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DIFFERENT SEASONAL PATTERNS IN SONG SYSTEM VOLUME IN WILLOW TITS AND GREAT TITS

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Running Head: Seasonal patterns in song system in two tit species

3 Figures

Key words: HVC, Area X, song control system, seasonal changes, Paridae, great tit, *Parus major*, willow tit, *Poecile montanus*

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1 **ABSTRACT**

2 In most species of seasonally breeding songbirds studied to date, the brain areas that control singing
3 (the song control system: SCS) are larger during the breeding season than at other times of the year.
4 In the family of titmice and chickadees (Paridae), one species (the blue tit, *Cyanistes caeruleus*)
5 shows the “traditional” pattern of seasonal changes, while another species (the black-capped
6 chickadee, *Poecile atricapillus*) shows at best much reduced seasonal changes in the SCS. To test
7 whether this pattern holds up in the two Parid lineages to which these two species belong, and to
8 rule out that the differences in seasonal patterns observed were due to differences in geography or
9 in laboratory, we compared the seasonal patterns in two song system nuclei volumes (HVC and Area
10 X) in willow tits (*Poecile montanus*; closely related to black-capped chickadees) and great tits (*Parus*
11 *major*; more closely related to blue tits) from the same area around Oulu, Finland. Both species had
12 larger gonads in the spring than during the rest of the year. Great tit males had a larger HVC in the
13 spring than at other times of the year, but their Area X did not change in size. Willow tits showed no
14 seasonal change in HVC or Area X size, despite having much larger gonads in the spring than the
15 great tits. Our findings suggest that the song system of willow tits and their relatives may be
16 involved in learning and producing non-song social vocalizations. Since these vocalisations are used
17 year round, there may be year-round demand on the song system. The great tit and blue tit HVC
18 may change seasonally because demand is only placed on the song system during the breeding
19 season, since they only produce learned vocalisations during this time. We suggest that changes
20 were not observed in Area X because its main role is in song learning, and there is evidence that
21 great tits do not learn new songs after their first year of life. Further study is required to determine
22 whether our hypothesis about the role of the song system in the learned, non-song vocalisations of
23 the willow tit and chickadee is correct, and to test our hypothesis about the role of Area X in the
24 great tit song system.

25 **INTRODUCTION**

26 The song control system (SCS) of many seasonally breeding songbirds undergoes seasonal plasticity
27 in size, as well as many other aspects of anatomy and physiology [e.g. De Groof et al., 2008, Meitzen
28 and Thompson, 2008, Meitzen et al., 2009, Ball and Balthazart, 2010]. In the rufous-collared sparrow
29 (*Zonotrichia capensis*), a tropical songbird, the song system is also larger when in breeding condition
30 [Moore et al., 2004]. Typically, brain areas of the SCS, especially the nucleus HVC (used as a proper
31 name, not an abbreviation), are larger at the time of year when birds sing the most, and these
32 effects have been found both in the lab and in the field. However, in a few species of seasonal
33 breeders, plasticity in the SCS has been either difficult to demonstrate or reduced in the field (wild
34 canaries (*Serinus canaria*), [Leitner et al., 2001]; and black-capped chickadees (*Poecile atricapillus*),
35 [Phillmore et al., 2006, Smulders et al., 2006, Phillmore et al., 2015]).

36 The black-capped chickadee follows the traditional seasonal songbird pattern in which males sing a
37 courtship/territorial song in the spring breeding season, at the same time as the gonads regrow and
38 testosterone levels increase [Smulders et al., 2006; Avey et al., 2008]. Nevertheless, in wild-caught
39 specimens of this species, seasonal changes in the SCS have been difficult to detect [Smulders et al.,
40 2006], except when grouping the animals by breeding condition (using testes size), rather than by
41 season, and even then the effect was very small. In one study, the effect was restricted to the
42 Robust nucleus of the Arcopallium (RA; [Phillmore et al., 2006]), but not HVC or Area X. In another
43 study, a breeding condition effect was found on HVC, but not on Area X (RA was not measured in
44 this study). In this case, the effect size was smaller than what has been observed in other songbird
45 species [Phillmore et al., 2015], and the sample birds had been kept in captivity for a period of time.
46 This could be problematic, since we know that captivity can have significant effects on another part
47 of the songbird brain: the hippocampus ([Smulders et al., 2000, LaDage et al., 2009, Tarr et al., 2009,
48 Calisi et al., 2009 (a review on the importance of differences between captive and wild species)]).
49 Photoperiod manipulations in captivity in black-capped chickadees do result in measurable changes

50 in HVC, as well as RA and Area X [MacDougall-Shackleton et al., 2003, Phillmore et al., 2005]. The
51 seeming lack of detectable seasonal changes in the SCS of wild black-capped chickadees contrasts
52 with the easily-detectable changes of close to 60% in HVC and RA in blue tits (*Cyanistes caeruleus*), a
53 relatively closely related species [Caro et al., 2005]. Area X was not measured in this study.

54 There are a number of possible explanations for why some studies fail to find seasonal changes in
55 the SCS, while others do. It could of course be coincidence, but repeated replication of either a
56 failure to find changes, or at least of very small changes compared to other species makes that
57 explanation unlikely. It is also possible that the environment in which the studies were performed
58 matters. The results from black-capped chickadees cited above came from populations in southern
59 Ontario [Canada; Phillmore et al., 2006], Nova Scotia [Canada; Phillmore et al., 2015] and central
60 New York State [USA; Smulders et al., 2006], while the blue tit results came from Corsica [French
61 Mediterranean; Caro et al., 2005]. It is therefore possible that the species differences in seasonal
62 HVC plasticity reflect the very different environments in which these studies were conducted.

63 Alternatively, the failure to detect seasonal changes may be because there are no seasonal changes
64 (or very small ones) in those species. This could be due to an unknown event in the past that has
65 removed the plasticity to change seasonally in particular phylogenetic groups. However, this is
66 unlikely to be a good explanation, since both canaries (at least domesticated ones, [Nottebohm,
67 1981]) and black-capped chickadees still show seasonal changes when tested in captivity
68 [Macdougall-Shackleton et al., 2003]. This suggests that the potential for seasonal changes is present
69 in these species. So why do we not see seasonal changes in the field? Even though wild canaries
70 change their repertoire across seasons, they do not change their singing intensity, singing (and
71 therefore using their SCS) year-round [Leitner et al., 2001]. If year-round use of the SCS is associated
72 with a lack of seasonal changes in SCS volumes, then one potential explanation for the Parid
73 situation is that the species differences could reflect the different vocalization repertoires and
74 seasonal uses of these repertoires in the two species: chickadees have a complex set of learned calls,

75 which are used year-round by both sexes, and a relatively simple breeding song [Avey et al., 2008];
76 while blue tits have a more complex song and lack the complex social calls of the chickadees [Bijnens
77 and Dhondt, 1984]. This would suggest chickadees and their relatives use the SCS year-round, while
78 blue tits and their relatives don't.

79 The current study aims to eliminate the possibility that different environments cause differences in
80 seasonal patterns, and to verify that the different patterns observed in chickadees and blue tits are
81 not specific to those species, but generalize to other species in their clades. We compared the
82 seasonal plasticity of two SCS nuclei, HVC and Area X, in two species exhibiting a different seasonal
83 pattern of vocalizations, but which were collected from the same environment: willow tits (*Poecile*
84 *montanus*) and great tits (*Parus major*).

85 Willow tits and great tits are sedentary hole-nesting passerines. In the Oulu area of northern Finland
86 (ca. 65°3'N, 25°27'E, average elevation 15 meters), the main singing period for great tits and willow
87 tits occurs in March and April when the breeding territories are established. The laying time of
88 genuine first clutches is May; the annual median onset of egg-laying range from 5th to 20th May in
89 the willow tit and from 7th to 30th May in the great tit [Vatka et al. 2011, 2014]. The earliest willow tit
90 and great tit nests have been commenced on 29 and 30 April, respectively. In both species the
91 earliest clutches start hatching in late May and the main nestling period is in June and early July.
92 Only females incubate, but both parents provide parental care for young. Both species can lay repeat
93 clutches if the first nesting fails. Parents go through postnuptial molt, willow tits in June–August and
94 great tits in late June–October [Orell & Ojanen, 1980].

95 Willow tits are closely related to the black-capped chickadee. They winter at their breeding grounds
96 in small, highly territorial and stable groups [Koivula & Orell, 1988, Ekman, 1989]. They have a set of
97 complex social vocalizations, which they use year-round, similarly to the chickadee [Haftorn, 1993,
98 Ficken et al., 1978, Ficken et al., 1985, Ficken et al., 1987, Miyasato and Baker, 1999, Baker et al.,
99 2000]. These non-song vocalisations have been shown to be learned, at least in the chickadee

100 [Mammen and Nowicki, 1981, Ficken and Weise, 1984, Ficken et al., 1987, Nowicki, 1989, Shackleton
101 et al., 1992, Kroodsma et al., 1995, Hughes et al., 1998]. Their territorial song, on the other hand, is
102 relatively simple compared to most songbird songs [Martens and Nazarenko, 1993].

103 Great tits are more closely related to blue tits, and equally lack the social vocalizations of the
104 chickadee [Johansson et al., 2013]. They follow the “traditional” songbird pattern of singing a
105 complex courtship and territorial song during the breeding season [Rost, 1990]. Great tits overwinter
106 in constantly changing non-territorial flocks outside their breeding territories [Ekman, 1989]. If the
107 production of song or song-like vocalisations is a potential mechanism of seasonal changes in the
108 SCS, then we should find seasonal changes in the SCS of great tits, but not of willow tits, even when
109 both are collected from the same environment.

110 **MATERIALS AND METHODS**

111 ***Animals***

112 Subjects were adult male great tits and willow tits, captured at Oulu under a licence from the North
113 Ostrobothnian Regional Environmental Centre. The birds were captured in two seasons: 2006-2007
114 and during April 2015. Great tits were caught using funnel traps baited with food, and willow tits
115 were caught using mist nets, song playback and decoy birds. All birds were aged in the hand based
116 on plumage. Great tits were sexed using the colour and pattern of their plumage, and wing length if
117 necessary, and willow tits by the observation of song production and wing length. Sex was confirmed
118 after the dissection of the gonads.

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		Number of samples collected						Total
		Breeding season		Rest of the year				
		March	April	Aug	Sept	Nov	Dec	
Species	Great tit	3	6	4	3	2	/	18
	Willow tit	/	10	3	4	1	1	19
Total		19		18				37

123

124 **Table 1:** total sample sizes from 2006/7 and 2015 of great tit and willow tit brains collected in the
 125 breeding season (March or April) or the rest of the year (August, September, November or
 126 December).

127 *2006 – 2007*

128 In the spring breeding season, male great tits were collected between 24th March and 30th March
 129 2007, and willow tits were collected between 16th April and 22nd April 2007. The average timing of
 130 the first clutches in 2007 was the 15th May for great tits, and the 10th May for willow tits. During this
 131 sampling period, our sample from the breeding season consisted of 8 birds: 3 great tits and 5 willow
 132 tits, and our sample from the rest of the year consisted of 18 birds: 9 great tits and 9 willow tits. For
 133 Area X analyses, 1 great tit sample and 3 willow tit samples were not included because of tissue
 134 damage.

135 *April 2015*

136 To increase our breeding season sample size, more birds were collected in April 2015. Great tits
 137 were collected between 8th April and 16th April, and willow tits were collected between 16th April
 138 and 22nd April. The average timing of the first clutches in 2015 was the 14th May for great tits, and
 139 the 10th May for willow tits. Our sample from this period consisted of 11 birds: 6 great tits and 5
 140 willow tits. Our exact sample sizes are indicated in Table 1.

141 ***Validating breeding condition***

142 To assess whether the birds were in breeding condition at the time of capture, their gonads were
 143 weighed after the birds had been humanely killed and the brain dissection had been performed. In

144 2006/07 gonads were rapidly frozen on dry ice after dissection. They were then shipped and
145 weighed back in Newcastle. To quantify gonad size, the frozen gonads were weighed in their
146 centrifuge tubes. The weight of the same empty frozen centrifuge tube was then subtracted from all
147 of these weights. In 2015, gonads were weighed fresh after dissection in Finland, and then
148 discarded.

149 ***Histology***

150 *2006 – 2007*

151 Birds were killed with rapid decapitation. One hemisphere of the brain was immersed in 4%
152 formaldehyde in PBS. After 48 hours of fixation, the hemispheres were cryoprotected in 30% sucrose
153 solution, embedded in O.C.T. (Optimal Cutting Temperature compound for cryosectioning), frozen
154 on dry ice and stored at -80°C. After all of the samples had been collected, they were shipped from
155 Oulu to Newcastle. They were all sectioned at 70 µm on a cryostat, and every other section was
156 thaw-mounted onto gelatin-coated slides. The sections were stained with cresyl violet and
157 coverslipped.

158 *April 2015*

159 Birds were anaesthetised using isoflurane before decapitation. Both hemispheres of the brain were
160 immersed in a solution of 4% formaldehyde in PBS. After 48 hours of fixation, the brains were
161 cryoprotected in 30% sucrose solution and stored in a cold room at 4°C. After all of the samples had
162 been collected, they were shipped from Oulu to Newcastle for further processing and histological
163 measurements. They were embedded in O.C.T., sectioned at 40 µm on a cryostat into PBS solution.
164 Every other free floating section was mounted onto gelatin-coated slides. The sections were then
165 stained with cresyl violet and coverslipped.

166 ***Brain region morphometry***

167 To quantify the size of the brain areas we were interested in, we outlined the nuclei in all of the
168 sections in which they could be seen. For HVC, nucleus Rotundus (Rt) and Telencephalon
169 measurements, we used StereoInvestigator® connected to a Leica DMLB microscope with a Prior
170 automated stage and an Optronics Microfire digital camera. For Area X measurements, we used
171 Zen® connected to a Nikon Eclipse microscope with a rotatable stage and a Zeiss Axiocam 105 colour
172 camera. Figure 1 displays representative examples of the nuclei we outlined in our morphometric
173 analyses. Outlines were drawn using a 2.5x or 2x objective, sometimes changing to the 10x objective
174 for clarification of boundaries. Only half brains were collected in 2006/07 (equal numbers of left and
175 right hemispheres), but we collected whole brains in 2015. For consistency, we outlined only one
176 hemisphere in the brains collected in 2015. We outlined equal numbers of left and right
177 hemispheres, which were randomly allocated beforehand.

178 Rt and the telencephalon (both used as control areas in our analysis) were outlined by different
179 people for the 2006-2007 samples vs. the 2015 samples. All HVC and Area X outlines were
180 performed by the same person (GKL). The outlines of the 2006/07 samples were performed blind to
181 species and season. It was not possible to be blind to the season of the 2015 samples since they
182 were all collected at the same time of year, however the outlines were performed blind to species.

183 *2006 – 2007 (70 µm sections, every other section taken)*

184 To calculate the volume of HVC, Area X and Rt, the area of each section was multiplied by 140 µm
185 (the distance between measurements). These volumes were added up for all the sections containing
186 the nucleus of interest. To calculate telencephalon volume, its surface area was measured on every
187 4th section on the slides, multiplied by 560 µm and added up.

188 *April 2015 (40 µm sections, every other section taken)*

189 To calculate the volume of HVC, Area X and Rt, the area of each section was multiplied by 80 µm.
190 These volumes were added up for all the sections containing the nucleus of interest. To calculate

191 telencephalon volume, its surface area was measured on every 14th section on the slides. This was
192 then multiplied by 560 μm . To provide a starting point and standardization of measurement, the first
193 section where the anterior commissure was present was always one of the sections measured.

194 ***Data analysis***

195 We investigated differences in seasonal patterns between species by testing whether an interaction
196 between season and species could explain variation in volumes of HVC, Area X and Rt, and in gonad
197 weight. All measurements were natural-log-transformed for data analysis (+ 1 to avoid negative
198 scaling). The design of our statistical models are classic factorial AN(C)OVAs. We implemented these
199 in the Generalized Linear Model function in SPSS version 22 for Windows, with a linear outcome
200 variable because this gave us a more flexible output, enabling pairwise comparisons between
201 means. The output from these models is Wald's χ^2 . All analyses were also run as classic AN(C)OVAs
202 and the outcomes were qualitatively the same.

203 Tests for HVC, Area X and Rt were run using two factors: species (willow tit/great tit) and season
204 (breeding season/rest of the year), and we tested for main effects of species and season, as well as
205 for the interaction between these factors. We included the volume of the telencephalon in the model
206 as a covariate, to control for any overall size differences between the samples because of the two
207 different methods used to process the tissue (see [Smulders 2002]). We included telencephalon as a
208 co-variate rather than analysing each nucleus as a percentage of the telencephalon, because ratios
209 conflate variation in the numerator with variation in the denominator. However, we will plot our
210 results as percentages of telencephalon, to enable readers to compare our results with other studies
211 which have used these ratios in their analyses of nuclei volume.

212 No other factors or interactions between factors and co-variate were included in the model. Results
213 were considered significant if $p < .05$.

214 **RESULTS**

215 ***Gonad mass***

216 We measured gonad mass as a proxy of breeding condition. In both species, testes were larger in birds
217 caught during the spring (March and April) than during the rest of the year (August – December; $\chi^2_1 =$
218 96.3, $p < 0.001$). We also found a significant interaction between species and season ($\chi^2_1 = 6.17$, $p =$
219 0.013; see Figure 2). During the spring, willow tits had larger testes than great tits ($p = 0.003$). This
220 effect was not present during the rest of the year, when birds were not in breeding condition ($p =$
221 0.593).

222 ***Brain morphometry***

223 We checked our calculation of nuclei volume was consistent with another measure used in the
224 literature: the formula for a cone frustum (see [Smith et al., 1995]). We observed similar results and
225 levels of significance, and the two measures were significantly correlated ($r = 0.944$, $p < 0.001$). The
226 analyses we report below use our original calculation of volume.

227 ***HVC volume***

228 We investigated whether the volume of the SCS nucleus HVC changed seasonally in both great tits and
229 willow tits. Telencephalon volume significantly predicted HVC volume ($\chi^2_1 = 42.84$, $p < 0.001$) and
230 there were no independent main effects of species ($\chi^2_1 = 2.32$, $p = 0.128$) or season ($\chi^2_1 = 2.434$, $p =$
231 0.119). There was, however, a significant interaction between species and season ($\chi^2_1 = 6.56$, $p = 0.01$;
232 Figure 3). In great tits, HVC volume was larger in the spring, than when birds were not in breeding
233 condition ($p = 0.006$). There was no seasonal difference in HVC volume in the willow tits ($p = 0.652$).
234 Comparing the species within each breeding season, we find that breeding great tits have significantly
235 larger HVC volumes than breeding willow tits ($p = 0.004$), but this effect was not present outside of
236 the spring ($p = 0.498$).

237 ***Area X volume***

238 We also investigated whether the volume of the SCS nucleus Area X changed seasonally in both great
239 tits and willow tits. Telencephalon volume significantly predicted Area X volume ($\chi^2_1 = 64.92$, $p < 0.001$)
240 and there were no independent main effects of species ($\chi^2_1 = 0.095$, $p = 0.758$) or season ($\chi^2_1 = 0.005$,
241 $p = 0.944$). In contrast to our analysis of HVC, we found no significant interaction between species and
242 season ($\chi^2_1 = 0.570$, $p = 0.450$; Figure 3).

243 ***Nucleus rotundus volume***

244 To ensure that the seasonal effect of the increase in HVC volume was specifically in the SCS, we
245 measured a control structure which is not involved in song control: the visual nucleus Rotundus (Rt;
246 [Laverghetta & Shimizu, 1999]). It is also easy to identify, making the quantification of its volume
247 reliable and repeatable across individuals.

248 Telencephalon volume significantly predicted Rt volume ($\chi^2_1 = 182.73$, $p < 0.001$). As expected, we
249 found no evidence of an effect of season on Rt volume ($\chi^2_1 = 1.76$, $p = .185$), nor any interaction
250 between species and season ($\chi^2_1 = 0.17$, $p = 0.679$). However, there was a significant main effect of
251 species: Rt was larger relative to telencephalon in great tits than in willow tits ($\chi^2_1 = 6.08$, $p = 0.014$;
252 Figure 3).

253 **DISCUSSION**

254 *Main findings*

255 Our results suggest seasonal stability in the size of the willow tit SCS, compared to seasonal plasticity
256 in the size of HVC in the great tit. The lack of seasonal change in the willow tit SCS in the field is
257 consistent with previous studies on the black-capped chickadee, a closely-related species, which have
258 reported either reduced seasonal change or seasonal stability in the SCS [Phillmore et al., 2006,
259 Smulders et al., 2006, Phillmore et al., 2015]. Ecology and behaviour of willow tits and black-capped
260 chickadees are very similar to each other. The finding of changes in HVC size in early spring in great
261 tits is also consistent with previous findings from blue tits [Caro et al., 2005]. Our results give support

262 to the hypothesis that the difference in seasonal plasticity between these two species is related to the
263 differences in vocalizations made by the two groups of birds [Smulders et al., 2006]. These differences
264 in turn relate to differences in winter social systems.

265 *Functional explanations of seasonal stability*

266 Although we measured the size of the SCS nuclei, previous research has observed seasonal changes
267 using alternative measures, including neuron number, neuronal density and incorporation of new
268 neurons into the nucleus (see [Tramontin & Brenowitz, 2000] for a review). One explanation for our
269 findings is that measuring volume was not sensitive enough to observe subtle seasonal changes in our
270 sample of birds. A previous study of the black-capped chickadee did find small seasonal changes in the
271 SCS, which were of a smaller magnitude to the changes observed in the majority of species [Phillmore
272 et al., 2015]. Measuring neuron number or neuronal density in our samples could therefore yield
273 different results than our measure of volume.

274 Nevertheless, in most songbirds, SCS volumes do change seasonally. We first consider why HVC
275 volume changes seasonally in great tits, but not in willow tits. Willow tits are closely related to black-
276 capped chickadees, and have a similar large and complex repertoire of social calls, which they
277 perform year-round [Haftorn, 1993]. Great tits, in contrast, are more closely related to blue tits, and
278 equally lack the learned social vocalizations, while possessing a complex courtship/territorial song,
279 like most other seasonally breeding songbirds studied [McGregor and Krebs, 1982]. Given these
280 differences in call repertoire and in the seasonal pattern of vocalizations, we hypothesise that HVC is
281 responsible for the learning and generation of the complex social call vocalizations year-round, just
282 like it plays a role in the learned zebra finch long call [Simpson and Vicario, 1990]. The extra singing
283 during the breeding season then does not add sufficient demand on HVC's circuitry to lead to a large
284 increase in volume during the breeding season. Interestingly, the size of the willow tit HVC is
285 equivalent to the great tit non-breeding HVC, and smaller than the breeding HVC. If our hypothesis is

286 correct, this suggests that the demands on HVC's circuitry are less for the learned social vocalizations
287 and simple song than they are for the complex great tit song.

288 In contrast to our results for HVC, we found no evidence of seasonal plasticity in Area X in either
289 species. The lack of seasonal plasticity in Area X volume in the willow tits is consistent with the lack
290 of seasonal plasticity in HVC. The finding is also consistent with several other studies on seasonal
291 songbirds which have found evidence of plasticity in one or two SCS nuclei (usually HVC) but not others.
292 Although the study on blue tits did not measure Area X [Caro et al., 2005], two studies of the black-
293 capped chickadee have found small seasonal plasticity in HVC but not in their Area X [Phillmore et al.,
294 2006, Phillmore et al., 2015]. HVC is involved in the motor production of song as well as in song
295 learning, whereas Area X is involved in learning, but not production. If great tits learn their song types
296 during an early life critical period and lose the ability to do so afterwards (close-ended song learning,
297 see [Marler., 1970]), this could explain why we have observed seasonal changes in the great tit HVC,
298 but not in Area X. However, there is not a definite consensus about when great tits learn their song.
299 Previous observations have suggested that great tits may learn songs from neighbours in adulthood
300 [McGregor & Avery., 1985, McGregor & Krebs., 1989, Franco & Slabbekoorn., 2009], while a recent
301 study suggests that these findings were due to methodological issues, and that great tit is actually a
302 close-ended learner [Rivera-Gutierrez et al., 2011].

303 To our knowledge, our study is the first to investigate seasonal changes in the great tit SCS. Additional
304 studies are required to understand the lack of changes we have observed in Area X, and to determine
305 when great tits learn their song. The fact that seasonal changes have been reported in HVC and RA,
306 but not in Area X in the white-crowned sparrow (*Zonotrichia leucophrys*), a confirmed close-ended
307 learner, supports our hypothesis [Smith et al., 1995, Smith et al., 1997, Tramontin et al., 1998]. Growth
308 of Area X has only been reported in this species after photoperiodic manipulations in experimental
309 settings [Brenowitz et al., 1998, Thompson & Brenowitz 2005]. Although we were unable to measure
310 SCS nucleus RA because of issues with our older samples' tissue quality, determining whether it

311 changes seasonally in the great tit could also add strength to our hypothesis about Area X, since RA is
312 mainly involved in motor production, which does change seasonally.

313 *The mechanisms of seasonal stability and plasticity*

314 The pattern we observed in our data is consistent with the idea that HVC size is driven by the amount
315 of vocalization performed by the birds [Sartor et al., 2005]. In great tits, the complex song is only used
316 intensively during the breeding season. This change in usage of the motor circuit could then drive the
317 change in HVC size. The social vocalizations in willow tits and chickadees, however, are used
318 intensively year-round [Avey et al., 2008], resulting in seasonal stability in HVC size. The fact that
319 breeding-condition-related changes in the SCS were detected in captive black-capped chickadees
320 [MacDougall-Shackleton et al., 2003] supports this argument. Captive chickadees produce the same
321 vocalizations as wild birds, but in much smaller quantities [Avey et al., 2011]. If vocal activity is indeed
322 responsible for the size of the SCS, then the lower level of vocalizations in captivity might lead to a
323 smaller SCS when birds are not in breeding condition (including Area X in that case; [MacDougall-
324 Shackleton et al., 2003]). The increase in fee-bee singing observed under increasing photoperiod in
325 the lab may then be a large enough change in the use of learned vocalizations to have a detectable
326 effect on SCS volumes, because the baseline vocal activity is so much lower than in the field. This
327 mechanism may also apply to other groups of songbirds, such as the European starling, where direct
328 effects of singing activity on SCS have been reported [Ball et al., 2004, Sartor & Ball., 2005].

329 The fact that HVC did not change in size in the willow tit, despite their large testes and their
330 presumably high levels of testosterone in the spring, suggests that in this species there is not a direct
331 effect of testosterone on HVC volume, as there is in other species [Brenowitz and Lent, 2000].
332 Although we did not directly measure testosterone in our population, several other studies have found
333 seasonal changes in testosterone levels in the willow tit [Silverin., 1984, Silverin et al., 1986], and the
334 great tit (plasma testosterone: [Van Duyse et al., 2003], testosterone metabolizing enzymes in the
335 brain: [Silverin & Deviche, 1991]), which suggests that there is a change in testosterone in our study

336 species during the breeding season which matches the change in gonad size that we have observed.
337 Interestingly, in blue tits, evidence also suggests that testosterone is not an important factor in driving
338 SCS seasonal plasticity, as SCS nuclei increase in size before the spring surge in testosterone [Caro et
339 al. 2005]. This may mean that in Parids in general, direct effects of testosterone on seasonal changes
340 in SCS nuclei size are less likely. This is similar to red-backed fairy wrens (*Malurus melanocephalus*) in
341 which a dissociation between testosterone levels and SCS nuclei size has been reported [Schwabl et
342 al., 2015], but unlike other groups of songbirds, where testosterone seems to be the driving force
343 behind seasonal plasticity in the SCS (e.g. the rufous collared sparrow [Small et al., 2015] and the
344 canary [Madison et al., 2015], see [Tramontin & Brenowitz, 2000] for a review). Additional studies of
345 seasonal changes in the Parid song system which directly measure and manipulate testosterone levels
346 (both systemically and locally [Tramontin et al., 2000, Brenowitz et al., 2007, Meitzen et al., 2007]) are
347 necessary to determine whether our hypothesis is correct.

348 *Conclusion*

349 In conclusion, this study is the first to directly compare seasonal changes in the song control system
350 between two Parid species from the same environment, exhibiting differences in song behaviour. Our
351 results confirm the lack of seasonal changes in HVC and Area X in the “atypical” species which
352 produces complex social vocalisations year-round in addition to its simple courtship song, and the
353 existence of seasonal plasticity in the HVC of the more “traditional” species, which has a much smaller,
354 simpler repertoire of non-song vocalisations, but a more complex courtship song. We suggest that
355 the willow tit HVC and Area X are stable in size throughout the year in the field because these nuclei
356 are involved in the learning and production of its social vocalizations, as well as its courtship song.
357 Area X may not change seasonally in the great tit because they are potentially close-ended learners.
358 Direct study of the role of HVC and Area X in the song and non-song vocalizations in different Parid
359 species will be required to test our hypotheses.

360

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545 **FIGURE CAPTIONS**

546 **Figure 1** Representative photomicrographs of HVC (**a**), Area X (**b**) and Rt (**c**) taken at 10 X magnification.

547

548 **Figure 2** Gonad mass for the two species at the different times of the year. We plotted the means for
549 the breeding season (March – April) and the rest of the year (August – December). Error bars represent
550 standard error. Asterisks indicate significant results ($*p < 0.05$, $** p < 0.01$).

551

552 **Figure 3** Volumes for the different brain regions, plotted by species and season. We plot the means
553 for nuclei volume calculated as a percentage of telencephalon volume. **a.** HVC (mean \pm SE): there is a
554 significant seasonal difference in the great tit HVC, but not in the willow tit HVC; **b.** Area X (mean \pm
555 SE): there is no seasonal difference in the great tit or the willow tit Area X; **c.** Nucleus Rotundus (mean
556 \pm SE): there is no seasonal difference in the great tit or the willow tit Rotundus, but the great tit
557 Rotundus is significantly larger than the willow tit Rotundus. Asterisks indicate significant results ($*p$
558 < 0.05 , $** p \leq 0.01$).