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Different Seasonal Patterns in Song System Volume in Willow Tits and Great Tits

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

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.

INTRODUCTION

The song control system (SCS) of many seasonally breeding songbirds undergoes seasonal plasticity

in size, as well as many other aspects of anatomy and physiology [e.g. De Groof et al., 2008, Meitzen

and Thompson, 2008, Meitzen et al., 2009, Ball and Balthazart, 2010]. In the rufous-collared sparrow

(*Zonotrichia capensis*), a tropical songbird, the song system is also larger when in breeding condition

[Moore et al., 2004]. Typically, brain areas of the SCS, especially the nucleus HVC (used as a proper

name, not an abbreviation), are larger at the time of year when birds sing the most, and these

effects have been found both in the lab and in the field. However, in a few species of seasonal

breeders, plasticity in the SCS has been either difficult to demonstrate or reduced in the field (wild

canaries (*Serinus canaria*), [Leitner et al., 2001]; and black-capped chickadees (*Poecile atricapillus*),

[Phillmore et al., 2006, Smulders et al., 2006, Phillmore et al., 2015]).

The black-capped chickadee follows the traditional seasonal songbird pattern in which males sing a

courtship/territorial song in the spring breeding season, at the same time as the gonads regrow and

testosterone levels increase [Smulders et al., 2006; Avey et al., 2008]. Nevertheless, in wild-caught

specimens of this species, seasonal changes in the SCS have been difficult to detect [Smulders et al.,

2006], except when grouping the animals by breeding condition (using testes size), rather than by

season, and even then the effect was very small. In one study, the effect was restricted to the

Robust nucleus of the Arcopallium (RA; [Phillmore et al., 2006]), but not HVC or Area X. In another

study, a breeding condition effect was found on HVC, but not on Area X (RA was not measured in

this study). In this case, the effect size was smaller than what has been observed in other songbird

species [Phillmore et al., 2015], and the sample birds had been kept in captivity for a period of time.

This could be problematic, since we know that captivity can have significant effects on another part

of the songbird brain: the hippocampus ([Smulders et al., 2000, LaDage et al., 2009, Tarr et al., 2009,

Calisi et al., 2009 (a review on the importance of differences between captive and wild species)].

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

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

2006 – 2007

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

April 2015

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

Validating breeding condition

To assess whether the birds were in breeding condition at the time of capture, their gonads were 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; χ^2_1 =

218 96.3, p <0.001). We also found a significant interaction between species and season (χ^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 (χ^2_1 = 42.84, p < 0.001) and

230 there were no independent main effects of species (χ^2_1 = 2.32, p = 0.128) or season (χ^2_1 = 2.434, p =

231 0.119). There was, however, a significant interaction between species and season (χ^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 (χ^2_1 = 64.92, p < 0.001)

240   and there were no independent main effects of species (χ^2_1 = 0.095, p = 0.758) or season (χ^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 (χ^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 (χ^2_1 = 182.73, p < 0.001).As expected, we

249   found no evidence of an effect of season on Rt volume (χ^2_1 = 1.76, p = .185), nor any interaction

250   between species and season (χ^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 (χ^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

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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

14

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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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, ** $p \leq 0.01$*).