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Dissociable effects on birdsong of androgen signaling in cortex-like brain regions of canaries

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1 **Title: Dissociable effects on birdsong of androgen signaling in cortex-like brain regions of**
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3 **Abbreviated Title: Hormonal control of vocal plasticity**

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42 **Abstract**

43

44 The neural basis of how learned vocalizations change during development and in adulthood

45 represents a major challenge facing cognitive neuroscience. This plasticity in the degree to

46 which learned vocalizations can change in both humans and songbirds is linked to the actions of

47 sex steroid hormones during ontogeny but also in adulthood in the context of seasonal changes in

48 birdsong. We investigated the role of steroid hormone signaling in the brain on distinct features

49 of birdsong using adult male canaries (*Serinus canaria*), which show extensive seasonal vocal

50 plasticity as adults. Specifically, we bilaterally implanted the potent androgen receptor antagonist

51 flutamide in two key brain regions that control birdsong. We show that androgen signaling in the

52 motor cortical-like brain region--the robust nucleus of the arcopallium--controls syllable and trill

53 bandwidth stereotypy, while not significantly affecting higher order features of song such

54 syllable-type usage (i.e., how many times each syllable type is used) or syllable sequences. In

55 contrast, androgen signaling in the pre-motor cortical-like brain region--HVC (acronym is proper

56 name)--controls song variability by increasing the variability of syllable-type usage and syllable

57 sequences, while having no effect on syllable or trill bandwidth stereotypy. Other aspects of

58 song, such as the duration of trills and the number of syllables per song, were also differentially

59 affected by androgen signaling in HVC versus RA. These results implicate androgens in

60 regulating distinct features of complex motor output in a precise and non-redundant manner.

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66 **Significance Statement**

67 Vocal plasticity is linked to the actions of sex steroid hormones, but the precise mechanisms are
68 unclear. We investigated this question in adult male canaries (*Serinus canaria*), which show
69 extensive vocal plasticity throughout their life. We show that androgens in two cortex-like vocal
70 control brain regions regulate distinct aspects of vocal plasticity. For example, in HVC (acronym
71 is proper name), androgens regulate variability in syntax but not phonology, while androgens in
72 the robust nucleus of the arcopallium regulate variability in phonology but not syntax. Temporal
73 aspects of song were also differentially affected by androgen signaling in HVC versus RA. Thus,
74 androgen signaling may reduce vocal plasticity by acting in a non-redundant and precise manner
75 in the brain.

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89 **Introduction**

90 Songbirds and humans share similarities in how they learn their vocalizations (Brainard
91 and Doupe, 2013; Brenowitz et al. 2010; Marler, 1970; Doupe and Kuhl 1999; Lipkind et al.,
92 2014; Tchernichovski and Marcus, 2014). Both go through a series of developmental changes
93 from stages that are characterized by a high degree of vocal variability to a less variable stage in
94 which vocal behavior is more stable (Brainard and Doupe, 2013; Marler, 1970; Doupe and Kuhl
95 1999). Vocal learning in humans involves a process by which vocal variability during the
96 babbling phase leads eventually to the production of clear words at approximately one year
97 (Brainard and Doupe, 2013). Vocalizations continue to be dynamic and subject to change based
98 on experience until puberty, after which some of the most dramatic changes in vocalizations
99 (e.g., the ability to learn other languages) are attenuated (Johnson and Newport, 1989). This
100 attenuation of vocal plasticity correlates with an increase in sex steroid hormones at puberty, and
101 testosterone (T) concentrations during early development (i.e., ~1-3 months of age) negatively
102 predict vocal plasticity at 18-30 months of age (Johnson and Newport, 1989; Kung et al., 2016).

103 A similar process occurs in songbirds who first go through a sensory stage when they are
104 nestlings and form an auditory memory of the tutor song (Reviewed in Hulsch and Todt, 2004).
105 They then experience a sensorimotor phase that in temperate zone songbirds occurs during the
106 following spring. The sensorimotor phase starts with a period of quiet variable vocalizations
107 labeled subsong (akin to babbling) followed by periods of highly variable song with adult
108 elements called plastic song that culminates in stable crystallized song. Experiments in sparrows
109 demonstrated that this process of moving from variable song to stable crystallized song is
110 dependent on T (Marler et al., 1988). Castrated male sparrows failed to transition out of the
111 plastic song state, while treatment with T induced crystallization; in some birds the T implant

112 was removed and the birds returned to a state of plastic song (Marler et al., 1988). Some
113 seasonally breeding songbirds such as male canaries (*Serinus canaria*) additionally undergo
114 substantial changes in vocal stability as adults: during the fall (i.e. non-breeding season) when T
115 concentrations are low, they repeat the sensorimotor phase, and can produce a new crystallized
116 song during the spring (i.e., breeding season) when T concentrations are high (Nottebohm et al.,
117 1986).

118 The neural basis of vocal behaviors transitioning from a variable to a stable state can be
119 investigated experimentally in songbirds. First, birdsong is controlled by a network of
120 telencephalic nuclei with distinct functions (See Figure 1). Throughout this telencephalic brain
121 circuit there is a distinct pattern of androgen receptor (AR) expression (Ball et al., 1992; Bernard
122 et al., 1999; Gahr, 2001). Recent research makes it clear that T has non-redundant effects on
123 song production in both the brain and the periphery (Reviewed in Alward et al., 2017). We
124 know for example that the motivation to sing can be activated by T acting outside the song
125 system in the medial preoptic nucleus (POM) (Alward et al 2013). However, song stimulated by
126 T action only in the POM lacks stereotypy and the simultaneous implantation in HVC rescues
127 the deficit (Alward et al., 2016). A gap in our knowledge concerns what the specific effects of T
128 are in two key forebrain song control nuclei that express AR, HVC and RA (See Figure 1); these
129 nuclei are essential for controlling fundamental aspects of song such as syllable acoustic
130 structure (RA; Sober et al., 2008; Yu and Margoliash, 1996), syllable sequencing, and which
131 syllable types are used (HVC; Basista et al., 2014; Bouchard and Brainard, 2013).

132

133 Insert figure 1 about here

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135 Here, we investigate these questions in canaries, which maintain their ability to alter their
136 song in adulthood (Nottebohm et al., 1986; Gahr et al., 2001), making them an excellent model
137 system in which to study the androgenic regulation of vocal plasticity. We blocked AR
138 selectively in HVC and RA to assess what aspects of song are supported by T acting in these
139 nuclei specifically.

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141 **Materials and Methods**

142 **Animals used and pre-experimental manipulations.** The protocols and procedures used here
143 were approved by the Johns Hopkins University Animal Care and Use Committee (Protocol #:
144 AV14A112) and followed the ASAB/ABS Guidelines for the use of animals in research.
145 Twenty-eight male canaries (*Serinus canaria*) of the Border strain were used for this study.
146 Canaries were obtained from a local breeder (Maryland Exotic Birds). All birds were 1-2 years
147 of age and had all experienced at least one breeding season before the beginning of the present
148 study. Upon entry into the laboratory, birds were placed on a short-day photoperiod (8L:16D) for
149 six weeks to induce photosensitivity (Nicholls and Storey, 1977; Hurley et al., 2008). Birds were
150 housed in mixed-sex groups.

151

152 **Bilateral implantation of flutamide targeting HVC or RA.** Birds were anesthetized with the
153 use of isoflurane gas (3-4%) and implanted subcutaneously with T-filled SilasticTM implants
154 (Dow Corning, Midland, MI, USA, outside diameter = 1.65 mm and inside diameter = 0.76 mm;
155 12 mm in length filled with 10 mm of T, Sigma Aldrich T-1500; RRID: CHEBI: 17347) in the
156 same fashion as in our previous studies (Alward et al., 2013, 2016c). We implanted all birds with
157 subcutaneous T given observations in our lab and others that some males sing at very low rates

158 (Calder, 1970; Alward et al., 2016b) and that laboratory held males have lower plasma T
159 concentrations than wild-caught individuals even when held on long days (e.g., Calder, 1970;
160 Meitzen et al., 2007). Implanting all birds with T thus increased experimental and statistical
161 power. Others have employed similar techniques in different songbird species to ensure high
162 rates of singing (Meitzen et al., 2007).

163 Immediately following subcutaneous implantation of T, birds were placed in a stereotaxic
164 apparatus modified for use in small birds such as canaries with the beak holder placed 45° below
165 the horizontal axis of the apparatus and then continued to be exposed to isoflurane gas. We
166 utilized an identical implant procedure as in our previous work but performed bilateral instead of
167 unilateral implants (Alward et al., 2013, 2016c). Birds were bilaterally implanted with 27-gauge
168 cannulae targeting HVC or RA filled with crystalline flutamide (Sigma Flutamide, F9397;
169 RRID: CHEBI: 5132) or left empty as a control. Needles were filled over a length of ~2 mm
170 with flutamide using a technique identical to Balthazart and Surlemont (1990). Implants were
171 cleaned using acetone and a Kimwipe to remove any flutamide that stuck to the outside of the
172 cannula.

173 Flutamide is a potent non-steroidal androgen receptor antagonist that has been used
174 extensively in songbirds and other species as a global and a local antagonist for androgen
175 signaling, with no reports of apparent toxicity or off-target effects (Balthazart and Surlemont,
176 1990; Bottjer and Hewer, 1992; Soma et al., 1999; Singh et al., 2000; Naghdi et al., 2001;
177 Brown, 2004; Van Duyse et al., 2005; Grisham et al., 2007; Meitzen et al., 2007; Fuxjager et al.,
178 2012; Hejmej et al., 2013).

179 In line with previous work, our goal was to place each cannula near but not into HVC or
180 RA (Alward et al., 2013, 2016c; Meitzen et al., 2007). Thus, implants were placed lateral to

181 HVC and dorso-lateral to RA. Of course, for HVC it would have been optimal to place the
182 cannulae directly dorsal to (i.e., above) the nucleus; however, this is particularly challenging
183 given that in all likelihood the cannulae would not remain in place and/or deliver flutamide into
184 the adjacent lateral ventricle. Moreover, it is paramount to avoid inserting the cannulae directly
185 into the nucleus, given the possibility of damage to the nucleus of interest. Indeed, lesions in
186 different parts of HVC lead to changes in neural activity in a highly topographical manner
187 (Stauffer et al., 2012) and medial versus lateral portions of HVC regulate distinct features of
188 song (Basista et al., 2014). This technique has been used previously in neuroendocrine studies
189 and shown to be effective in delivering the compound of interest while avoiding damage to the
190 nucleus of interest, which could lead to confounding effects (Alward et al., 2013, 2016c; Meitzen
191 et al., 2007).

192 Coordinates for targeting HVC were as follows: -0.6 mm from the dorsal surface of the
193 brain; anterior-posterior: -2.3 mm from the rostral tip of the cerebellum; and medial-lateral: ± 3.0
194 mm from midline. Coordinates for RA were: -2.40 mm from the dorsal surface of the brain;
195 anterior-posterior: -0.9 mm from the rostral tip of the cerebellum; and medial-lateral: ± 2.70 mm
196 from midline. 15 canaries were implanted with cannulae bilaterally targeting HVC: 12 of these
197 birds were implanted with flutamide (HVC-flut) and 3 birds were implanted with empty
198 cannulae (HVC-noflut). 13 canaries were implanted bilaterally with cannulae targeting RA: 10 of
199 these birds were implanted with flutamide (RA-flut) and 3 birds were implanted with empty
200 cannulae (RA-noflut). The relatively lower numbers for the ‘empty’ controls were chosen based
201 on expected variation in implant sites and the fact that AR blockade in HVC and RA affects
202 singing behavior in a specific manner only when the implants are near the edge of the nucleus
203 (Meitzen et al., 2007). Moreover, the implant procedure we used here has been shown to produce

204 highly localized effects on the brain nucleus of interest (Alward et al., 2013, 2016c). This is
205 largely explained by the fact that in all songbirds in which AR expression patterns have been
206 studied, including canaries, ARs are expressed in HVC and RA but not in the surrounding
207 nidopallium and arcopallium (Balthazart et al., 1992; Bernard et al., 1999; Gahr and Metzdorf,
208 1997; Smith et al., 1996; Soma et al., 1999a). Data from birds with flutamide cannulae that were
209 found to be located too distant from HVC or RA (see below) were pooled with data from the
210 corresponding control group after confirming by statistical analyses that they did not differ.

211 **Animal Housing and Care.** Immediately following stereotaxic implantation, birds were placed
212 individually in sound-attenuating recording chambers (41 cm x 48 cm x 51 cm) set to a long-day
213 photoperiod (14L:10D) to simulate breeding conditions (Nicholls and Storey, 1977; Hurley et al.,
214 2008; Alward et al., 2014). Chambers contained a wooden perch and birds could move freely,
215 perform wing stretching, dusting, and self-preening behavior. Each chamber was outfitted with a
216 recording device that captured video and audio recordings (recording parameters are described
217 below). All of these cameras were connected to a computer that allowed continuous real-time
218 audio-visual observations of the individual birds. Birds were housed alone given our previous
219 work demonstrating that male canaries sing when housed alone on long days but reduce singing
220 substantially and sometimes do not sing at all when housed with a female (Alward et al., 2014).

221 **Song recording and analysis.** Isolation chambers were outfitted with a microphone (BT-
222 MP8087 Mini microphone; B&H Photo and Electronics Corp, New York, NY) and camera
223 (KPC-600 Pinhole Camera 3.6 mm; B&H Photo and Electronics Corp, New York, NY)
224 connected to a computer running DVRserver (V6.33b; Mammoth Technologies, Austin, TX)
225 designed for real-time video and audio surveillance recording. Each day, the DVRserver
226 captured song behavior from 0800h to 1030h (lights on at 0800 h) in .wav files sampled at

227 22,050 Hz which translated to a frequency range of 0–11 kHz. Song files were run through a
228 high-pass filter set to a threshold of 900 Hz to remove low-frequency noise and converted to a
229 digital format using Goldwave™ (Version 5.55; GoldWave, St. John's, NF, Canada) before they
230 were visualized into sound spectrograms using Avisoft (SASlab Pro, Berlin, Germany; RRID:
231 SCR_014438), a Windows application for investigating animal acoustic communication. For the
232 spectrograms, the fast Fourier transform length was set to 512 with an overlap of 75% for the
233 temporal resolution. Songs were defined as vocalizations having a duration greater than 1 second
234 with no gaps longer than 500 milliseconds (Voigt and Leitner, 2008; Alward et al., 2013, 2014,
235 2016a, 2016b, 2016c). Spectrograms were visually inspected to further eliminate noise that
236 escaped the filter.

237 Based on work by Meitzen and colleagues (2007) and Alward and colleagues (2013), we
238 intended to analyze song during an early (e.g., day 10), middle (e.g., day 14), and later time point
239 (e.g., day 21, the last day of treatment). However, we found that on some of the early and middle
240 days some birds did not sing during our recording time from 800 to 1030 am. These observations
241 are very similar to what we observed in a recent study from our lab (Alward et al., 2016b) and
242 the time course for the increase in song rate for all birds regardless of hormone treatment was
243 also very similar to what we observed our previous work (Alward et al., 2016b). Therefore, we
244 analyzed songs during time blocks over days 10-12 and days 13-14, which allowed us to include
245 all birds in our analyses and to perform repeated-measures ANOVAs which increased our
246 statistical power. Meitzen and colleagues (2007) used nearly identical sampling periods in their
247 study on singing in white-crowned sparrows when males were implanted with AR or estrogen
248 receptor (ER) antagonists targeting HVC. Other studies have also binned across days when
249 analyzing changes in song (Madison et al., 2014; Pytte et al., 2012).

250 **Quantification of song rate and whole song bandwidth stereotypy.** Song rate (# songs/hour)
251 was determined by dividing the total number of songs sung by each bird during each time bin by
252 the total number of hours analyzed within that time bin. We also quantified whole song
253 bandwidth stereotypy. We have shown previously that the stereotypy of whole song bandwidth
254 (bandwidth=the difference between the minimum and maximum frequency) changes
255 substantially as a function of T action in canaries (Alward et al., 2013, 2016c). Using the Avisoft
256 software package, we quantified the bandwidth of each whole song on each analysis day. We
257 then computed whole song bandwidth coefficient of variation (CV), which is the standard
258 deviation of bandwidth across individual songs produced by a given bird, divided by the average
259 bandwidth across those songs multiplied by 100. CV is a measure of consistency of an acoustic
260 feature over renditions of songs and has been used extensively in birdsong studies as a measure
261 of song stereotypy (Meitzen et al., 2007; Sakata et al., 2008; Alward et al., 2013, 2016a-c). A
262 higher value of whole song bandwidth CV means that over song renditions, the bandwidth of
263 whole song is less stereotypic.

264 **Quantification of trill and syllable bandwidth stereotypy.** We were also interested in
265 quantifying the bandwidth stereotypy of units of song that vary over distinct temporal domains.
266 Syllables are considered to be in the shortest temporal domain, and are the minimal units of
267 production of birdsong that, when strung together, form phrases. Phrases are then strung together
268 to form whole songs (Catchpole, 2003). Previous work suggests that these distinct temporal
269 domains are controlled differentially by HVC and RA (Margoliash, 1997).

270 A particular type of canary phrase is referred to as a trill, which is the fast repetition of a
271 syllable type (Catchpole, 2003). We analyzed the bandwidth stereotypy of trills, given previous
272 work showing seasonal changes in trill production in wild canaries (Voigt and Leitner, 2008).

273 Trills were quantified similarly as in Alward et al. (2016b). Briefly, we visually inspected songs
274 for trills sung within a bout of continuous singing of five minutes. Using this visual inspection
275 method, we were able to identify particular trill types. To quantify the bandwidth of each trill, we
276 dragged the cursor over each trill, manually labeled each trill, and then computed bandwidth.
277 Bandwidth stereotypy was analyzed for each trill separately and then collapsed into an overall
278 trill stereotypy value. We and others analyzing canary song have also used a visual inspection
279 method for the quantification of individual song components such as trills and syllables (Alward
280 et al., 2016b; Markowitz et al., 2013).

281 Previous studies assessing different components of canary song have analyzed syllable
282 acoustic stereotypy based on the features of a common syllable type; i.e., a syllable type sung by
283 most or all birds (Iserbyt et al., 2017). While quantifying trills across birds, it became apparent
284 that each bird sang a common syllable type that possessed distinct acoustic and temporal features
285 that permitted an automatic analysis of this particular syllable type for all subjects (See Figure
286 2A).

287

288 Insert figure 2 about here

289

290 Therefore, we quantified in all birds this particular syllable type to determine syllable bandwidth
291 stereotypy. These syllables were sung in succession as part of a trill. To conduct an unbiased
292 analysis of the stereotypy of these syllables, 40 of these syllables were randomly selected for
293 each bird and each day from the total number of syllables detected and they were quantified
294 based on the same features we employed for trills and overall songs. An alternative approach
295 would be to attempt to analyze the acoustic features of all syllables across the songs of the birds

296 included in this study. However, the strategy we employed in this study is valid and produced
297 meaningful and interpretable results. Indeed, our goal was to assess how androgen signaling in
298 HVC versus RA may regulate distinct features of canary song and previous investigators have
299 successfully employed approaches similar to ours to investigate different features of canary
300 songs, trills, and syllables (Nottebohm et al., 1986; Leitner et al., 2001; Alward et al., 2013;
301 Markowitz et al., 2013; Alward et al., 2016a-c; Iserbyt et al., 2017).

302 **Quantification of the variability of syllable-type usage and syllable sequencing.** Canaries
303 sing a variety of syllable types (Nottebohm et al., 1986; Catchpole, 2003). Syllable types were
304 labeled manually in Avisoft by placing brackets around the whole rendition of that syllable type.
305 Within a given year, canaries do not change the number of different syllables they produce, but
306 the variability of how often each syllable type is used (hereon called “syllable-type usage
307 variability”) and how they are sequentially arranged has been shown to change seasonally
308 (Nottebohm et al., 1986). For each bird on the days on which song was analyzed, we manually
309 labeled syllables over 10-20 songs or about 180-300 syllables. Different syllables can be labeled
310 reliably by visual means, and manual labeling methods have been used by us and others when
311 quantifying different syllable types in canary song (Nottebohm et al., 1986; Leitner et al., 2001;
312 Markowitz et al., 2013; Alward et al., 2016b; Iserbyt et al., 2017).

313 To assess syllable-type usage variability, we quantified the zero-order entropy of syllable
314 usage, which is defined as:

$$315 \text{Zero order entropy} = -\sum p_i \times \log_2(p_i)$$

316 where the sum is over all of the different syllables and p_i is the probability of each syllable
317 occurrence. Zero order entropy values were standardized across birds to the maximum amount of
318 entropy possible by the formula:

319
$$\text{Syllable-type usage variability} = \text{zero order entropy} / \log_2(y)$$

320 where y is the number of different syllable types produced by each bird, as done in previous
321 work (Parker, 2009). This yielded syllable-type usage variability values between 0 and 1, where
322 1 is the maximum possible entropy (i.e., maximum randomness or variability of syllable-type
323 usage). The higher this number, the more variable is syllable-type usage.

324 We measured syllable sequence variability by methods similar to others (Nottebohm et
325 al., 1986; Sakata et al., 2008; Matheson et al., 2015). We analyzed for each bird transitions from
326 its dominant (i.e., most frequently used) syllable type to all other types. We first determined the
327 dominant syllable type from the labeled syllables that were used for quantifications of syllable-
328 type usage variability and then quantified first order entropy for the dominant syllable type by:

329
$$\text{First order entropy} = -\sum p_i \times \log_2(p_i)$$

330 where the sum is over all over possible transitions and p_i is the probability of the i th transition
331 from the dominant syllable type. As for syllable-type usage variability, we standardized this
332 measure with the formula:

333
$$\text{Syllable sequence variability} = \text{First order entropy} / \log_2(z)$$

334 Where z is the number of different syllables that followed the dominant syllable type. This
335 yielded syllable sequence variability values between 0 and 1, where 1 is the maximum possible
336 entropy (i.e., maximum randomness or variability of syllable sequence). In some cases, the
337 dominant syllable type was the first syllable in a part of a syllable sequence that occurred >95%
338 of the time. These types of transitions are considered to be fully stereotyped sequences (e.g.,
339 similar to motifs in zebra finch songs). For these situations, we treated the transitions from these
340 stereotyped sequences as “branch points” (e.g., in the fully stereotyped sequence A-B, the
341 transitions from B were used for calculating first order entropy) as done in previous studies

342 (Sakata et al., 2008; Hampton et al., 2009; Tchernichovski and Marcus, 2014; Matheson et al.,
343 2015). There was no effect of treatment on the presence of dominant syllable types that were
344 defined as branch points.

345 **Brain extraction, fixation, and cloacal protuberance measurements.** 21 days after treatment
346 initiation, birds were deeply anesthetized (4% Isoflurane), weighed, rapidly decapitated, and
347 their brain was extracted and fixed in acrolein. Brains were agitated in 5% acrolein for 2 hours,
348 then washed for 15 minutes four times in phosphate buffered saline and cryoprotected in 30%
349 sucrose overnight. Brains were flash frozen in dry ice for 5 minutes and then placed into a -70° C
350 freezer. We also measured the length and width of the cloacal protuberance (CP), an androgen-
351 sensitive organ (Meitzen et al., 2007; Alward et al., 2016a). If flutamide did not enter into the
352 general circulation, all birds should experience a similar increase in the size of the CP after being
353 exposed to long days and a Silastic™ T implant.

354 **Cryosectioning and histological verification of implant site.** Brains were sectioned on a
355 cryostat from the olfactory bulb to the brainstem in four series of 30 micrometer-thick transverse
356 sections that were stored in cryoprotectant into a -20° C freezer. One series was later mounted on
357 gelatin-coated slides and exposed to air for a day. These mounted sections were then Nissl
358 stained and coverslipped using Permount (Fisher Scientific).

359 Photomicrographs of HVC and RA were taken at 2.5x magnification in the Nissl-stained
360 sections using an AxioCam attached to a Zeiss Axioskop. HVC and RA are easily identifiable in
361 Nissl stained sections and the cannula tracts were also readily apparent as in our previous studies
362 (Alward et al., 2013, 2016c). We determined the implant locations under microscopic analysis
363 and took images of the implant tracts. Using Image J (NIH; RRID: SCR_003070) we quantified

364 the distance from the cannula tip to either HVC or RA in the rostro-caudal, dorso-ventral, or
365 medio-lateral plane for all birds that received bilateral flutamide implants.

366 Specificity of action for flutamide-filled cannulae was determined by correlating the
367 functional response (whole song bandwidth stereotypy) with the position of the cannulae relative
368 to the brain region of interest. As mentioned above, for HVC, the goal was to place the cannula
369 tips adjacent (lateral) to the nucleus to minimize damage to the nucleus (Meitzen et al., 2007).
370 For RA, the goal was to place cannula tips directly above or adjacent to the nucleus for the same
371 reason (Meitzen et al., 2007; Larson et al., 2013). For HVC, only distance of the cannula tip
372 lateral to HVC was used to determine specificity because most cannula tips did not occur ventral
373 to HVC, making a meaningful analysis of variation based on dorso-ventral distance to HVC and
374 whole song stereotypy impossible. For RA, only variation in the dorso-ventral plane was used as
375 a reliable indicator of cannula distance, given that only one bird had a cannula tip that did not
376 occur directly dorsal to RA (i.e., values for distance in the medial-lateral plane for cannula tips
377 were mostly “zero”).

378 Distances were determined for each hemisphere separately. There were no differences
379 between the left and right hemispheres in cannula distances from the nucleus of interest for HVC
380 (unpaired t-test, $p=0.28$) or RA (unpaired t-test, $p=0.11$). Therefore, for each bird we averaged
381 over both hemispheres the implant distances from each nucleus. Because all flutamide cannula
382 that missed were substantially caudal to the caudal portion of the nucleus of interest, they were
383 not included in a correlational analysis relating distance to HVC or RA and the effect on whole
384 song bandwidth stereotypy.

385 **Statistical Analyses.** Mixed-design ANOVAs were used to determine the effects of AR
386 antagonism in either region on song measures, using day (days 10-12, 13-14, and day 21) as the

387 within-subjects factor and treatment (flutamide versus empty) as the between-subjects factor.
388 Greenhouse-Geisser corrected p values were used. Following significant interactions in the
389 omnibus ANOVA, post-hoc Scheffe's tests were used to determine the differences driving the
390 interaction effects. A mixed-design ANOVA was also used to assess the effects of treatment on
391 CP size, wherein time (pre-treatment versus post-treatment) was the within-subjects factor and
392 treatment (flutamide versus empty) was the between-subjects factor. Correlational analyses were
393 conducted using Pearson's r. Effects were considered significant at $p \leq 0.05$. Effect sizes were
394 calculated as Partial Eta Squared (η_p^2) to describe the importance of a significant effect in the
395 omnibus ANOVA.

396

397 **Results**

398 **Specificity of flutamide treatment.** Some birds had a lesioned HVC (3 birds) or lesioned RA (1
399 bird) or only had a unilateral implant located near HVC (1 bird) or RA (2 birds) instead of
400 bilateral implants. Due to the sparse nature of this variation, these birds could not be placed into
401 a specific treatment group and were thus excluded from statistical analysis.

402 Two birds treated with a flutamide implant targeting HVC that missed the nucleus
403 (implant tip more than 600 microns caudal to the nucleus) were shown to have song stereotypy
404 values that were not different from controls (unpaired t-test, $p=0.66$); they were thus added to the
405 control group. A similar observation was made for two birds treated with flutamide targeting but
406 missing RA (implant tip was more than 400 microns dorsal to the nucleus; unpaired t-test,
407 $p=0.37$); they were similarly added to the control group. Therefore, the final sample sizes were as
408 follows—HVC: Control, $n=5$; Flutamide, $n=9$; RA: Control, $n=5$; Flutamide, $n=5$ (Total $N=24$).

409 The distance from the flutamide-filled cannula tip to the nucleus of interest was
410 negatively correlated with the overall song stereotypy of the birds (Figure 2B-E; for HVC:
411 distance lateral to HVC versus day 21 Song Bandwidth CV, $n=9$, $r=-0.83$, $p<0.05$; for RA:
412 distance dorsal to RA versus day 21 Song Bandwidth CV, $n=5$, $r=-0.82$, $p<0.05$).

413 The CP size did not differ between birds treated with flutamide or empty cannula (Figure
414 2F; $t_{22}=0.69$, $p=0.49$), indicating that flutamide did not leak into the general circulation (Bottjer
415 and Hewer, 1992; Meitzen et al., 2007; Alward et al., 2016c).

416

417 **Androgen signaling in HVC and RA regulates whole song bandwidth stereotypy but not**
418 **the motivation to sing.** Song rate was not significantly affected by antagonism of ARs in either
419 HVC ($p>0.20$ for all sources of variation in the mixed-design ANOVA; Overall Mean \pm SEM for
420 HVC-Ctrl=28.24 \pm 5.76 and HVC-Flut=32.68 \pm 7.41) or RA ($p>0.21$ for all sources of variation in
421 the mixed-design ANOVA; Overall Mean \pm SEM for RA-Ctrl=47.75 \pm 13.32 and RA-
422 Flut=27.75 \pm 10.79), which is consistent with previous observations (Brenowitz and Lent, 2002;
423 Meitzen et al., 2007; Alward et al., 2016c). The higher mean value in the RA-Ctrl group appears
424 to be most likely due to one active singer (without this bird in the calculation the mean value for
425 RA-Ctrl birds drops to 37.34).

426 Bilateral blockade of androgen signaling with flutamide in HVC caused whole songs to
427 become less stereotyped as assessed by the whole song bandwidth CV (Figure 3A-B:
428 representative sonograms; Figure 3C-E: quantitative analysis; treatment, $p=0.17$; time, $p=0.03$,
429 $\eta_p^2=0.25$; time*treatment, $p=0.001$, $\eta_p^2=0.44$). The significant interaction resulted from a
430 difference between flutamide-treated and control birds on day 21 of treatment (Scheffe's post-
431 hoc tests, $p<0.05$). Birds treated with flutamide bilaterally in RA also sang whole songs with

432 lower whole song bandwidth stereotypy than controls (Figure 4A-B: representative sonograms;
433 Figure 4C-E: quantitative analysis; time, $p=0.14$; treatment, $p=0.01$, $\eta_p^2=0.56$; time*treatment,
434 $p=0.12$).

435

436 Insert figures 3 and 4 about here

437

438 **Androgen signaling in HVC versus RA controls distinct features of song bandwidth**

439 **stereotypy.** Whole canary songs are composed of syllables, which are strung together to form
440 phrases and trills. Canary song plasticity could thus be modulated in terms of not only the
441 bandwidth stereotypy of whole songs, but also the stereotypy of individual song components
442 such as syllable or trill bandwidth stereotypy, syllable sequencing, and/or overall syllable-type
443 usage variability (i.e., the probability of specific syllable types being produced). Changes in one
444 or more of these different components of song could give rise to an overall change in whole song
445 bandwidth stereotypy. For instance, the decrease in whole song bandwidth stereotypy observed
446 by day 21 in HVC-Flut birds could be driven not only by changes in bandwidth stereotypy of
447 individual syllables, but also by their arrangement (i.e., ordering or syntax). Some of these
448 features have been shown to change seasonally (Voigt and Leitner, 2008), suggesting a
449 modulation by gonadal steroids.

450 Blockade of androgen signaling in HVC had no effect on trill or syllable bandwidth
451 stereotypy (Figure 5A-B; trills: time, $p=0.91$; treatment, $p=0.42$; time*treatment, $p=0.10$;
452 syllables: time, $p=0.32$; treatment, $p=0.88$; time*treatment, $p=0.34$). In contrast, antagonism of
453 AR in RA caused birds to sing trills (Figure 5C; time, $p=0.14$; treatment, $p=0.002$, $\eta_p^2=0.71$;
454 time*treatment, $p=0.91$) and syllables (Figure 5D; time, $p=0.23$; treatment, $p=0.01$, $\eta_p^2=0.56$;

455 time*treatment, $p=0.18$) with reduced bandwidth stereotypy. Representative sonograms are
456 shown in Figure 5E-J. Syllable, trill, and whole song bandwidth stereotypy all correlated strongly
457 with one another in males treated with flutamide in RA and their controls (Figure 5K-M).

458

459 Insert figure 5 about here

460

461 Antagonism of androgen signaling in HVC resulted in a substantial increase in syllable
462 sequence variability (Figure 6A; time, $p=0.74$; treatment, $p=0.009$, $\eta_p^2=0.44$; time*treatment,
463 $p=0.81$; Figure 6B-C illustrates examples of syllable transitions in control versus flutamide
464 subjects). Changes in syllable-type usage variability was similarly affected (increased syllable-
465 type usage variability in flutamide birds; Figure 6D; time, $p=0.29$; treatment, $p=0.43$;
466 time*treatment, $p=0.04$, $\eta_p^2=0.25$) but this effect developed more slowly and only became
467 significant on day 21 (Scheffe's contrasts, $p<0.05$). Figure 6E-F presents distribution charts
468 showing the more variable use of different syllable types in flutamide birds compared to
469 controls. Syllable-type usage variability correlated positively with whole song bandwidth
470 stereotypy, as measured by the whole song bandwidth CV, on day 21 (Figure 6G-I), which could
471 explain the decrease in whole song bandwidth stereotypy on this day. On the other hand, there
472 was no effect of AR antagonism in RA on syllable sequence variability (Figure 6J; time, $p=0.76$;
473 treatment, $p=0.81$; time*treatment, $p=0.73$) or syllable-type usage variability (Figure 6K; time,
474 $p=0.74$; treatment, $p=0.09$; time*treatment, $p=0.43$).

475

476 Insert figure 6 about here

477

478 **Temporal properties of song are differentially regulated by androgen signaling in HVC and**
479 **RA.** Aspects of song timing, such as the duration of different units of song, may also be
480 differentially controlled by HVC versus RA (Arnold, 1975; Long and Fee, 2008). HVC-Flut
481 birds did not differ from control birds in terms of whole song duration (Figure 7A; time, $p=0.14$;
482 treatment, $p=0.08$; time*treatment, $p=0.36$); however, HVC-Flut birds sang longer trills
483 compared to controls (Figure 7B; time, $p=0.65$; treatment, $p=0.04$, $\eta_p^2=0.30$; time*treatment,
484 $p=0.38$). Antagonism of AR in RA caused these birds to sing longer whole songs than controls
485 (Figure 7C; time, $p=0.43$; treatment, $p=0.02$, $\eta_p^2=0.50$; time*treatment, $p=0.20$). This effect was
486 driven by an overall increase in the number of individual syllables per whole song (Figure 7D;
487 time, $p=0.68$; treatment, $p=0.01$, $\eta_p^2=0.59$; time*treatment, $p=0.77$). At the individual level,
488 whole song duration and number of syllables per song were positively correlated in birds treated
489 with flutamide in RA and their controls (Figure 7E; $r=0.80$, $p=0.005$).

490

491 Insert figure 7 about here

492

493 **Discussion**

494 By selectively blocking androgen action in HVC and RA we have provided insight into
495 how the steroid hormone T regulates specific features of learned birdsong in a localized manner.
496 It is often assumed that T modulates behavior in rather general ways, such as changing the
497 probability and intensity of a behavioral response to a given stimulus. HVC and RA are clear
498 candidates for a marked regulation by T based on the enriched localization of AR protein
499 (Balthazart et al., 1992) or mRNA (Gahr and Metzdorf, 1997; Bernard et al., 1999) within these
500 nuclei. However, HVC and RA are not involved in the motivation to sing as was clear from the

501 initial lesions studies of song nuclei (Nottebohm et al., 1976), where lesions to HVC blocked
502 song production but not movements associated with trying to sing. The current study indicates
503 that androgens act on specific aspects of song related broadly to stereotypy and temporal features
504 of song organization. In particular, androgen signaling in RA regulates the bandwidth stereotypy
505 of syllables and trills (Figure 5C-M), while androgen signaling in HVC regulates the variability
506 of syllable sequencing and syllable-type usage (Figure 6A-I). Androgen signaling in both HVC
507 and RA regulates the duration of different components of song (Figure 7): androgens in HVC
508 regulate trill duration while androgens in RA reduce whole song length by regulating the number
509 of notes per whole songs. Flutamide may have preferentially affected the lateral HVC; however,
510 since syllable sequencing was affected by flutamide implanted lateral to HVC and this song
511 feature is thought to be regulated by medial HVC (Basista et al., 2014), this suggests that medial
512 HVC was also exposed to flutamide, albeit possibly at a lower concentration. Nonetheless,
513 effects obtained by exposing HVC and RA to the same compound were very different, which
514 indicates anatomical specificity in androgen action on these two nuclei. In this discussion, we
515 will try to link concisely known cellular and neurophysiological effects of T in HVC and RA to
516 our behavioral effects of AR blockade.

517

518 **Possible neuronal and molecular mechanisms underlying the androgenic regulation of song**
519 **stereotypy: linking cellular actions of T with behavioral outcomes.**

520

521 AR are expressed in both HVC and RA and AR-positive neurons in HVC project to either RA or
522 Area X (Sohrabji et al., 1989; see Bottjer and Johnson 1997 for review). Androgen signaling has
523 been implicated in regulating several neural features in HVC and RA (Reviewed in Brenowitz,

524 2014; Brenowitz and Larson, 2015), including overall changes in nuclei volumes, neuronal soma
525 size and neuron density, and in HVC the incorporation of new neurons that mostly project to RA.
526 All these changes in HVC are enhanced by T action via its androgenic and estrogenic
527 metabolites (Smith et al., 1995, 1997a, 1997b; Tramontin et al., 2000; Brenowitz and Lent, 2002;
528 Sartor et al., 2005; Meitzen et al., 2007; Yamamura et al., 2011; Alward et al., 2016). The
529 addition of new neurons in HVC of adult zebra finches is positively associated with song
530 stereotypy (Pytte et al., 2012). Thus, effects of AR antagonism in HVC on syllable sequence
531 variability, syllable-type usage variability, and temporal features of song (i.e. trill duration)
532 might be due to a reduction in the addition of new neurons in HVC, which would explain the
533 rather long latency between beginning of treatment and some of the observed effects. HVC
534 neurons fire sparsely, in a chain-like manner during song and this activity is thought to underlie
535 song timing (Hahnloser et al., 2002; Long and Fee, 2008). HVC neurons also show coding
536 properties that relate to the ordering of song syllables (Bouchard and Brainard, 2013) that were
537 likely also altered by the blockade of AR in HVC.

538 It took 21 days to reduce whole song bandwidth stereotypy and enhance syllable-type
539 usage variability in HVC-flut birds compared to HVC-ctrl birds. Similarly, in male white-
540 crowned sparrows T-treated males increased song stereotypy by day 21 compared to day 13, but
541 treatment with AR and ER antagonists in HVC prevented this increase from occurring (Meitzen
542 et al., 2007). The enhancement in syllable sequence variability caused by the blockade of
543 androgen signaling in HVC occurred much sooner. These differences may reflect HVC's
544 topographical specializations in terms of function and hodology (Basista et al., 2014). The
545 topographical specificity of HVC's afferents or function may cause a dissociation in the manner
546 in which certain song features are regulated by androgen signaling within HVC.

547 This dissociation might alternatively originate in specific effects of AR antagonism in
548 HVC on RA function. In white crowned sparrows treatment of HVC with AR or ER antagonists
549 leads to a decrease in soma area and spontaneous firing rate within RA by day 21 (Meitzen et al.,
550 2007). Moreover, systemic T and exposure to LD photoperiod caused an increase in spontaneous
551 firing rate at day 21 compared to day zero. However, days 4, 11, and 14 were not different from
552 day zero. A similar pattern was observed for RA neuron size. Importantly, RA neurons transform
553 HVC input that is relayed to the hindbrain and then to the syrinx to produce song (Spiro et al.,
554 1999). It can thus be hypothesized that blockade of AR signaling in HVC leads to a perturbation
555 in RA's ability to transform HVC input on day 21, specifically as it relates to syllable usage
556 variability, thus leading to the observed effects.

557 Multiple studies have also shown that firing patterns in RA neurons strongly correlate
558 with the acoustic structure of individual syllables (Yu and Margoliash, 1996; Margoliash, 1997;
559 Sober et al., 2008). Since blockade of AR in RA reduces spontaneous firing rate and soma size
560 (Meitzen et al., 2007), it can be assumed that this perturbation in neural firing contributes to the
561 decreased bandwidth stereotypy in syllables, trills, and overall song observed here.

562 Alternatively, the speed of NMDA-excitatory post synaptic potentials (EPSPs) in RA
563 inversely correlates with the potential for vocal plasticity (Livingston et al., 2000). Androgens
564 can speed up NMDA-EPSPs leading to prematurely crystallized song in juvenile songbirds
565 (White et al., 1999; Livingston and Mooney, 2001), suggesting that androgens attenuate vocal
566 plasticity circuitry. Therefore, AR antagonism in RA might have decreased the speed of NMDA-
567 EPSPs, thus increasing vocal variability as observed here (Livingston et al., 2000; Livingston
568 and Mooney, 2001).

569 LMAN is also known to regulate acoustic but not syntactical stereotypy (reviewed in
570 Brainard and Doupe, 2013). LMAN is thought to introduce acoustic variability into song via RA.
571 We found here that antagonism of androgen receptors in RA reduced bandwidth stereotypy of
572 individual song units (i.e., syllables) without affecting syntactical features. This suggests that AR
573 antagonism in RA potentially perturbed LMAN-RA synapses or significantly influenced RA's
574 ability to transform LMAN input (Spiro et al., 1999). However, according to this hypothesis,
575 blocking AR in RA should have affected features regulated by HVC as well, unless there is
576 segregation of afferent input in androgen-sensitive RA neurons. Data from Stark and Perkel
577 (1999) support this contention. HVC input to RA is mediated by NMDA and AMPA receptors
578 while LMAN input to RA is mediated almost exclusively by NMDA receptors. HVC-RA and
579 LMAN-RA synapses are thus partially segregated based on the different complements of
580 glutamate receptors. The partial segregation of HVC-RA and LMAN-RA synapses may help to
581 explain some of the current findings.

582 We also observed that AR antagonism in HVC enhanced syllable-type usage variability
583 and on days 13-14 AR antagonism in RA led to a non-significant increase in syllable-type usage
584 variability, but this difference disappeared by day 21. These results suggest that variation in
585 syllable-type usage is encoded in HVC, but retrograde feedback from RA can affect this process
586 due to its regulation of the addition of new neurons into HVC (e.g., Larson et al., 2013).

587

588 **Role for estrogenic signaling in the regulation of song stereotypy.** While this study did not
589 investigate the role of estrogen signaling, we can make predictions about its role in regulating
590 some of the song features affected here. Meitzen et al (2007) have shown that blockade of both
591 ARs and ERs in the HVC of white crowned sparrows reduces song stereotypy and also

592 spontaneous firing rate in RA. Moreover, acute inhibition of aromatase, the enzyme that converts
593 T to estradiol, can rapidly and reversibly reduce overall song bandwidth stereotypy in canaries
594 (Alward et al., 2016a). ER is only expressed in HVC, so it is possible that estrogen signaling in
595 HVC regulates aspects of syllable-type usage variability, syntax, and temporal aspects of trills.
596

597 **Conclusion: Working model on the regulation of birdsong by steroid hormones.** It is an old
598 idea that steroids can modulate physiological state to change the probability of producing a
599 particular behavior, and this applies to birdsong in that T action in the hypothalamus regulates
600 the motivation to sing (Alward et al., 2013). However, in this study we showed by blocking
601 androgen signaling in two cortical-like brain regions that T regulates complex features of song
602 performance such as syllable bandwidth stereotypy, syllable sequencing, syllable-type usage, and
603 the temporal organization of song in a non-redundant fashion. These findings indicate that
604 steroids regulate behavior in a more precise manner than previously thought. E.O. Wilson opined
605 that hormones would prove to be less important for our understanding of the neural control of
606 behavior because they represented “crude tuning devices” (Wilson, 1975; p. 6) that lacked
607 explanatory power (Wilson, 1975). The present demonstration of specific actions of androgens
608 on cognitive aspects of birdsong controlled by cortical-like brain regions shows the limitation of
609 such claims. Combining the current results with past observations and other models (Ball et al.,
610 2002; Meitzen et al., 2007; Meitzen and Thompson, 2008; Balthazart and Ball, 2016; Alward et
611 al., 2017), we propose a working model illustrating how steroid hormones may regulate birdsong
612 at multiple levels (Figure 8). This model includes the highly localized, non-redundant effects of
613 sex steroid hormones in the regulation of brain and behavior, but also considers the interactions
614 between individual hormone-sensitive nuclei of the song control system.

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915 **Figure Legends:**

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917 **Figure 1. Steroid hormone receptors are expressed throughout the vocal motor pathway of**
918 **the song control circuit.** This simplified schematic shows the distribution of androgen receptors
919 (AR), estrogen receptors (ER), and aromatase (AROM), the enzyme that converts T to estradiol,
920 in the vocal motor pathway (VMP) of the song control system. HVC (acronym is proper name)
921 projects to the robust nucleus of the arcopallium (RA). RA controls motor neurons in the
922 tracheosyringeal portion of the twelfth cranial nerve (nXIIIts), which in turn controls the muscles
923 of the syrinx, the avian vocal organ, to generate birdsong. AR are expressed at every level of the
924 VMP, while ER are expressed only in HVC. *ER is expressed in HVC only in some species,
925 including canaries (Ball et al., 2002). **AROM mRNA is not found in HVC, but AROM protein
926 has been observed in presynaptic boutons in HVC in zebra finches (Peterson et al., 2005).

927

928 **Figure 2. Flutamide specificity and syllable of interest for analysis of bandwidth stereotypy.**
929 (A) The white arrow indicates an example of the syllable used in the automated syllable analysis.
930 (B) Effects of flutamide-filled implants targeting the area directly adjacent to HVC were
931 correlated to their distance medial to the lateral part of HVC (IHVC). All birds with flutamide-
932 filled implants presented here were in the “Flut” group as their cannula tip was in each case near
933 HVC. (C) Photomicrograph of Nissl-stained section illustrating an implant located near HVC
934 (delineated by dashed border). (D) Flutamide-filled implants targeting RA were also highly
935 spatially effective depending on their distance dorsal to the dorsal edge of RA (dRA). (E)
936 Photomicrograph of Nissl-stained section illustrating an implant located near RA (delineated by
937 dashed border). (F) Cloacal protuberance (CP) was not different in birds treated with empty

938 cannula (Ctrl) or flutamide-filled cannula (Flut), suggesting flutamide did not leak into the
939 general circulation (Alward et al., 2016c; Tramontin et al., 2003). In (C and E) Scale bars
940 represent a distance of 200 μm .

941

942 **Figure 3. Androgen receptor antagonism in HVC bilaterally decreases whole song**
943 **bandwidth stereotypy.** (A and B) Examples of canary whole songs on day 21 from birds treated
944 bilaterally with control (Ctrl) or flutamide-filled (Flut) cannula targeting HVC (the scale bar
945 represents 1 second). (C) By day 21, androgen receptor (AR) blockade in HVC led to an increase
946 in whole song bandwidth coefficient of variation (CV), a measure of song stereotypy (the higher
947 the CV, the lower the stereotypy). Symbols represent means \pm SEM. * $p<0.05$ compared to Ctrl on
948 day 21. (D and E) Histograms show the highly variable distribution of the proportion of whole
949 songs sung at different bandwidths for birds with AR blocked in HVC (blue) or controls (gray)
950 on day 21.

951

952 **Figure 4. Androgen receptor antagonism in RA bilaterally decreases whole song bandwidth**
953 **stereotypy.** (A and B) Examples of canary whole songs on day 21 from birds treated bilaterally
954 with control (Ctrl) or flutamide-filled (Flut) cannula targeting RA (the scale bar represents 1
955 second). (C) Androgen receptor blockade in RA led to a rapid, large increase whole song
956 bandwidth coefficient of variation (CV), a measure of song stereotypy (the higher the CV, the
957 lower the stereotypy). Symbols represent means \pm SEM. ** at the top of a graph indicates $p=0.01$
958 for the overall effect of treatment. (D and E) Histograms show the highly variable distribution of
959 the proportion of whole songs sung at different bandwidths for birds with androgen receptors
960 blocked in RA (red) or controls (dark gray) on day 21.

961

962 **Figure 5. Androgen signaling in RA but not in HVC controls bandwidth stereotypy of**
963 **syllables and trills.** There were no differences between birds treated with flutamide-filled
964 cannula in HVC (Flut) and controls (Ctrl) in terms of trill (**A**) and syllable (**B**) bandwidth
965 coefficient of variation (CV), a measure of stereotypy (higher CV, lower stereotypy). (**C** and **D**)
966 Androgen receptor (AR) blockade in RA led to an increase in trill and syllable bandwidth CV.
967 Symbols represent means \pm SEM. *** at the top of a graph indicates $p < 0.01$ for the overall effect
968 of treatment; ** indicates $p = 0.01$ for an overall effect of treatment. (**E-G**) Three successive trills
969 sung by a control bird showing high stereotypy (i.e. low variability) from trill to trill as well as
970 from syllable to syllable, compared to trills in birds with AR blocked in RA (**H-J**), which show
971 lower stereotypy (i.e., more variability) from trill to trill and syllable to syllable. (**K-M**) All
972 measures of stereotypy correlated significantly with one another in birds treated with flutamide
973 near RA and their controls. Symbols for individual birds are color-coded like in panels C-D.

974

975 **Figure 6. Androgen receptors in HVC control syllable sequence variability and syllable-**
976 **type usage.** (**A**) Blocking androgen receptors (AR) in HVC led birds to sing with enhanced
977 syllable sequence variability (first order entropy; see Materials and Methods). (**B-C**) Birds
978 treated with flutamide in HVC (Flut) showed more random (i.e., variable) transitions from their
979 dominant syllable type compared to controls (Ctrl). (**D**) By day 21, antagonism of AR in HVC
980 also caused birds to sing with high syllable-type usage variability (zero order entropy; see
981 Materials and Methods). (**E-F**) Pie charts showing the proportion of use of different syllables
982 (each slice=different syllable). Use of different syllables was more equally distributed (i.e.,
983 closer to a random distribution) in flutamide birds compared to control birds. (**G-I**) Syllable-type

984 usage variability correlated positively with whole song bandwidth coefficient of variation (CV;
985 the higher the bandwidth CV, the less stereotypic) only on day 21 (I) but not on the two previous
986 time points (G-H), which is when birds with their AR in HVC blocked sang with significantly
987 more whole song bandwidth CV compared to controls (see Figure 2C-E). There were no
988 differences between birds treated with flutamide-filled cannula in RA (Flut) and controls (Ctrl)
989 in terms of syllable sequence variability (**J**) or syllable-type usage variability (**K**) (first order and
990 zero order entropy; see Materials and Methods). In A and D, symbols represent means \pm SEM.
991 *** at the top of a graph indicates $p < 0.01$ for the overall effect of treatment in A; * $p < 0.05$
992 comparing Flut to Ctrl on day 21 in B. (G-I) Symbols for individual birds are color-coded like in
993 panels A-B.

994

995 **Figure 7. Blocking androgen receptors in HVC causes birds to sing trills of longer duration,**
996 **while blockade of androgen receptors in RA extends song duration by causing birds to sing**
997 **more syllables per song. (A)** Androgen signaling in HVC does not appear to control whole song
998 duration; **(B)** however, blockade of androgen signaling in HVC with flutamide (Flut) leads birds
999 to produce trills of longer duration compared to controls (Ctrl). **(C)** Birds with their androgen
1000 receptors in RA blocked (Flut) sang longer songs than controls (Ctrl), **(D and E)** an effect driven
1001 by these birds singing more syllables per song. Bars represent mean \pm SEM. * $p < 0.05$ for an effect
1002 of treatment. Symbols in **(C)** for individual birds are color-coded like in panels A-B.

1003

1004 **Figure 8. Working model of the regulation of birdsong by androgen signaling.** This model
1005 posits that increased androgens and estrogens (e.g., during the breeding season) act at multiple
1006 sites in the songbird brain to regulate distinct features of birdsong by modulating

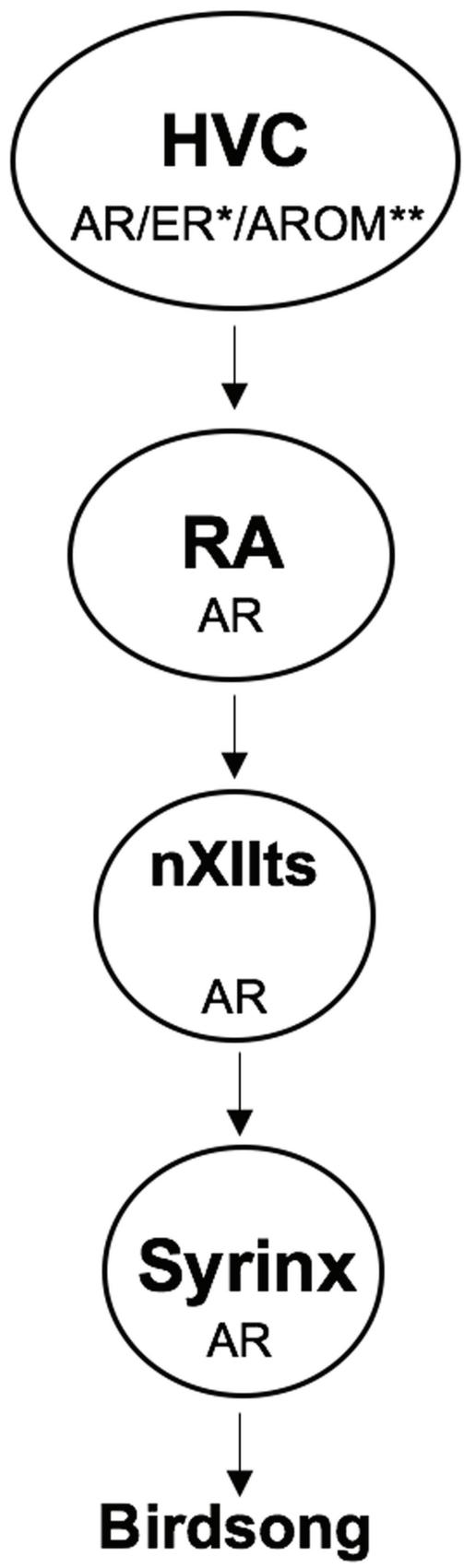
1007 neurophysiological features. Androgens act in an anatomically distinct non-redundant manner to
1008 regulate specific features of birdsong, but these effects are also modulated by the transsynaptic
1009 feedback between these nuclei. Previous studies indicate androgens and estrogens acting within
1010 the medial preoptic nucleus (POM) may regulate the motivation to sing (Alward et al., 2013;
1011 Alward et al., 2016c). The POM likely influences the song control system indirectly, via
1012 projections to the ventral tegmental area (VTA) and/or periaqueductal gray (PAG) (Riters and
1013 Algers, 2004), which project to the HVC (acronym is proper name), robust nucleus of the
1014 arcopallium (RA), and Area X (Appeltants et al., 2000; Appeltants et al., 2002; Castelino et al.,
1015 2007). There is also evidence that estrogens acting within HVC regulate song stereotypy
1016 (Meitzen et al., 2007). Aromatase (AROM) expression has been found within the POM in
1017 multiple songbirds, including canaries (Shen et al., 1995; Fusani et al., 2000), while AROM
1018 protein (in the absence of detectable AROM mRNA) has been found within presynaptic boutons
1019 within HVC in zebra finches (Peterson et al., 2005). Therefore, in addition to the potential
1020 actions of estrogens of a gonadal origin acting in the POM and HVC to regulate song, actions of
1021 estrogens generated from testosterone within these nuclei is also possible. There is also evidence
1022 that neural activity/hormone action in one region can influence morphological or
1023 neurophysiology characteristics in downstream or upstream brain regions (Brenowitz and Lent,
1024 2002; Meitzen et al., 2007; Larson et al., 2013; Brenowitz, 2014; Alward et al., 2016c), which
1025 could affect control of song. All song features listed in *italics* are predicted to be controlled by
1026 androgens and/or estrogens based on the results of past studies (Spiro et al., 1999; Long and Fee,
1027 2008; Picardo et al., 2016). HVC is colored as a gradient to represent the topographical nature of
1028 its functions: lateral HVC controls aspects of syllable usage, while medial HVC controls aspects
1029 of syntax (Basista et al., 2014). RA is also colored as gradient to represent the fact that it receives

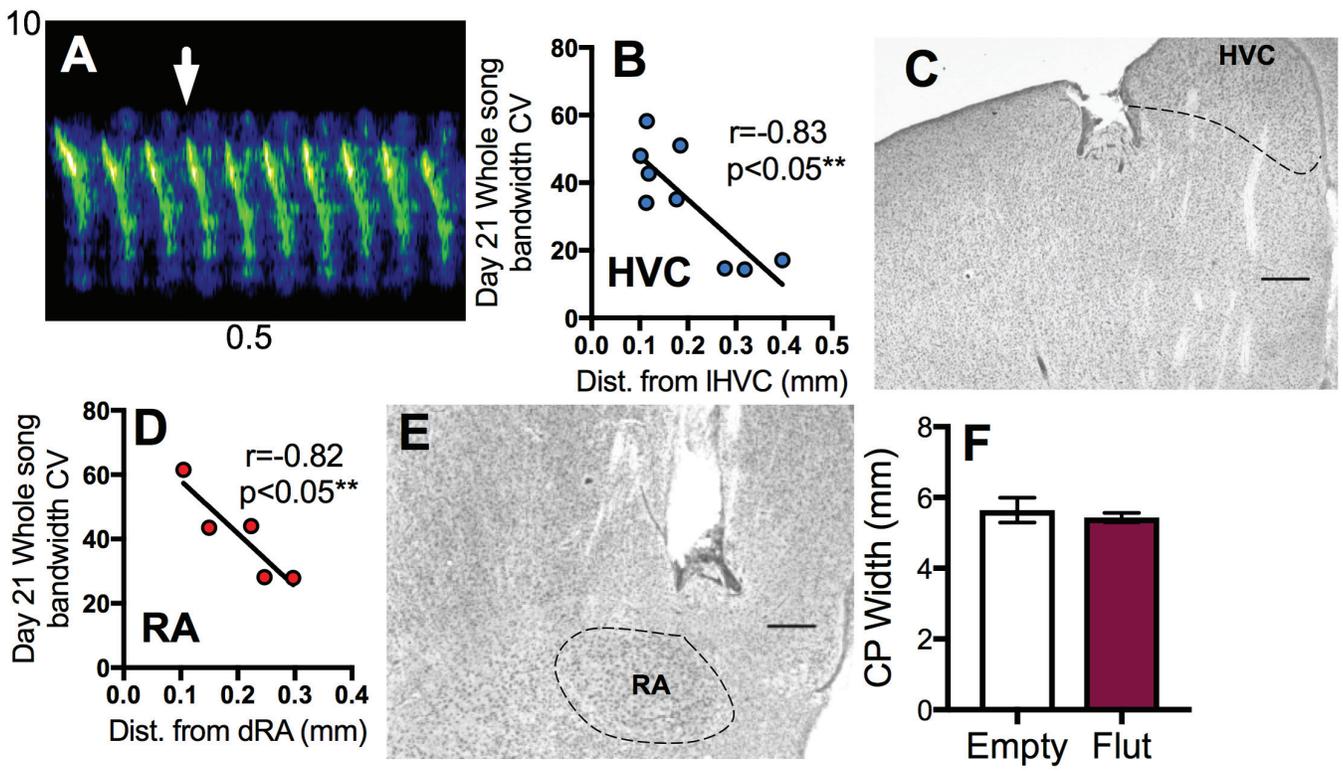
1030 input from both HVC and lateral magnocellular nucleus of the anterior nidopallium (LMAN), but
1031 then transforms these signals into firing patterns whose variability correlates to the variability of
1032 individual acoustic units of song (e.g., syllables) (Spiro et al., 1999). LMAN is part of a circuit
1033 including Area X and the dorsolateral nucleus of the medial anterior thalamus (DLM) that is
1034 critical for song learning. RA projects to the tracheosyringeal portion of the twelfth cranial nerve
1035 (nXIIIts), a hindbrain nucleus that contains androgen-sensitive motor neurons (Harding, 2008).
1036 nXIIIts neurons control muscles of the syrinx, which are also androgen sensitive, to generate
1037 song. We have shown previously that androgens at the syrinx regulate aspects of vocal
1038 performance without affecting the motivation to sing or song stereotypy (Alward et al., 2016b).
1039 The basic principle of steroid hormone action in the regulation of a complex behavior presented
1040 here—that steroid hormones act in a non-redundant manner throughout the brain and periphery
1041 to regulate behavior—may be applicable to a wide-range of behaviors and systems.
1042 AR=androgen receptors; ER=estrogen receptors; SCS=song control system.

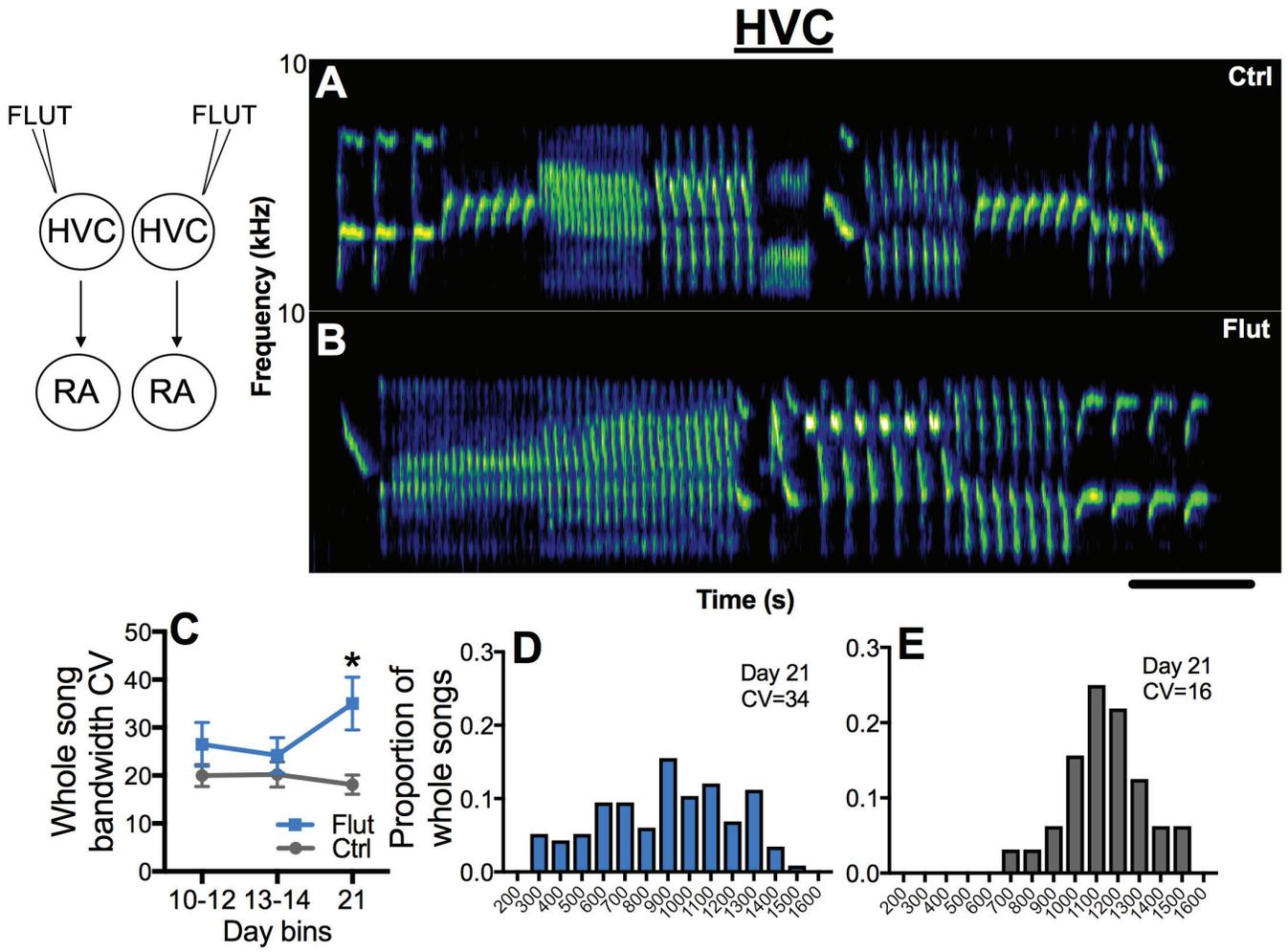
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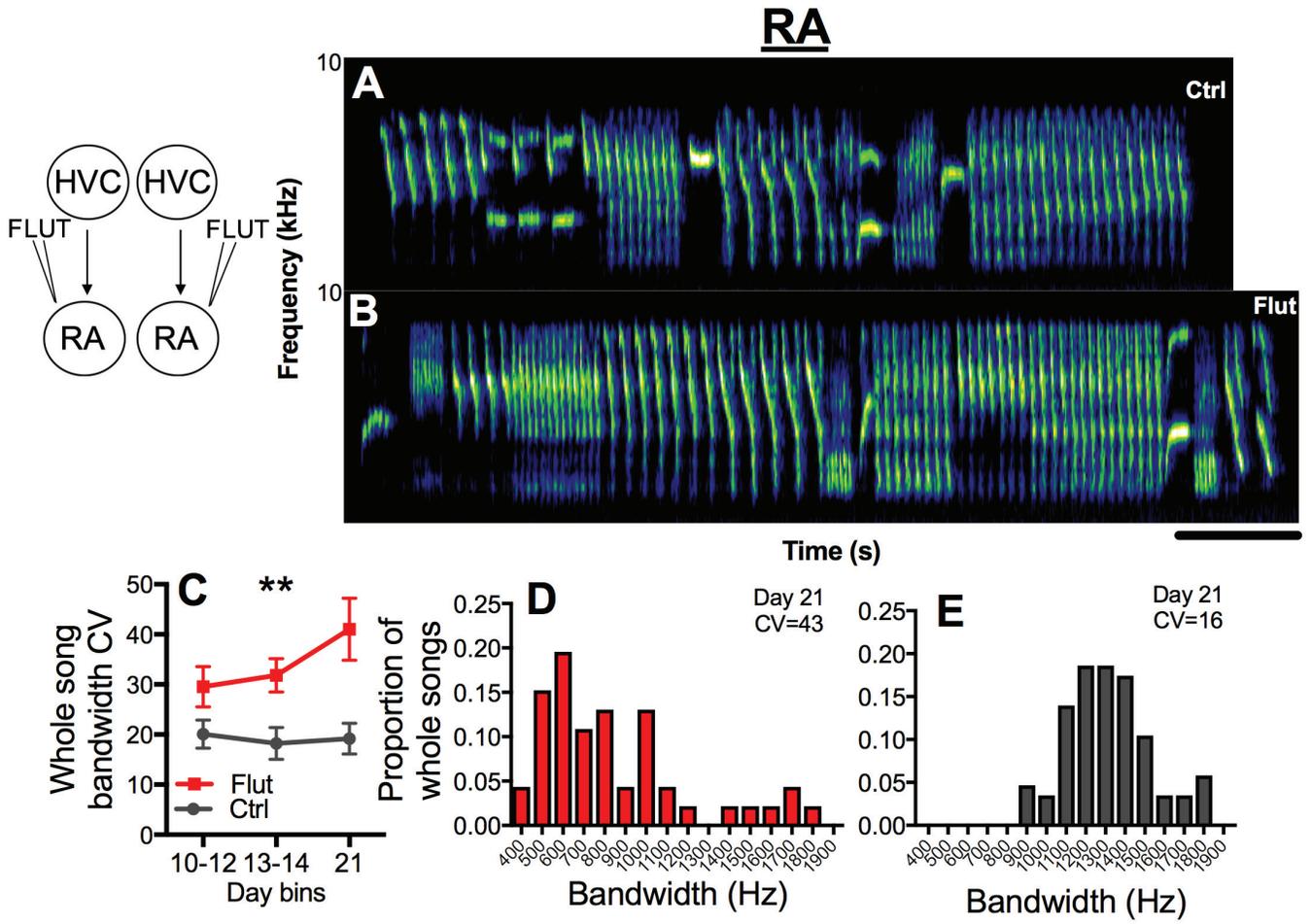
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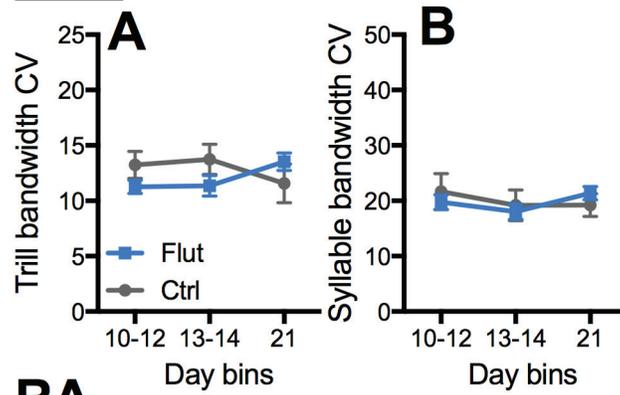




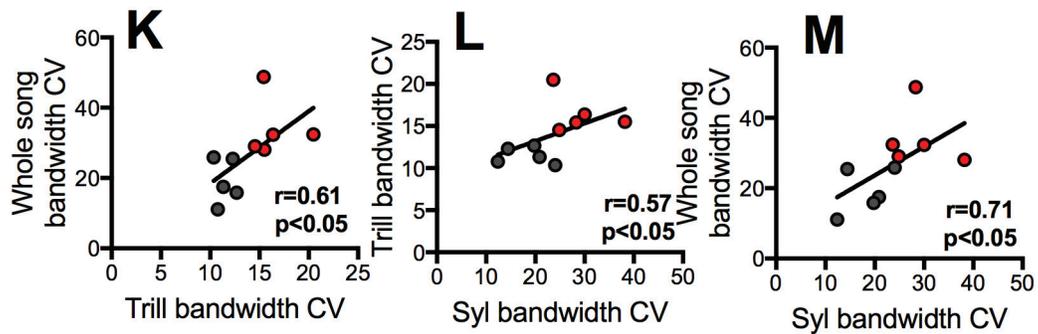
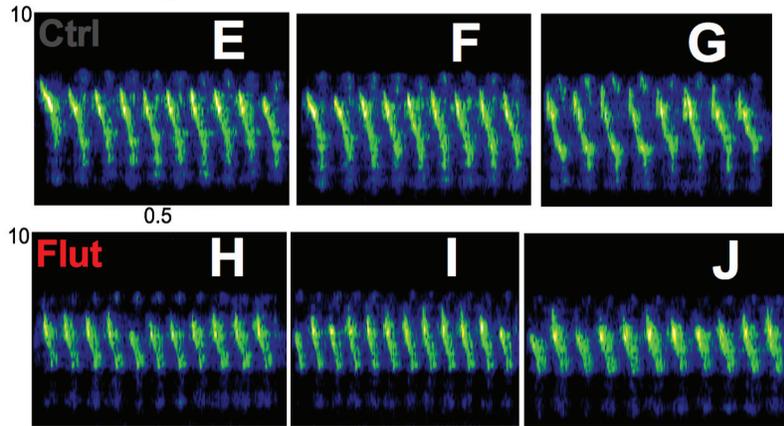
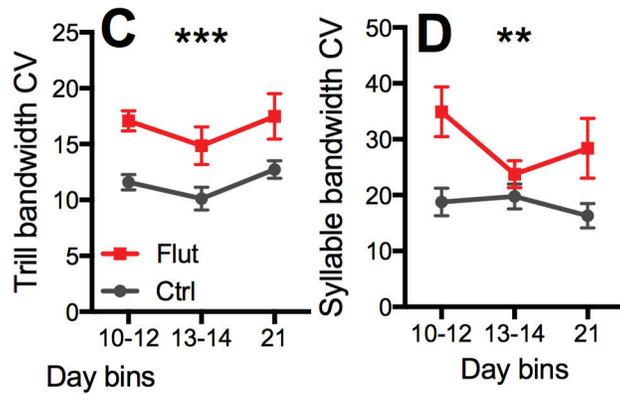




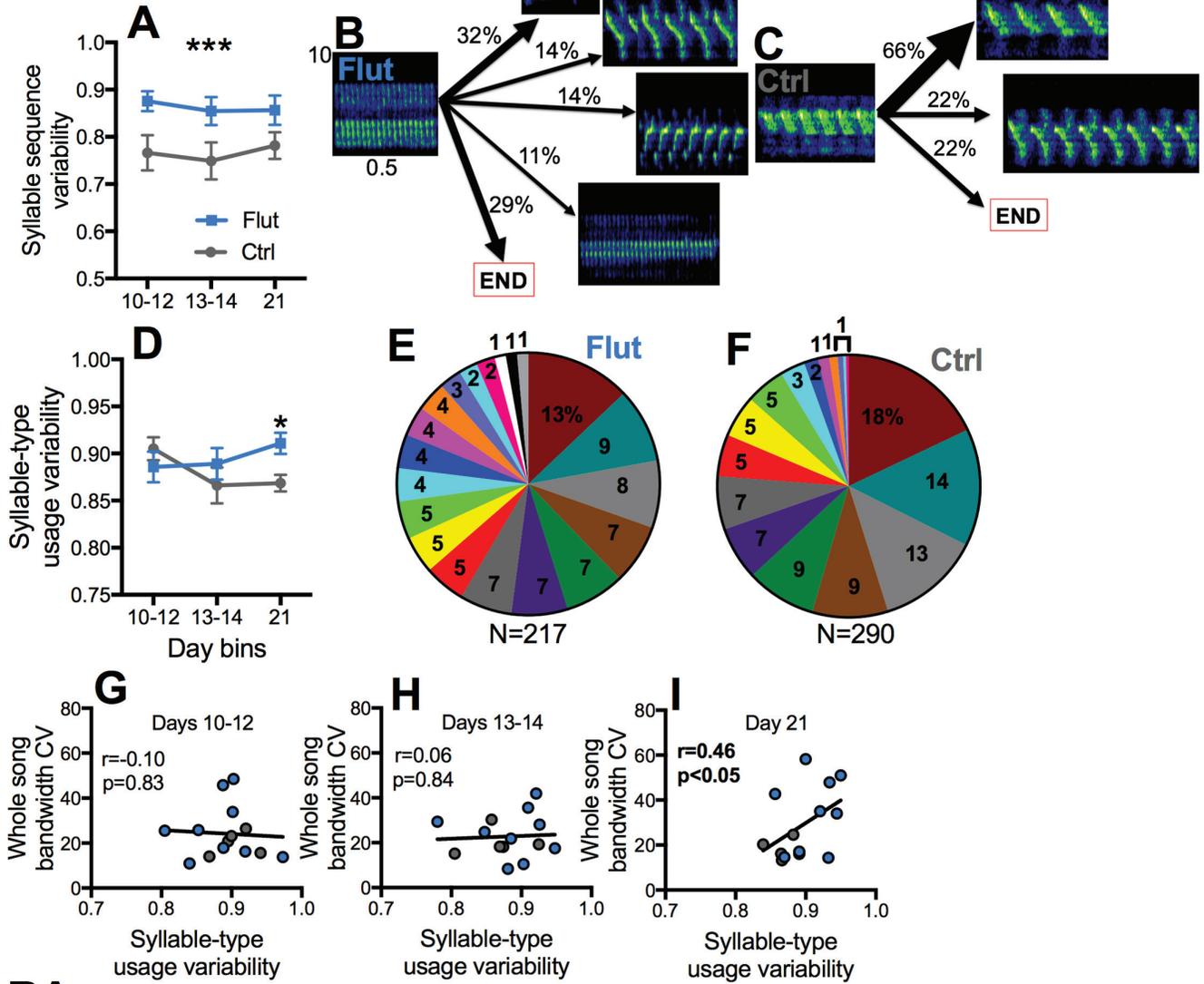
HVC



RA



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