Experimental exposure to traffic noise affects brain development and song learning in juvenile zebra finches (*Taenopygia guttata*)

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Recently, numerous studies have observed changes in bird vocalizations – especially song - in urban habitats. These changes are often interpreted as being adaptive, since they increase the active space of the signal in its environment. However, the proximate mechanisms driving cross-generational changes in song are still unknown. We performed a captive experiment to identify whether urban traffic noise affects song learning in young birds, which could drive cultural evolution of urban song. We used male zebra finches (*Taeniopygia guttata*) that were bred exposed, or not exposed, to traffic noise, recorded their songs and compared these to fathers' songs. We also measured baseline corticosterone to determine if traffic noise acts as a chronic stressor, and investigated the effects of noise on development of song-control brain regions in males. While individuals tended to learn syllables accurately from tutors regardless of noise environment, syntax (the ordering of syllables within songs) was affected by noise. Noise did not affect baseline corticosterone, however brain regions associated with song learning were smaller in males that had experienced noise in early development. These findings provide a possible mechanism by which noise affects behaviour, leading to potential population differences between wild animals occupying noisier urban environments compared with those in quieter habitats.

1   **Experimental exposure to traffic noise affects brain development and song learning in**

2   **juvenile zebra finches (*Taenopygia guttata*)**

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14   Running Headline: Traffic noise affects song learning

20 **Abstract**

21 Recently, numerous studies have observed changes in bird vocalizations – especially song - in

22 urban habitats. These changes are often interpreted as being adaptive, since they increase the

23 active space of the signal in its environment. However, the proximate mechanisms driving cross-

24 generational changes in song are still unknown. We performed a captive experiment to identify

25 whether urban traffic noise affects song learning in young birds, which could drive cultural

26 evolution of urban song. We used male zebra finches (*Taeniopygia guttata*) that were bred

27 exposed, or not exposed, to traffic noise, recorded their songs and compared these to fathers'

28 songs. We also measured baseline corticosterone to determine if traffic noise acts as a chronic

29 stressor, and investigated the effects of noise on development of song-control brain regions in

30 males. While individuals tended to learn syllables accurately from tutors regardless of noise

31 environment, syntax (the ordering of syllables within songs) was affected by noise. Noise did not

32 affect baseline corticosterone, however brain regions associated with song learning were smaller

33 in males that had experienced noise in early development. These findings provide a possible

34 mechanism by which noise affects behaviour, leading to potential population differences

35 between wild animals occupying noisier urban environments compared with those in quieter

36 habitats.

37 **Keywords**

38 noise, birdsong, zebra finch, HVC, song learning, urban ecology

39

40   **Introduction**

41

42   Research into the effects of traffic noise on the behaviour of wild animals has increased

43   over the past decade. We now know that several species of bird change their song in association

44   with anthropogenic noise. Specifically, birds appear to alter the frequency (Brumm 2006a;

45   Potvin et al. 2011; Slabbekoorn & Peet 2003), amplitude (Kight & Swaddle 2015), timing

46   (Brumm 2006b; Cartwright et al. 2014; Fuller et al. 2007), meme use (Cardoso & Atwell 2011;

47   Potvin & Parris 2013), and tempo (Potvin et al. 2011; Slabbekoorn & den Boer-Visser 2006) of

48   their songs in noisy environments.

49   While some of these adjustments are immediate responses to the sound environment

50   (Halfwerk & Slabbekoorn 2009; Kight & Swaddle 2015; McMullen et al. 2014; Potvin &

51   Mulder 2013; Verzijden et al. 2010), others show less short-term flexibility and suggest

52   increased efficacy of acoustic communication in a specific habitat over the course of

53   generations—in agreement with the Acoustic Adaptation Hypothesis (Morton 1975). For

54   example, the differential occurrence in noisy areas of particular memes or dialects is evidence of

55   cross-generational cultural evolution (Luther & Baptista 2010; Luther & Derryberry 2012;

56   Potvin & Parris 2013). However, the proximate mechanisms that contribute to this process - that

57   is, the developmental, physiological, neurological, or mechanical changes resulting in song

58   differences between generations - are still unknown.

59   One theory suggests that noise may be a source of developmental or chronic stress

60   (Wright et al. 2007). Birds living in noisy areas may chronically engage their stress response,

61   allowing them to cope with the stressor but paying a longer-term cost. While there is currently

62   some evidence for this, results have so far been inconsistent (Blickley et al. 2012; Bonier 2012;

63 Crino et al. 2013; Partecke et al. 2006). If chronic stress is affecting young songbirds in noisy

64 environments, cognitive development may be affected, resulting in altered songs (Buchanan et

65 al. 2004; Nowicki et al. 2002; Schmidt et al. 2013; Spencer & MacDougall-Shackleton 2011).

66 This would likely have an impact on the complexity and/or syllable content of songs (Brumm et

67 al. 2009; Schmidt et al. 2014; Schmidt et al. 2013; Spencer et al. 2003; Zann & Cash 2008).

68 Documenting connections among anthropogenic noise, biomarkers of developmental chronic

69 stress, and adult song in individual birds would be consistent with this theory.

70   Even if birds do not respond to noise as a chronic stressor, noise may still affect the song-

71 learning process in birds through direct effects on neural and cognitive development (Iyengar &

72 Bottjer 2002; Kujala & Brattico 2009). These changes in neuroanatomy may occur through the

73 interruption or masking of tutor-tutee communication, through impairing auditory feedback

74 during the song-learning process, or through other as yet unknown mechanisms (Dooling &

75 Blumenrath 2013; Kight & Swaddle 2011). In the classic model of zebra finch (*Taenopygia*

76 *guttata*) song learning, tutees (young males) listen to songs sung by tutors (their fathers) for

77 approximately 20 days (post-hatch day (PHD) 15 - 35), then subsequently attempt to mimic

78 sounds and develop song through the production of subsong (PHD 35-50) and plastic song (PHD

79 50-80; Catchpole & Slater 2008). Noise may mask components of tutor song that may then not

80 be heard or learned by tutees. Since anthropogenic noise is known to interfere with parent-

81 offspring communication in other contexts (Leonard & Horn 2008; McIntyre et al. 2014;

82 Schroeder et al. 2012) it is reasonable to assume that a process as intricate as song learning may

83 also be affected by such interference. Furthermore, noise may impair necessary auditory

84 feedback in tutees, slowing song development (Tschida & Mooney 2012; Zevin et al. 2004):

85 evidence for this effect can be found in the increase in individual song variation shown in some

86 wild birds occupying noisy environments (Gough et al. 2014).

87   We aimed to experimentally determine how anthropogenic noise affects song learning,

88 song development, and development of the song-control regions of the brain, as well as whether

89 noise may act as a developmental stressor by affecting circulating glucocorticoid hormone levels.

90 By conducting an experiment under laboratory conditions we isolated the effects of

91 anthropogenic noise and controlled for other characteristics associated with urban habitats that

92 may induce a stress response or affect song-learning in the wild, such as lighting (Kempenaers et

93 al. 2010), breeding density (Hamao et al. 2011), diet (Gavett & Wakeley 1986), or parasite load

94 (Bonier et al. 2007). If nestling birds perceive anthropogenic noise as a chronic stressor, we

95 predicted that noise would elevate baseline corticosterone levels. This in turn could affect

96 development of song-control brain regions, resulting in under-developed song-control regions in

97 noise-treated birds. Furthermore, we predicted that noise would hamper the development of song

98 among juvenile male zebra finches either due to developmental stress, interference with auditory

99 feedback, or masking songs sung by tutors. If developmental stress from noise occurred, we

100 predicted similar results to those shown in previous studies: tutees' songs in noise would be

101 developmentally delayed, would have reduced similarity to tutors' songs, and would be less

102 complex. If song masking and/or auditory feedback disruption occurred, we predicted that tutees

103 subjected to noise would sing higher frequency songs (reducing the effect of masking on lower

104 frequencies) compared to tutors and tutees learning under quiet conditions.

105

106 **Methods**

107

108   *Experimental protocol and data collection*

109

110   *Group 1*

111   We obtained at random twenty adult breeding pairs of zebra finches from a previously

112   established domestic flock at the Advanced Facility for Avian Research at the University of

113   Western Ontario (Group 1). These birds were also used as part of parallel studies on the effects

114   of noise on adult song and nestling growth rates and survival (Potvin and MacDougall-

115   Shackleton 2015a, 2015b). Prior to subjecting pairs to experimental conditions, we recorded

116   each adult male's song using a standard protocol, as each male would be a potential tutor for the

117   future brood. Each male was put in isolation in a sound attenuation chamber (modified

118   audiometric testing booth) for a period of 24 hours, after which an adult female was introduced,

119   inducing males to sing in all cases. These songs were recorded for five minutes using a Marantz

120   Solid State PMD671 recorder and a Sennheiser ME67 microphone. Once each male had been

121   recorded, they were placed with a female in a cage in a similar isolation chamber, along with 2-3

122   other breeding pairs for six months. We gave all birds water and premium finch seed *ad libitum*,

123   along with a daily tablespoon of eggfood (boiled egg, bread and cornmeal mixture). Birds were

124   kept on a 14:10 h light:dark photoperiod at approximately 22°C to maintain birds in a breeding

125   state. Three of the chambers (10 zebra finch pairs) were used as a control group ("silent" group;

126   no noise played), while the other three chambers (10 pairs) were exposed to traffic noise during

127   all daylight hours. Traffic noise was recorded by DAP at a busy urban park (Melbourne,

128   Australia), using the recording equipment described above, and combined with soundtracks of

129   trains, cars, motorcycles and lawnmowers downloaded from Soundbible.com. This traffic

130   soundtrack was played inside the chambers using an ipod touch connected to amplified computer

131 speakers (Logitech S11). Sound pressure levels were regularly checked using a Realistic 332050

132 Sound Level Meter, using A weighting. Each testing booth contained 3-4 cages (pairs), one of

133 which was adjacent to the speakers, with average background noise levels ranging from 60-80

134 dBA SPL at the centre of the cage; another one (or two, in the case of chambers that contained

135 four cages) which was placed at a mid-range distance from the speakers (average background

136 noise range 50-75 dBA SPL at cage centre), and the last cage furthest from the speakers, which

137 experienced noise at an average sound level of 40-70 dBA SPL. We therefore generated four

138 treatment groups for subsequent statistical analyses: Loud Noise, Moderate Noise, Soft Noise,

139 and Silent (no traffic noise).

140   We used a randomized cross-fostering protocol with nests that were synchronous for the

141 date of first egg-lay, in order to limit any potential genetic or maternal effects. This involved

142 moving one egg from each (donor) nest into a paired (recipient) nest, and vice versa (reciprocal

143 cross-fostering so as not to alter the brood size of either nest). All nests were subject to cross-

144 fostering. For nests with more than four eggs, a second egg was to be cross-fostered, however

145 due to chance (only two nests with larger brood sizes were in fact synchronous) we were only

146 able to do a second cross-fostering once. Cross-fostering was done both within and between

147 treatments.

148   Of the twenty pairs, four did not reproduce successfully, and one further pair only

149 produced female young. All other nests (N = 15) successfully fledged at least one male nestling,

150 resulting in 24 juvenile males total that could be used in the study. As we could not track which

151 individual was cross-fostered in each nest, we did not attempt to account for genetic relatedness

152 in analyses. The songs of all juvenile males (tutees) were recorded at PHD 40, PHD 60, and PHD

153 100. These time periods were selected as representative of the three major song-learning stages

154   in zebra finches, with the aim of recording an example of sub-song (PHD 40), plastic song (PHD

155   60), and crystallized song (PHD 100) (Catchpole & Slater 2008; Slater et al. 1988). For the sub-

156   song and plastic song recordings we placed the focal male in a smaller cage (~30x25x25cm)

157   adjacent to the original larger cage (~60x40x40cm) that contained his parents and siblings, all

158   inside an acoustic chamber. In this manner we could use a directional microphone (Sennheiser

159   ME67) to record the focal male's vocalizations without measuring the vocalizations of the other

160   birds. We recorded males using this protocol for 2-4 hours during the hours of 09:00-13:00, and

161   checked that all recordings had examples of subsong or plastic song, before putting males back

162   into their original home cages and chambers. For PHD 100 song, we used the same protocol to

163   record the crystallized song from the juvenile males as used for the adult males prior to the

164   experiment (see above).

165   In order to ensure that adult males' songs were not changing due to the noise, or to

166   account for this in subsequent analyses, we re-recorded each of the 20 adult males (father, tutor)

167   when their offspring were PHD 60 using the protocol described above. Since adult males' songs

168   did change slightly throughout the experiment (Potvin & MacDougall-Shackleton 2015a), we

169   used recordings taken from tutors once juveniles had fledged at PHD60 as tutor songs for

170   comparison analyses (see below).

171

172   *Corticosterone sampling and analysis*

173   On PHD 90, we took a small blood sample from each male offspring (N= 24) by puncturing the

174   brachial vein using a 26-gauge needle and collecting approximately 50 µl of blood into a

175   heparinized microhematocrit capillary tube. All blood samples were collected within three

176   minutes of opening the door of the isolation chamber, therefore ensuring that we could analyze

177   baseline corticosterone levels rather than acute stress responses associated with disturbance and

178   handling (Romero & Reed 2005). Blood was then centrifuged at 13G for 10 minutes and the

179   supernatant plasma was collected and then kept frozen (-30 °C) until assay. Plasma was assayed

180   for total corticosterone using a specific and sensitive radioimmunoassay kit (ImmuChem 07-

181   120103, MP Biomedicals, Orangeburg, NY, USA). All samples were measured in a single assay.

182   Sensitivity of the assay was 12.5 ng ml^{-1} and within-assay coefficients of variation were

183   acceptably low at 9.6% and 3.9% for low and high controls.

184

185   *Group 2*

186   At a separate location (College of William and Mary, Williamsburg, Virginia, USA), a

187   second group (Group 2) of zebra finches was subject to similar protocols. Twenty-four pairs of

188   zebra finches were housed in breeding cages, randomly selected from a large outbred stock

189   population, in two separate rooms (12 pairs in each room). Both rooms experienced a 14:10

190   light:dark photoperiod at approximately 20 °C, and were identically set up (room effects on

191   reproductive success and other physiological factors have been previously tested and ruled out;

192   Swaddle *et al.* unpublished data). All cages were visually but not acoustically separated from

193   each other within each room. Each pair was provided with Volkman Avian Science Super finch

194   seed, grit, cuttlebone, and vitamin-enriched (Vitasol) drinking water ad libitum, as well as two

195   wooden perches, a plastic nest box and sufficient hay for nest building. Breeding checks were

196   conducted every other day, and the number of eggs and hatchlings was recorded throughout the

197   experiment.

198   In the experimental room, a small speaker (Memorex ML622) was attached to the back of

199   each cage in the center and connected to an mp3 player (Sandisk Sansa). Noise was played

200   through each speaker starting on PHD1and continued for the remaining duration of the

201   experiment. The treatment noise was a 0.1 - 3 kHz pink noise (white noise bandpass filtered at 3

202   kHz), played back at 75 dBA SPL at the center of each cage for 24 h per day. Speaker

203   functioning was checked every other day and amplitude of the noise was confirmed every two

204   weeks with an Extech instruments Digital Sound Level Meter (407727), using A weighting. The

205   control room had some background noise from the surrounding animal facility, but this remained

206   between 50 to 55 dBA SPL (measured in the center of each cage) throughout the study.

207   Birds were housed in these conditions for six months and allowed to breed throughout.

208   All offspring produced were banded with numbered metal bands before fledging. Female

209   offspring and female parents were removed after the first clutch in that cage had fledged. All

210   pairs except for three (two in the noise treatment, one in the control room) produced a viable

211   clutch. In total the pairs in the experimental (noise) room produced 29 male offspring across 8

212   pairs (i.e., four pairs did not produce male offspring). The pairs in the control room produced 28

213   male offspring across 7 families (five pairs did not produce male offspring). From these male

214   offspring, we were able to record songs at PHD200 from five in each of the noise and control

215   treatments, where each male came from different parents. The sample size was lowered because

216   of premature deaths and occasional failure to solicit sufficient song on PHD200. On the day

217   following each offspring (tutee) recording we also recorded their fathers (tutors). Song

218   recordings followed similar protocols as described above. A male was placed in a quiet room

219   (ambient noise < 50 dBA SPL) in a small cage (approximately 20 x 20 x 30 cm) adjacent to an

220   unrelated adult female in a separate small cage. Using a directional microphone (Sennheiser

221   ME67) we recorded 10 clear directed songs from each male (tutees and tutors) onto a Marantz

222   PMD661MKII recorder.

223

224   *Song analysis (Both Groups)*

225   We used RavenPro 1.4 software (Cornell Lab of Ornithology) to create spectrograms of

226   all recordings in order to identify and extract 5 random examples of song from each tutor (N =

227   25) and tutee (N = 24 at PHD 100 and N=10 at PHD 200) song recording. We also used

228   RavenPro to identify all periods of singing behaviour in the PHD 40 and PHD 60 recordings for

229   Group 1 birds.

230   For juvenile subsong (PHD 40) we visually identified the number of fully-formed distinct

231   syllables by comparing all syllables in the subsong to those in the same individual's crystallized

232   song. We used the number of these crystallized syllables that were present in PHD 40 subsong as

233   an indicator of song development (Tchernichovski & Mitra 2002; Tchernichovski et al. 2001).

234   We also used RavenPro to measure the minimum (lowest) frequency and maximum (highest)

235   frequency of subsong over the entire PHD 40 recording, using the minimum and maximum

236   frequency peaks at a threshold of >30 dB as identified by power spectra (Beecher 1988).

237   For plastic song (PHD 60) we used Sound Analysis Pro 2011 software (Tchernichovski et

238   al. 2000) to compare each juvenile male's PHD 60 song to their crystallized song (PHD 100). For

239   crystallized song (PHD 100) we used the same software to compare each male's song to its

240   respective tutor's (social father's) song. We ran a similarity batch analysis using an MxN matrix

241   to compare all possible combinations of song-pair comparisons (5 from tutor compared with 5

242   from tutee), giving an output of estimates of song-similarity. We used the following estimates: %

243   similarity (the percentage of tutors' sounds included in the final tutee song), accuracy (the

244   similarity of each sound produced within songs between tutor and tutee), % sequence similarity

245   (the similarity of the tutor and tutee sequence of sounds within the song), and pitch difference.

246   We used the mean estimates of similarity for each individual tutee in subsequent statistical

247   analyses.

248   For the offspring male crystallized songs (i.e., PHD 100 and 200), we also extracted the

249   following song parameters independently using RavenPro. The number of notes per song and

250   song complexity (number of different note types) were counted manually. Minimum frequency,

251   maximum frequency, peak frequency (the frequency with the most energy), and song duration

252   were calculated by RavenPro based on the visual discrimination of the spectral space in the

253   recording occupied by the song, using power spectra and confirmation with spectrograms, this

254   time using a power threshold of >20dB. Tempo (notes per second) was calculated using the

255   number of notes and song duration. These crystallized song analyses were conducted for Group 1

256   and Group 2 separately.

257

258   *Brain histology and analysis*

259   Once juvenile males in Group 1 were recorded on PHD 100, they were euthanized by an

260   overdose of isoflurane and their brains extracted immediately from the skull. Brains were fixed

261   by storing them in 4% paraformaldehyde for 24 h, then cryoprotected in 30% sucrose (in

262   phosphate-buffered saline, PBS) for 48 h. They were then frozen on powdered dry ice and kept at

263   -80 °C until sectioning. We sectioned one hemisphere (sagittal plane, 30 μm sections) using a

264   cryostat, collecting every second section into 0.1M PBS, then mounted sections onto microscope

265   slides. We Nissl-stained the sections with thionin, then serially dehydrated them in graded

266   ethanol solutions, cleared the sections in solvent (Neo-clear) then affixed a coverslip onto the

267   slide with Permount (Fisher Scientific). Slides were subsequently examined under a Zeiss

268   Axiophot microscope and photomicrographs of the song-learning brain regions Area X, HVC,

269   and RA (robust nucleus of the arcopallium) were captured with a Spot Insight 5-megapixel

270   microscope camera. Images of the entire telencephalon were captured using a high resolution

271   (2400 dpi) flat-bed scanner with transparency adapter. To calculate the volume of the these

272   song-control regions as well as the telencephalon as a whole we traced the cross-sectional area of

273   the regions of interest using ImageJ software (Schneider et al. 2012) and volumes were

274   calculated by combining the cross-sectional areas and the sampling interval (60 μm) using the

275   formula for a frustum (truncated cone). All tracing was done blind to treatment group.

276

277   *Statistical analyses*

278   We performed all statistical analyses using a Bayesian framework in WinBUGS 1.4.3.

279   For group 1, we created a regression model to estimate the effect of noise treatment (silent, quiet,

280   moderate and loud noise) on baseline corticosterone levels, including uninformative priors

281   (McCarthy 2007). Since the number of siblings in a nest affects nestling condition, and therefore

282   may also affect brain and song development in zebra finches (Gil et al. 2006) we also included

283   number of brothers as a covariate. We estimated the mean and standard deviation from 200,000

284   samples from the posterior distribution, discarding the first 100,000 samples as a burn-in, and

285   used the 95% credible intervals (CI) for our estimations. Following common Bayesian statistical

286   procedures, we considered effects important if their 95% CIs did not overlap zero or if the 95%

287   CIs were highly skewed and effect sizes were large (McCarthy 2007).

288   We used a similar model to estimate the effect of noise on all song variables of interest

289   (number of crystallized syllables at PHD 40, similarity measurements of PHD 60-100 and

290   PHD100 song, minimum, maximum and peak frequency, bandwidth, duration, number of notes,

291   complexity and tempo), as well as total telencephalon volume and relative volumes of RA, HVC,

292   and Area X (volume of structure divided by telencephalon volume). To confirm our results, we

293   repeated analyses on brain structures using the absolute brain structure volume with total

294   telencephalon volume minus the structure volume as a covariate. We did not have brain

295   measurements or corticosterone measurements from Group 2 in the study, therefore only the

296   effect of noise (two categories) on crystallized song features and on father-son song similarity

297   was estimated for Group 2.

298   To determine whether song similarity to father was predicted by noise exposure, by brain

299   structure volumes, or any interaction effects, we ran similar regression models using the

300   following independent variables: noise treatment group, telencephalon volume, RA relative

301   volume, Area X relative volume, HVC relative volume and number of brothers. We used the

302   DIC (Deviance Information Criterion) tool in WinBUGS to compare all models and determine

303   the model that best predicted the variability in song similarity between tutor and tutee (lowest

304   DIC by at least 2; Spiegelhalter et al. 2002).

305   **Ethical Note**

306   All birds in Group 1 were kept and treated in accordance with guidelines set by the

307   Canadian Council on Animal Care, and all procedures in this study were approved by the

308   University of Western Ontario Animal Use Subcommittee (protocol number 2007-089). Group 2

309   protocols were approved by the College of William and Mary Institutional Animal Care and Use

310   Committee (IACUC-2012-11-23-8173-jpswad).

311

312   **Results**

313   Baseline corticosterone was not affected by the noise treatment (Table S1).

314   On PHD 40, treatment noise did not affect the number of fully-formed syllables (Table

315   S2). However, both minimum frequency (mean effect = 37.51Hz; 95% CIs = -14.4, 84.72) and to

316   a greater extent maximum frequency (mean effect = -258.0Hz; 95% CIs = -320.3, -196.1) were

317   affected by treatment at this early subsong stage, as indicated by the large skew in CI. The effect

318   of noise treatment on minimum frequency was primarily driven by the group experiencing the

319   loudest noise, as they sang approximately 113Hz (approximately 30%) higher than the other

320   groups. Maximum frequencies were approximately 774Hz lower (approximately 6%) in subsong

321   sung by males from the moderate and loudest cages compared to those in the silent or quiet

322   cages.

323   Songs recorded at PHD 60 had high similarity to songs recorded at PHD 100,

324   demonstrating well-developed song by PHD 60. Similarity measurements at these stages were

325   unaffected by treatment, indicating songs were developing at the same rate in birds across all

326   treatment groups (Table S3).

327   There was no effect of noise on a variety of parameters of crystallized songs recorded at

328   PHD 100 including the number of notes in a song, song duration, or tempo (Table S4).

329   Additionally, the effect of noise on lowest frequencies identified at PHD 40 was no longer

330   detected at PHD 100 (Table S4, Figure 1). However, maximum frequency was still

331   approximately 20% lower in songs sung by males from the moderate and loud noise cages than

332   those in the quiet or silent cages (mean = -874.9Hz; 95% CIs = -934.8, -814.7; Figure 1). Peak

333   frequency was also affected by treatment, with the loudest cages having the lowest peak

334   frequency (mean = -69.81Hz; 95% CIs = -129.5, -9.972; Figure 1).

335   The PHD 200 songs of birds in Group 2 were also affected by noise. Similar to Group 1 birds at

336   PHD100, there were no differences in minimum frequency (Table S5, Figure 1). However, while

337   peak frequencies were also lower in this noise group (mean = -228.9Hz; 95% CIs = -404.3, -

338   54.08; Figure 1), birds in the noise treatment in Group 2 sang higher, not lower, maximum

339   frequencies (mean = 117.7Hz, 95% CIs = -17.65, 271.1; Figure 1; all effects in Table S5).

340   While telencephalon volume and RA were unaffected by treatment, the noise treatment

341   negatively affected HVC volume and Area X volume (using either method of correction for total

342   telencephalon volume). The number of brothers also had a negative impact on brain structure

343   volume (HVC mean = -0.0338mm^3; 95% CIs = -0.057, -0.010; Area X mean = -0.011mm^3, 95%

344   CIs = -0.237, 0.017; Figure 2; all effects in Table S6).

345   DIC analysis identified the model incorporating noise treatment, number of brothers, and

346   area X volume as being the model with best fit for all three measures of song similarity to father

347   (% Similarity DIC score = 217.225; Accuracy DIC score = 196.356; % Sequence similarity DIC

348   score = 219.638; all other scores for comparison in Table S7a). Area X itself was not important

349   in the models predicting overall % Similarity or Accuracy, however it was important in

350   predicting % Sequence similarity as was noise (larger Area X and higher noise levels were both

351   correlated with lower % Sequence similarity), although their interaction was not important

352   (Table S7b, Figure 3).

353   At PHD 200 for Group 2 birds, although we did not have brain measurements for this

354   group of birds, we identified noise as having an effect on % Sequence similarity between tutor

355   and tutee (mean = 12.05%, 95% CIs = -1.055, 25.07) with other similarity measurements being

356   unaffected (Figure 3, all effects in Table S8).

357

358 **Discussion**

359   We found that while noise during development did not affect baseline corticosterone in

360 young male zebra finches, it did affect HVC and Area X volume—brain regions that are crucial

361 to song learning. These neuroanatomical effects were accompanied by behavioural

362 consequences. The similarity of song between the tutor (father) and tutee (son) was decreased by

363 the combined effects of noise treatment, Area X volume, and number of brothers. Specifically,

364 the similarity in the sequence of notes in a song, comparing father to son, decreased with

365 increasing noise. This latter result was observed in two independent experiments (i.e., in both

366 Groups 1 and 2) with different populations of zebra finches. In contrast to predictions made from

367 observations of free-living birds singing in urban habitats, songs that developed in the noise

368 treatments were consistently lower in peak frequency, and not higher in frequency range or

369 minimum frequency. Furthermore, maximum frequencies showed inconsistent changes in

370 response to noise treatments—in Group 1 crystallized songs (at PHD100) had a lower maximum

371 frequency, whereas in Group 2, songs (at PHD200) had a higher maximum frequency. These

372 mixed results make it difficult for us to support the hypothesis that putatively adaptive frequency

373 changes observed in wild urban birds are due to an effect of noise on song frequency

374 development in early life stages.

375   While we attempted to identify whether chronic stress might be a mechanism by which

376 noise affects song development in birds living in artificially noisy environments, our results do

377 not show that baseline corticosterone in young birds was elevated under such conditions.

378 Similarly, a previous study on nestling white-crowned sparrows (*Zonotrichia leucophrys*

379 *oriantha)* found that young birds experiencing chronic traffic noise had lower baseline

380 glucocorticoid levels than those in quiet conditions (Crino et al. 2013), while another showed

381   similar results to our own (Heiss et al. 2009). At the moment, most studies on the effects of

382   anthropogenic noise on corticosterone levels have been conducted on wild adult birds, and have

383   produced varied results. While there is some evidence that certain species might experience

384   chronically elevated glucocorticoid levels in urban or noisy areas (Blickley et al. 2012; Bonier et

385   al. 2007; Zhang et al. 2011) other species do not (Fokidis et al. 2009; Partecke et al. 2006; Potvin

386   & MacDougall-Shackleton 2015a). Many of these studies have attempted to isolate the effect of

387   noise from other anthropogenic impacts on birds that might induce chronically elevated

388   glucocorticoids; however it is clear that further research is needed to better understand how some

389   species might be better able to acclimate or adjust their stress response to chronically noisy

390   environments than others, and whether or how this acclimation may depend on age and social or

391   genetic environment.

392   We did find an effect of noise environment on male brain structures associated with song

393   learning. Telencephalon volume was unaffected, as was RA volume, but both Area X and HVC

394   volumes were proportionally smaller in males from the noise treatment. The size of song-control

395   brain regions is often correlated with song quality within- and between-species. We found that

396   Area X, in particular, was related to the similarity of experimental males' song (at PHD 100) to

397   their fathers' songs, along with noise treatment and number of brothers. Finding an effect of

398   noise on corticosterone levels might have provided a mechanism by which noise could impact

399   the size of Area X and HVC (Buchanan et al. 2004; Schmidt et al. 2013). However, it is likely

400   that HVC is sensitive to environmental factors that may not instigate a chronic elevation in

401   corticosterone. For example, noise may have been only transiently stressful to the birds at times

402   other than those at which we sampled. Alternatively, reduced singing behaviour itself may have

403   led to altered brain development. Noise and deafening has been shown to affect auditory and

404   song learning circuits in previous studies of zebra finches (Iyengar & Bottjer 2002), and neural

405   plasticity of HVC is regulated by singing and social housing in canaries (Alward et al. 2014).

406   Determining the mechanisms by which noise affected neural and song development in our study

407   would require further experiments.

408   The similarity of birds' songs to their fathers' songs was generally high across treatment

409   groups, however noise did appear to specifically affect sequence similarity, or syntax, in both

410   experiments. Noise has been shown to affect certain aspects of song learning in previous studies

411   due to auditory disruption (Tschida & Mooney 2012; Zevin et al. 2004). Traffic noise in

412   particular has been shown to disrupt or mask other forms of parent-offspring communication in

413   birds (Leonard & Horn 2008; McIntyre et al. 2014; Schroeder et al. 2012), therefore its impact

414   on the accuracy of song learning, and especially the ability to copy long strings of syllables (even

415   if the syllables themselves are accurate) is unsurprising. Zebra finch song is made up of common

416   elements some of which are also expressed as calls (Price 1979). Hence, while the learning of

417   individual elements is important for communication in general, the accurate sequencing of these

418   elements is likely particularly important for song construction (Menyhart et al. 2015; Riebel

419   2009; Zann 1993). The fact that this characteristic was impacted by noise therefore indicates a

420   significant disturbance to the song learning process in this species. This disruption was observed

421   in both experiments.

422   While we found that learning was impacted by noise, we found no evidence of the

423   putatively adaptive changes in song that have been reported in wild populations living along

424   urban-rural gradients (i.e., singing higher minimum frequencies in environments subject to

425   anthropogenic noise). While at PHD 40, Group 1 birds in noise sang higher minimum

426   frequencies, by day 100 the only effect of traffic noise was on maximum frequency, which was

427   slightly lower than in quiet treatment birds. In contrast, birds from Group 2 showed higher

428   maximum and peak frequencies after chronic pink (1 – 3 kHz) noise exposure. Combined, these

429   results are inconsistent and do not support the hypothesis that zebra finches alter their song in the

430   long-term to improve transmission in a noisy environment. All birds were recorded in relative

431   silence, which could mean that young birds were adjusting their song frequency to the current

432   acoustic environment only (i.e., they may have sung at higher frequencies in the experimental

433   chambers but not in the recording chamber). We do not know whether zebra finches possess the

434   vocal flexibility to spontaneously alter the frequency of their songs, but it seems likely given that

435   it has been observed in other species (Potvin & Mulder 2013; Verzijden et al. 2010).

436   Nevertheless, we interpret our results to show that in this species, the masking of lower acoustic

437   notes in the transfer from tutor to tutee, resulting in only higher notes being learned, is not the

438   underlying mechanism by which acoustic adaptation occurs in this environment.

439   One unsuspected novel result from our study was our finding that the number of brothers

440   an individual has may have an impact on song-learning accuracy. The number of siblings has

441   been shown previously to affect some aspects of nestling condition (Gil et al. 2006) and mate

442   preferences (Holveck & Riebel 2009), however brood size did not appear to influence metrics of

443   song learning in a previous study (Gil et al. 2006). A possible explanation for the effect of brood

444   size on song learning that we observed is that more brothers may increase the noise in a nest, and

445   therefore provide additional noise effects separate from already present chronic traffic or

446   background noise. This more immediate source of auditory disruption may limit the amount a

447   juvenile bird is able to practice its song, leading to higher numbers of discrepancies among birds

448   that have to compete with siblings. Zebra finches also require a sensorimotor phase whereby

449   there is one-on-one interaction between tutor and tutee (Derégnaucourt 2011); a large number of

450   brothers could modify the nature of interactions between a bird and its father, and brothers may

451   serve as potential tutors for each other, thus affecting the song learning process. Having many

452   siblings has also been shown to negatively affect offspring quality (growth rate, biometry; Gil et

453   al. 2006; Potvin & MacDougall-Shackleton 2015b)), which may in turn result in poorer song

454   learning ability. Further investigation into how brood size or, more specifically, the number of

455   tutees in a group might impact song development over more specific time periods may shed

456   more light on this finding.

457   We provide the first experimental findings for the impact of anthropogenic noise on song

458   learning structures in the avian brain. We also found that noise affects the learning of song

459   element sequences in particular. Both findings indicate that noise, along with brood size, is a

460   crucial aspect of an individual's early environment with long-term consequences, despite noise

461   not being identified as a physiological stressor. These results may also contribute to our current

462   understanding of some of the difference in urban and rural birdsong. Of course, such conclusions

463   do not rule out other processes that may be contributing to song changes in urban environments,

464   such as sexual selection for effective urban songs or elements (Candolin & Heuschele 2008). We

465   suggest that future research focus on female preference of putatively urban-adapted song in

466   urban and rural environments to disentangle whether sexual selection, rather than environmental

467   pressures on song learning, might be the defining selective process behind song changes

468   commonly observed in wild urban populations.

469   **Acknowledgments**

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637   Figure 1. Effect of noise treatment during development on crystallized song frequency

638   characteristics (maximum, peak and minimum frequencies) in each of the two groups of zebra

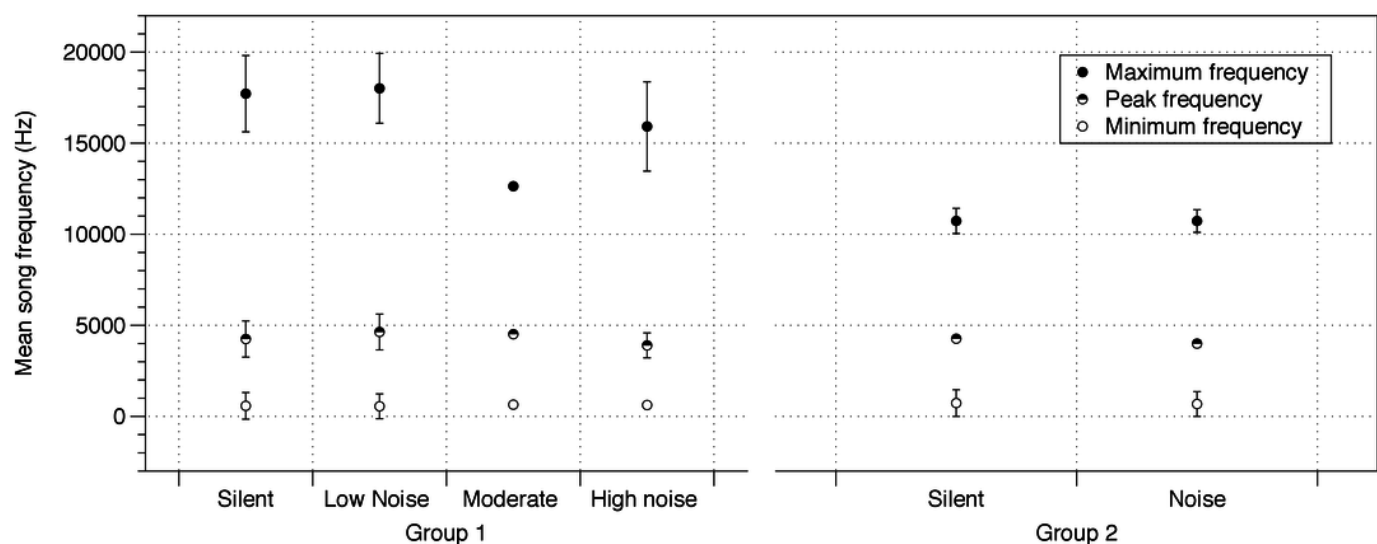639   finches used in the study. Error bars denote 95% confidence intervals.

640   Figure 2. Mean brain structure volume (HVC and Area X) relative to total brain size from zebra

641   finches in group 1 under each traffic noise treatment condition. Error bars denote 95%

642   confidence intervals.

643   Figure 3. Mean percent sequential similarity of tutee's crystallized song to tutor's song in each of

644   the two groups of zebra finches used in the study. Higher values denote a better copy of the

645   sequence of syllables (syntax) by the tutee. Error bars denote 95% confidence intervals.
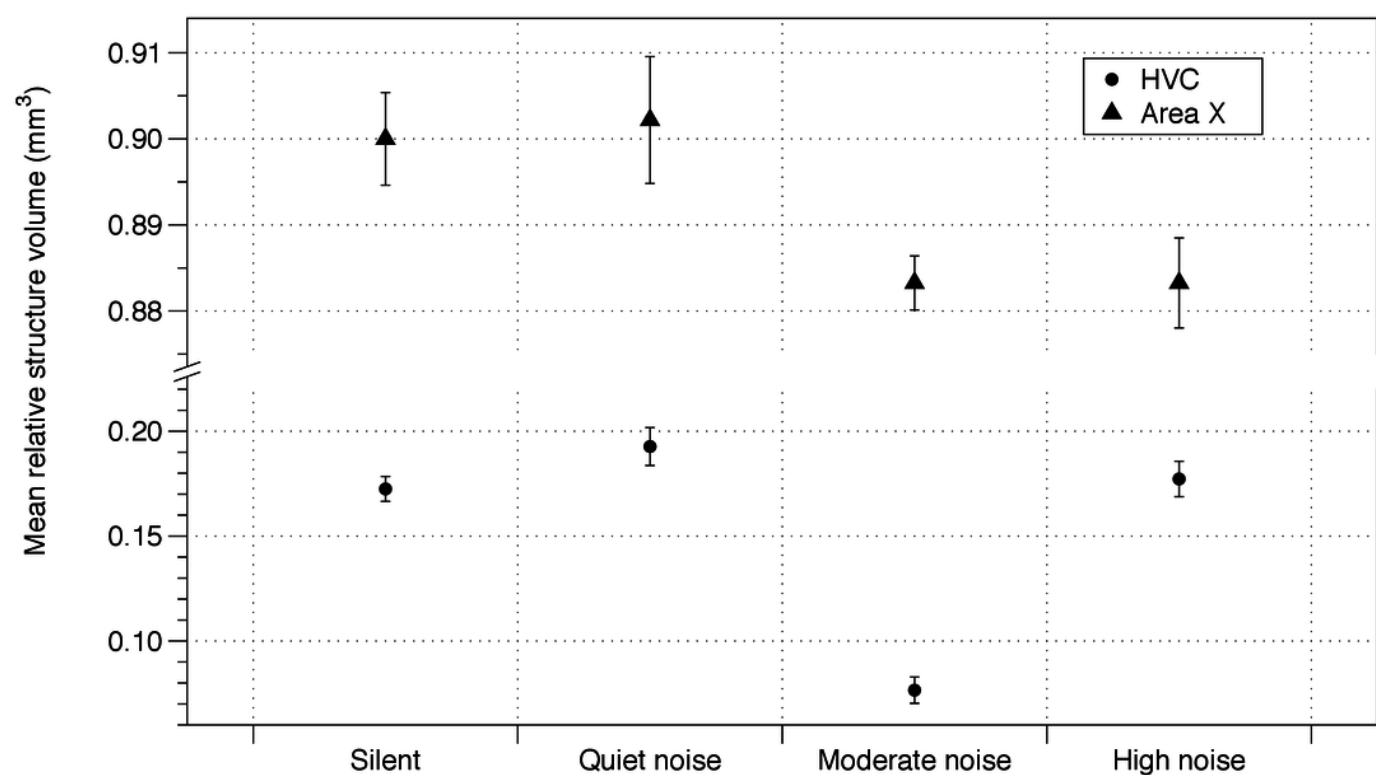
1

Noise effect on song frequency

Figure 1. Effect of noise treatment during development on crystallized song frequency characteristics (maximum, peak and minimum frequencies) in each of the two groups of zebra finches used in the study. Error bars denote 95% confidence intervals.

2

Noise effect on HVC and Area X regions of brain

Figure 2. Mean brain structure volume (HVC and Area X) relative to total brain size from zebra finches in group 1 under each traffic noise treatment condition. Error bars denote 95% confidence intervals.

3

Noise effect on sequential similarity between tutee and tutor song

Figure 3. Mean percent sequential similarity of tutee's crystallized song to tutor's song in each of the two groups of zebra finches used in the study. Higher values denote a better copy of the sequence of syllables (syntax) by the tutee. Error bars denote 95% confidence intervals.