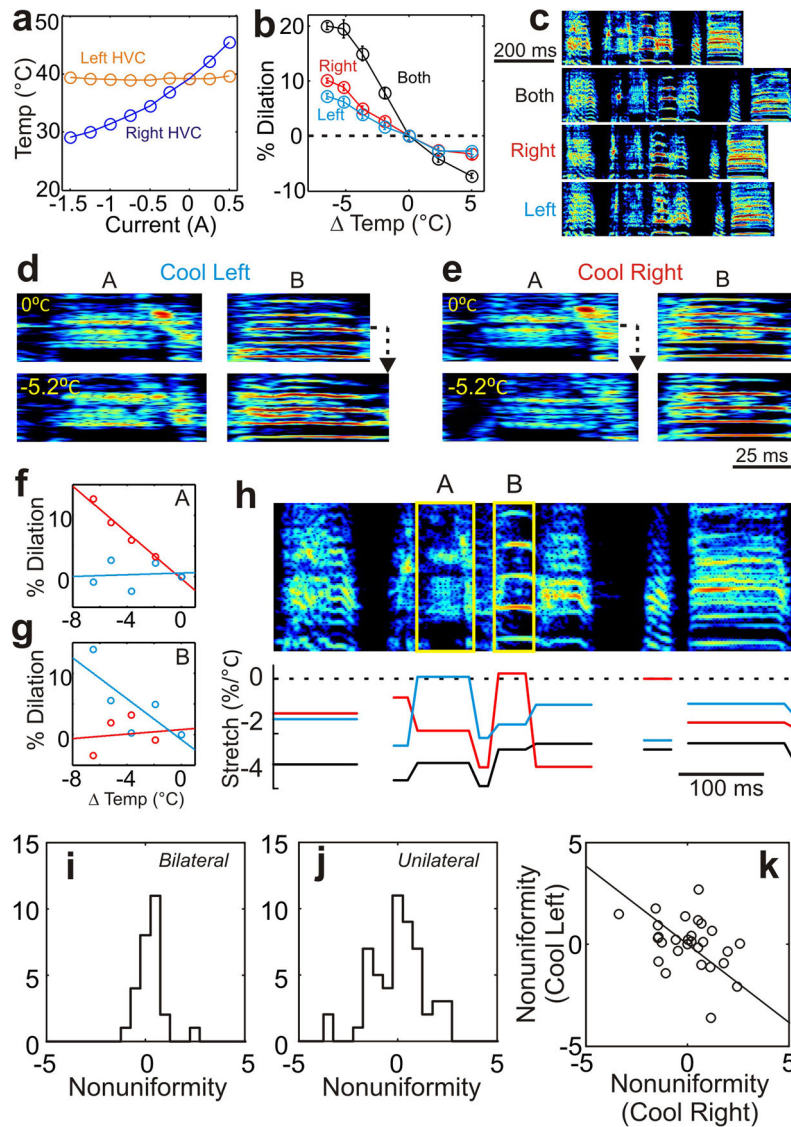
Figure 5. Effect of unilateral HVC cooling on song timing. a
, Simultaneous temperature measurements from HVC in both hemispheres when the Peltier device was configured for right HVC cooling. **b**, Change in motif duration as a function of HVC temperature change during unilateral and bilateral cooling in bird #11. **c**, Spectrograms of song motif during control, bilateral, left and right HVC cooling. **d**, Selective dilation of subsyllabic element B, but not A, during left HVC cooling. **e,** Selective dilation of element A, but not B, during right HVC cooling. Dilation of subsyllabic element A (**f**) and B (**g**) during left (blue) and right (red) HVC cooling. **h**, Stretch metric of identified song segments during cooling of left, right, or both HVCs. Distributions of stretch nonuniformity values following bilateral (**i**) and unilateral (**j**) cooling. **k**, Nonuniformity values during left and right cooling show significant anticorrelation (p=0.026). Solid line shows the first principal component of the distribution.