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Experimental inhibition of a key cellular antioxidant affects vocal communication

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Running headline: Song rate and oxidative stress

Summary

- 1 1. There is substantial interest of evolutionary ecologists in the proximate
2 mechanisms that modulate vocal communication. In recent times, there has been
3 growing interest in the role of oxidative stress as a mediator of avian song
4 expression.
- 5 2. Here we tested whether the experimental inhibition of the synthesis of a key
6 cellular antioxidant (glutathione) reduces song rate metrics of male European
7 starlings (*Sturnus vulgaris*). We measured the effect of our treatment on total song
8 rate and on its two components, undirected and nest-box oriented song, outside
9 the breeding season.
- 10 3. Treated males that did not own a nest-box (subordinate males likely to be of lower
11 quality) suffered increased oxidative stress relative to untreated males, while
12 treated males that owned a nest-box (dominant males likely to be of higher
13 quality) did not. Treated non-owners also reduced their undirected song rate,
14 whereas treated nest-box owners did not suffer any reduction in song rate.
- 15 4. Our results revealed that inhibition of a key cellular antioxidant results in
16 decreased vocal communication in a social vertebrate, and that this effect is
17 dependent on its social status (nest-box owner versus non-owner).
- 18 5. This work provides support for the hypothesis that acoustic signals may honestly
19 convey information about the individual oxidative status and capacity to regulate
20 the oxidative balance. Our findings raise the possibility of hitherto unexplored
21 impacts of oxidative stress on fitness traits in social species.
- 22

- 23 **Key-words:** antioxidants, constraint, glutathione, honest-signalling, life history,
24 oxidative damage, sexual selection, song rate, *Sturnus vulgaris*

25 **Introduction**

26 Production of visual and non-visual signals is a key component of social interaction.
27 Sounds, conspicuous colourations and odours are some of the most notable ways
28 animals use to communicate with conspecifics. The expression of these signals largely
29 varies among individuals. One reason for this variation lies with the individual quality,
30 where high quality individuals are expected to express more exaggerated signals than
31 low quality individuals. This is because either production or maintenance of honest
32 signals carry costs that only high quality individuals would be able to afford (Lindström
33 *et al.* 2009; Pike *et al.* 2010; Garratt & Brooks 2012).

34 Acoustic signals are condition-dependent in a variety of invertebrates and
35 vertebrates and advertise to conspecifics their social or mating status (e.g., Hunt *et al.*
36 2004; Ball *et al.* 2006; Koren 2006; Humfeld 2013). Avian song is a renowned acoustic
37 trait that may convey several attributes of individual quality (Gil & Gahr 2002). For
38 example, it has been found that different traits of song output are related to immune
39 function (Duffy & Ball 2002), to food availability and body mass (Ritschard & Brumm
40 2012) and to stress response and survival (MacDougall-Shackleton *et al.* 2009). Avian
41 song is also linked with dominance and territory ownership. For example, male
42 European starlings *Sturnus vulgaris* that own a nest-box sing at higher rates compared
43 to individuals that do not own a nest-box (Kelm-Nelson *et al.* 2011; DeVries *et al.*
44 2016).

45 There are clearly constraints that limit song expression. For example, it has been
46 shown that immunization can reduce song rate in collared flycatchers *Ficedula*
47 *albicollis* (Garamszegi *et al.* 2004) and in white-browed sparrow weavers (York *et al.*
48 2016), or rattle duration in barn swallows *Hirundo rustica* (Dreiss *et al.* 2008). Recent

49 studies in male European starlings found that inflammatory processes significantly
50 decreased song rate (Casagrande *et al.* 2015) and that the antibody production caused a
51 moderate reduction of one particular mode of singing (undirected song rate, i.e. song
52 produced away from the nest-box; Costantini *et al.* 2015). Song rate can also be
53 constrained by sex steroid hormones. Experimental manipulations of testosterone in
54 male European starlings showed that this hormone can influence song rate (De Ridder,
55 Pinxten & Eens 2000; Pinxten *et al.* 2002; Ball *et al.* 2006; Van Hout *et al.* 2012).
56 Finally, there is also evidence that the size of certain song nuclei limit the expression of
57 song (reviewed in Garamszegi & Eens 2004).

58 Which cellular mechanisms may constrain signalling has been a central question
59 in the study of animal communication in recent years (Hill 2011). In this regard, one
60 cellular mechanism thought to be particularly important is oxidative stress (von Schantz
61 *et al.* 1999; Garratt & Brooks 2012; Casagrande, Pinxten & Eens 2016), a complex
62 biochemical condition of the organism that is dependent on the rate of oxidative damage
63 generation and oxidation of non-protein and protein thiols that regulate the cell
64 oxidative balance (Jones 2006; Halliwell & Gutteridge 2007; Costantini & Verhulst
65 2009; Sohal & Orr 2012). Dysfunctional regulation of the oxidative balance might be a
66 significant handicap for the expression of sexual signals in low quality individuals
67 (Garratt & Brooks 2012).

68 Recent studies found significant correlations between song rate and some
69 metrics of either oxidative damage or non-enzymatic antioxidants in European starlings
70 and snow buntings (*Plectrophenax nivalis*) (reviewed in Casagrande, Pinxten & Eens
71 2016). Although these studies suggest that there might be a link between song
72 production and oxidative stress, the role of oxidative stress as a constraint on song

73 production and, hereby, the role of song as a signal of oxidative status, has never been
74 experimentally tested.

75 In this study, we tested whether a change in individual oxidative balance through
76 experimental inhibition of the synthesis of a key cellular antioxidant (glutathione; Jones
77 2006) reduces song rate metrics of male European starlings. We also tested whether the
78 role of oxidative stress as a constraint on song rate differs between birds that either own
79 or do not own a nest-box. Male European starlings are an excellent study system to
80 address these questions. In male starlings the acquisition of a nest site/nest-box is a
81 good indicator of individual quality and dominance status. Males that acquire a nest-box
82 chase other males from perches or feeding sites more frequently and exhibit higher song
83 rate on or inside the nest-box (i.e., nest-box oriented song rate) than other males (Eens
84 1997; Riters *et al.* 2000; Spencer *et al.* 2004; Sartor & Ball 2005; Kelm-Nelson *et al.*
85 2011; Cordes *et al.* 2014; DeVries *et al.* 2016). The nest-box oriented song rate is also
86 used by starling males outside the breeding season for the acquisition and defense of a
87 nest site (Gwinner, Gwinner & Dittami 1987; Eens 1997). European starlings also sing
88 away from the nest-box throughout the year; this undirected song is used for
89 maintaining group cohesion and social order (Eens 1997). It has been shown that song
90 rate metrics in this species are associated with levels of antioxidants and oxidative
91 damage during both the breeding and non-breeding season (Van Hout, Eens & Pinxten
92 2011; Casagrande *et al.* 2014; Costantini *et al.* 2015). In contrast to most other songbird
93 species, starling males sing at high levels throughout most of the year (apart from the
94 moulting period; Eens 1997; Riters *et al.* 2000; Van Hout *et al.* 2009).

95

96 **Materials and Methods**

97 HOUSING CONDITIONS

98 Adult male starlings used for the experiment had been previously captured in the
99 Antwerp district and housed in large single-sex outdoor aviaries on the grounds of
100 Campus Drie Eiken of the University of Antwerp (Wilrijk, Belgium). At the start of the
101 experiment, all starlings had been kept in captivity for at least one year and were
102 between 2 and 9 years old.

103 To exclude any potential confounding effect of testosterone, we conducted our
104 study during the fall season when testosterone levels are basal, as indicated by the black
105 beak colouration of our experimental birds, and when all birds had completed the moult
106 (Eens 1997; Riters *et al.* 2000). On October 26, 2015, 32 starlings were moved in two
107 adjacent outdoor aviaries (L × W × H; 27.0 × 7.0 × 2.75 m). Birds were randomly
108 allocated to the two aviaries (16 birds in each aviary). In each aviary, there were both
109 control birds and birds going to be treated (8 control and 8 treated birds in aviary A and
110 8 control and 8 treated birds in aviary B). However, one control bird from aviary A died
111 before the start of the treatment, limiting the number of birds used in that aviary to 15
112 (and 31 in total). Each aviary had 16 nest-boxes and each nest-box had a perch in front
113 of it. All starlings were marked with a unique combination of coloured bands and a
114 metal ring, which allowed individual recognition. Food (Orlux, Deinze, Belgium; Nifra
115 Van Camp, Boechout, Belgium) and water were provided *ad libitum*.

116

117 EXPERIMENTAL DESIGN

118 The experimental treatment started about four weeks after the starlings were moved in
119 the new aviaries in order to allow them to get accustomed with the new environment.
120 The experiment was performed according to the timescale in Fig. 1. During 12-21

121 November, the song of all males was scored and males were also identified as owners or
122 non-owners of a nest-box (see below). On November 23, the manipulation of individual
123 oxidative balance was started. Starlings were given intramuscular injections of 200 μ l of
124 a solution containing 50 mg of DL-buthionine-(S,R)-sulfoximine (Sigma-Aldrich
125 B2640) per ml of saline solution (PBS). The birds were injected five times on alternate
126 days (Fig. 1). The control individuals were subjected to the same regime, but were
127 injected with PBS only. Further details are given in the paragraph “Glutathione
128 synthesis inhibition”.

129 Immediately before the first injection and the day after the last injection, at a
130 comparable time of day (13:30-15:00), a sample of blood (ca. 500 μ l) was collected by
131 venipuncture of the wing vein using heparinized microvettes (Sarstedt, Nümbrecht,
132 Germany). Body mass was also recorded. Blood samples were maintained at around
133 +4°C while on the field. When back in the laboratory, tubes were centrifuged in order to
134 separate plasma from red blood cells. Both plasma and red blood cell sample were
135 stored at -80 °C. In order to capture starlings, we took advantage of the small (entrance)
136 aviary attached to each of the two large outdoor aviaries. On the capture day, the door of
137 this small aviary was opened and then two of us, standing on the opposite side of the
138 aviary, made the birds fly into the small aviary. Inside this small aviary, it was easy to
139 capture birds with a butterfly net without causing any damage to them. Some birds were
140 also captured using a butterfly net while they were flying to the small aviary. The whole
141 procedure took a maximum of 10 minutes for each of the two aviaries.

142

143 **QUANTIFICATION OF SONG RATE AND OWNERSHIP**

144 All behavioural observations were made by the same person (using a binocular) from
145 behind a shelter located ca. 5 m from each aviary. Using a one-zero sampling technique
146 (Martin & Bateson 2007), we monitored the behaviour of all starlings within one aviary,
147 in uninterrupted sessions of 60 minutes, between 10h00 and 12h00 (when singing
148 activity of starlings during the day is highest, Eens 1997). Every minute, the aviary was
149 scanned from the left to the right side to register which males were singing. All the
150 observations were registered using an automatic voice recorder, which enabled us to
151 keep looking at the birds without any distractions. We alternated the order of the
152 aviaries between subsequent days in order to have a balanced distribution of the timing
153 of observations. Behavioural observations were made for 10 consecutive days (12-21
154 November) before birds were given the first injection. The average value of these data
155 was used as pre-treatment value. Thereafter, the collection of behavioural data
156 continued from the day after the first injection until the third day after the last injection
157 (Fig. 1). On days of injections, the song behaviour of the males was always registered
158 before the injections. During all behavioural observations, both the nest-box oriented
159 song rate and the undirected song rate were scored for each individual. Given that
160 European starlings, while singing, adopt a characteristic upright stance, with an
161 upturned bill and the throat feathers and beak can be seen moving (Fear 1984), singing
162 behaviour can be easily quantified. It is also easy to score males singing inside a nest-
163 box because they sing with their head looking out from the nest-box hole, which makes
164 them visible. The ring of birds singing inside a nest-box was either identified while the
165 bird entered the nest-box or while it left it. The nest-box oriented song rate was
166 quantified as the proportion of samples per session during which a male was singing
167 while inside a nest-box, on the top of it or on the perch connected to the nest-box. The

168 undirected song rate was quantified as the proportion of samples per session when a
169 male was singing away from nest-boxes, for example on perches away from nest-boxes
170 or on the ground (Pinxten *et al.* 2002; Casagrande *et al.* 2015; Costantini *et al.* 2015).
171 Based on behavioural observations conducted before the treatment (12-21 November),
172 birds were also classified as either owners or non-owners of a nest-box. Owners were
173 birds observed occupying the same nest-box (singing at or in it and repeatedly entering
174 and leaving it) and chasing other males away from it (Gwinner, Van't Hof & Zeman
175 2002; Spencer *et al.* 2004; Kelm-Nelson, Stevenson & Ritters 2012) for at least 8 days
176 out of the 10 observation days. In case birds were seen to sing on the roof of a nest-box
177 but did not perform other ownership-linked behaviours, they were considered as non-
178 owners. All the birds that were considered owners before the start of the treatment were
179 still owners in the period after the start of the treatment (observed singing, entering and
180 leaving the same nest-box and chasing other males away from it for at least 10 out of 12
181 days). Note that we relied on a rather conservative definition of nest-box ownership,
182 implying that non-owners may also have occupied a nest-box (and hence produced nest-
183 box oriented song) during a short period. Male captive European starlings vigorously
184 defend a nest-box not only during the breeding season, but also after the moult has been
185 completed from September onwards. Although abundant nest-boxes were provided in
186 the aviaries, not all males became nest-box owners because some males occupied and
187 defended more than one nest-box. The captive set-up enabled us to distinguish between
188 dominant and subdominant males. Social status (in the form of nest-box possession) has
189 previously been shown to affect song rate (e.g., Eens 1997; Ritters *et al.* 2000, 2012,
190 2014).
191

192 GLUTATHIONE SYNTHESIS INHIBITION

193 To induce a state of oxidative stress, starlings were given intramuscular injections of
194 200 µl of a pure solution containing 50 mg of DL-buthionine-(S,R)-sulfoximine (Sigma-
195 Aldrich B2640) per ml of saline solution (PBS). Sulfoximine is a non-toxic drug that
196 reduces the synthesis of glutathione, a key cellular antioxidant, by inhibiting the activity
197 of the enzyme gamma-glutamylcysteine synthetase (Griffith & Meister 1979; Griffith
198 1982; Galván & Alonso-Alvarez 2008; Costantini *et al.* 2016; Koch & Hill 2016). The
199 injections were done on alternate days for a total of five times (Fig. 1). The total amount
200 of sulfoximine given to each treated individual corresponded to 50 mg; this amount was
201 chosen based on work with other songbird species (Galván & Alonso-Alvarez 2008;
202 Romero-Haro & Alonso-Alvarez 2015; Costantini *et al.* 2016). This resulted in variable
203 doses (i.e., amount of sulfoximine per gram of body mass) given that there was
204 variation in body mass among birds (range of body mass of treated individuals from 75
205 to 95 grams), but the body mass did not differ significantly between treatment groups
206 nor was it affected by the treatment (see Table 1). The control individuals were
207 subjected to the same regime, but injected with PBS only.

208

209 ANALYSIS OF BLOOD OXIDATIVE STATUS

210 To validate the effect of treatment on glutathione concentration and oxidative damage,
211 two methods commonly applied to vertebrates were used. First, high-performance liquid
212 chromatography with electrochemical detection was applied for simultaneous
213 determination of reduced glutathione (GSH, tripeptide synthesized by the organism that
214 has antioxidant properties) and oxidized glutathione (GSSG, it is the molecule of GSH
215 that has been oxidised, for example, after reaction with a free radical) in red blood cells

216 by a Reversed-Phase HPLC of Shimadzu (Hai Zhonglu, Shanghai). We applied the
217 protocol as described in Sinha *et al.* (2014). Concentrations of GSH and GSSG were
218 expressed as $\mu\text{mol g}^{-1}$ fresh weight of red blood cells. We calculated the GSH/GSSG
219 ratio that was used as an index of redox state (higher values indicate lower oxidative
220 stress; Jones 2006). Second, the d-ROMs assay (Reactive Oxygen Metabolites; Diacron
221 International, Grosseto, Italy) was used to measure plasma oxidative damage
222 metabolites (mostly organic hydroperoxides) that are generated early in the oxidative
223 cascade. Our work relied on this metric of oxidative damage because GSH is used to
224 detoxify the organism from organic hydroperoxides (Halliwell & Gutteridge 2007).
225 Hence, a decrease of GSH is expected to result in an increase of ROMs. Analyses of
226 ROMs were done according to manufacturer's instructions as in previous studies (e.g.
227 Costantini *et al.* 2015). Quality controls (Diacron International) were also assessed in
228 each assay. Values of ROMs have been expressed as mM of H_2O_2 equivalents.

229

230 STATISTICAL ANALYSES

231 Linear mixed models with a repeated measures design (SAS Version 9.3, Cary, NC,
232 USA) were used to assess the effect of treatment on reduced glutathione (GSH),
233 oxidised glutathione (GSSG), the GSH/GSSG ratio, plasma reactive oxygen metabolites
234 and body mass. In each full model, treatment group (control and treated), ownership
235 (owner and non-owner of a nest-box), sampling day (pre- and post-treatment) and all
236 their interactions were included as fixed factors; aviary and individual nested within
237 aviary were both entered as random factors. Pre- and post-treatment values of oxidative
238 status metrics refer to blood samples collected on 23 November and 2 December,
239 respectively (Fig. 1). Non-significant interactions were sequentially removed starting

240 from the three-way interaction; main factors were always retained in the reduced models
241 irrespective of their statistical significance. Post-hoc Tukey tests were used to explore
242 further any significant interactions.

243 Generalized linear mixed models with a binomial error distribution (lmer in
244 package ‘lme4’, R version 3.1.1; R Core Team, 2013) were used to test the effect of
245 treatment on total song rate, nest-box oriented song rate and undirected song rate,
246 respectively. We relied on binomial models because we had count data (i.e., number of
247 times a bird was seen to sing over 60 minutes) that were bounded (i.e., ranging from 0
248 to 60), hence the use of percentages as response variable raises a few concerns (chapter
249 16 in Crawley 2007). For graphical purposes, we have, however, shown data of each
250 song rate metric as percentages. In each full model, treatment groups (control and
251 treated), ownership (owner and non-owner of a nest-box), sampling day (13 days in
252 total) and all their interactions were included as fixed factors; aviary and individual
253 nested within aviary were both entered as random factors. To account for
254 overdispersion, which is common in Poisson models, an observation level random effect
255 was also added (Harrison 2014). As pre-treatment (i.e., sampling day 1) values of each
256 song metric, we used the average of the values recorded from 12 to 21 November (Fig.
257 1), i.e., before the first injection. The following twelve days of collection of song rate
258 were those from 23 November to 5 December (Fig. 1). Non-significant interactions
259 were sequentially removed starting from the three-way interaction; main factors were
260 always retained in the reduced models irrespective of their statistical significance. Post-
261 hoc tests were used to explore further any significant interactions.

262 Linear mixed models were also used to assess whether any of the song rate
263 metrics was associated with d-ROMs values. To do so, we pooled all males together

264 because the sample size was not adequate to test covariation within each treatment
265 group. Within each model, both pre- and post-treatment values of d-ROMs were pooled
266 together and were included as a single fixed predictor; aviary and individual nested
267 within aviary were both entered as random factors because pre- and post-treatment
268 values are not independent from each other. Of each song metric, we included within
269 each model the pre-treatment values (those recorded on day 1, see Figure 1) together
270 with values recorded on sampling day 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13, respectively.

271 Preliminary linear models showed that at the time of the first injection control
272 and treated owner or non-owner birds were similar for age, body mass and tarsus length
273 in both aviaries (treatment group \times ownership \times aviary, p-values ≥ 0.24). In the two
274 aviaries, the number of owners (8 each) and non-owners (7 and 8, respectively) was
275 similar, as well as the proportion of owners and non-owners was evenly distributed in
276 control and treated birds. All the birds that were considered owners before the start of
277 the treatment were still owners in the period after the start of the treatment.

278

279 **Results**

280 Irrespective of ownership, the concentration of GSH in red blood cells increased
281 significantly in control individuals, while it was stable in treated birds (Table 1, Fig. 2).
282 This indicates that sulfoximine prevented upregulation of GSH synthesis. The
283 concentration of GSSG in red blood cells and the GSH/GSSG ratio respectively
284 increased (estimate \pm SE: 0.48 \pm 0.10) and decreased (estimate \pm SE: -1.44 \pm 0.46) with time
285 in all birds, irrespective of treatment group and ownership (Table 1, Fig. 2). The
286 difference in plasma ROMs between control and treated individuals depended on
287 whether or not a bird was owner of a nest-box (experimental group \times ownership \times

288 sampling day: $p = 0.03$). To investigate this further, the effect of treatment on plasma
289 ROMs was tested separately for owners and non-owners, using similar mixed models
290 including the treatment \times sampling day interaction (Table 1, Fig. 3). In owners, there
291 was no effect of treatment or sampling day, nor of their interaction. In non-owners,
292 plasma ROMs increased in treated birds as compared to control birds (Table 1, Fig. 3).
293 There was no effect of treatment nor of ownership on body mass (Table 1); there was
294 only a significant decrease of body mass (estimate \pm SE: -2.32 ± 0.46) with time.

295 The reduced model of the total song rate shows that it increased over the
296 experiment irrespective of treatment or ownership (Table 2, Fig. 4) and that it was
297 higher in owners than non-owners (Table 2). The difference in either nest-box oriented
298 or undirected song rate between control and treated individuals depended on whether a
299 bird was or was not owner of a nest-box (experimental group \times ownership \times sampling
300 day, p -values < 0.001). To investigate this further, the effect of treatment on these two
301 song rate metrics was tested separately for owners and non-owners, using similar mixed
302 models including the treatment \times sampling day interaction. In owners, the reduced
303 models showed that the undirected song rate increased over the experiment irrespective
304 of treatment, while the nest-box oriented song rate was stable over the experiment and
305 did not differ between treatment groups (Table 2, Fig. 4). In non-owners, the undirected
306 song rate was lower in treated birds than in control birds toward the end of the
307 experiment as shown by post-hoc tests (Fig. 4). At the same time, post-hoc tests showed
308 that the nest-box oriented song rate was significantly lower in treated birds than in
309 control birds during the first part of the experiment (Table 2, Fig. 4).

310 There was generally a negative covariation between total song rate and d-ROMs
311 values, but only the model including total song rate on day 2 was significant (Table 3).

312 Seven out of 12 models showed a significant positive covariation between undirected
313 song rate and d-ROMs values (Table 3). Eleven out of 12 models showed a significant
314 negative covariation between nest-box oriented song rate and d-ROMs values (Table 3).

315

316 **Discussion**

317 Experimental inhibition of the production of a key cellular antioxidant (glutathione)
318 enabled us to reveal a causal effect of a deregulation of oxidative balance on song
319 production. Our data also showed that the effect of the treatment on song was dependent
320 on nest-box ownership, with only non-owner birds suffering a reduction of song rate.
321 Considering that nest-box ownership reflects social dominance, our results appear to
322 indicate that subordinate males (non-owners) suffered increased oxidative damage as
323 compared to dominant males (owners). This result provides experimental support to the
324 hypothesis that the individual oxidative balance may influence the expression of
325 condition-dependent signals, hereby underlying their honesty. Analysis of individual
326 variation in song behaviour showed that a high song activity away from the nest-box
327 was associated with higher oxidative damage, while singing more at the nest-box was
328 associated with lower oxidative damage, respectively.

329 Irrespective of the ownership, our treatment prevented the upregulation of GSH
330 synthesis in all treated birds. However, only non-owner treated birds showed an
331 increased level of plasma oxidative damage metabolites. It might be that individuals of
332 high social rank were capable of upregulating their antioxidant defences to buffer any
333 oxidation induced from the inhibition of glutathione, thus avoiding any costs for the
334 production of vocalizations. On the other hand, low rank individuals were unable to
335 avoid increased oxidative damage, which came at a cost for the expression of song.

336 Overall, these results support the hypothesis that song rate may signal the individual
337 capacity to withstand oxidative stress (Van Hout, Eens & Pinxten 2011; Casagrande *et*
338 *al.* 2014; Baldo *et al.* 2015), supporting the idea that the song rate might reflect
339 individual quality (Garamszegi *et al.* 2004).

340 Our results indicate that dominant individuals have higher resistance to oxidative
341 stress than subordinate individuals during the non-mating season. Previous correlational
342 work on the link between social status and oxidative status in males before the start of
343 the mating season has provided controversial evidence. For example, higher-ranking
344 male rhesus macaques (*Macaca mulatta*) or mandrills (*Mandrillus sphinx*) had lower
345 levels of oxidative damage (Beaulieu *et al.* 2014; Georgiev *et al.* 2016). Conversely,
346 prior to breeding, male white-browed sparrow weavers (*Plocepasser mahal*) and male
347 Seychelles warblers (*Acrocephalus sechellensis*) did not show rank-related differences
348 in markers of oxidative damage or antioxidant protection (van de Crommenacker *et al.*
349 2011; Cram, Blount & Young 2015). What are the exact mechanisms via which
350 dominance status is linked to resistance to oxidative stress and why such a link varies
351 among and within species remain open questions.

352 Our experimental inhibition of glutathione caused suppression of undirected
353 song rate in non-owner birds only. The undirected song of male European starlings is
354 tightly coupled to a positive (or less negative) physiological state (Riters & Stevenson
355 2012; Kelm-Nelson & Riters 2013; Riters *et al.* 2014) and it is used for maintaining
356 group cohesion and social order (Eens 1997). It might be that the prolonged suppression
357 of glutathione and increased generation of oxidative damage were responsible for an
358 organism physiological deregulation that led non-owner birds to invest less in social
359 communication. Suppression of undirected song rate might also indicate higher

360 metabolic costs associated with it as suggested by the higher oxidative damage in those
361 males that sang more away from the nest-box. On the other hand, after an initial
362 decrease due to our experimental treatment, the nest-box-oriented song rate of non-
363 owners was no longer affected by our treatment. This result suggests that resources
364 invested to regulate the oxidative balance were not taken away from song production.
365 The nest-box-oriented song rate was always negatively associated with oxidative
366 damage at individual level. The nest-box-oriented song rate is mainly used for the
367 acquisition and defense of a nest site (Gwinner, Gwinner & Dittami 1987; Eens, Pinxten
368 & Verheyen 1990; 1991). We have, for example, observed repeatedly non-owner males
369 singing on a perch of an occupied nest-box, in an attempt to challenge its owner. The
370 bird owning the nest-box reacted by singing and chasing it from the nest-box. These
371 results might indicate that non-owners prioritized investment in this song rate metric in
372 order to achieve a dominance status. Previous work conducted during the breeding
373 season also showed that, when facing an immune challenge, male starlings appear to
374 prioritize preservation of the nest-box oriented song (Costantini *et al.* 2015).

375 Our results also revealed temporal changes in baseline metrics of oxidative
376 balance. The concentration of GSH in red blood cells increased significantly in all
377 control individuals, while it remained stable due to the effect of sulfoximine in treated
378 birds. In both control and treated birds, concentration of GSSG increased and the
379 GSH/GSSG ratio decreased during the experiment compared to pre-manipulation
380 values, respectively. Previous studies on passerine birds also found seasonal increases
381 and decreases of GSH and GSH/GSSG ratio (*Hirundo rustica*, Raja-aho *et al.* 2012;
382 *Passer domesticus*, Pap *et al.* 2015). The reasons for seasonal changes in the glutathione
383 system (GSH, GSSG or GSH/GSSG ratio) are currently unknown.

384 In a previous work on male starlings (Costantini *et al.* 2015) it was found that
385 immunization did not affect the ratio GSH/GSSG. This is in agreement with the present
386 study, possibly suggesting that any perturbation in one of the two molecules (GSH or
387 GSSG) is somehow compensated in order to keep the ratio, and so the redox balance of
388 the cell (Jones 2005), constant.

389

390 **Conclusions**

391 Our study has provided evidence that experimental inhibition of an important cellular
392 antioxidant (glutathione) resulted in a decreased song rate and an increased oxidative
393 damage in males that did not own a nest-box (subordinate individuals) but not in males
394 that owned a nest-box (dominant individuals). Our results also suggest that different
395 metrics of song rate might provide different information about the individual oxidative
396 balance because they differ in how they are associated with individual quality. These
397 results provide experimental support for the hypothesis that acoustic signals may
398 honestly convey information about the individual oxidative status.

399

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407 This study was done in agreement with the Belgian and Flemish legislation and was
408 approved by the ethical committee of the University of Antwerp (code 2013-28).

409

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587

588 Table 1. Outcomes of both full and reduced linear mixed models of factors affecting
 589 reduced glutathione, oxidised glutathione, the GSH/GSSG ratio, reactive oxygen
 590 metabolites and body mass of starlings.

| Variable | Effect | Full model d.f. | F | P | Reduced model d.f. | F | P |
|--|--|--------------------|-------|--------|-----------------------|-------|--------|
| Reduced glutathione (GSH) | Treatment group | 1,27 | 15.65 | <0.001 | 1,28 | 16.28 | <0.001 |
| | Ownership | 1,27 | 0.64 | 0.43 | 1,28 | 0.67 | 0.42 |
| | Sampling day | 1,27 | 6.66 | 0.02 | 1,29 | 6.69 | 0.02 |
| | Treatment group x ownership | 1,27 | 0.05 | 0.82 | | | |
| | Treatment group x sampling day | 1,27 | 7.72 | 0.01 | 1,29 | 7.72 | 0.01 |
| | Ownership x sampling day | 1,27 | 0.01 | 0.92 | | | |
| Oxidised glutathione (GSSG) | Treatment group | 1,54 | 0.03 | 0.87 | 1,58 | 0.02 | 0.88 |
| | Ownership | 1,54 | 0.19 | 0.67 | 1,58 | 0.17 | 0.68 |
| | Sampling day | 1,54 | 21.4 | <0.001 | 1,58 | 22.67 | <0.001 |
| | Treatment group x ownership | 1,54 | 0.40 | 0.53 | | | |
| | Treatment group x sampling day | 1,54 | 0.04 | 0.83 | | | |
| | Ownership x sampling day | 1,54 | 1.07 | 0.31 | | | |
| GSH/GSSG | Treatment group | 1,54 | 1.56 | 0.22 | 1,58 | 1.60 | 0.21 |
| | Ownership | 1,54 | 1.38 | 0.25 | 1,58 | 1.41 | 0.24 |
| | Sampling day | 1,54 | 9.45 | 0.003 | 1,58 | 9.87 | 0.003 |
| | Treatment group x ownership | 1,54 | 0.28 | 0.60 | | | |
| | Treatment group x sampling day | 1,54 | 0.002 | 0.97 | | | |
| | Ownership x sampling day | 1,54 | 0.001 | 0.98 | | | |
| Reactive oxygen metabolites owners | Treatment group | 1,13 | 0.07 | 0.80 | 1,13 | 0.07 | 0.80 |
| | Sampling day | 1,14 | 1.70 | 0.21 | 1,15 | 1.80 | 0.20 |
| | Treatment group x sampling day | 1,14 | 0.18 | 0.68 | | | |
| Reactive oxygen metabolites non-owners | Treatment group | 1,13 | 6.92 | 0.02 | 1,13 | 6.92 | 0.02 |
| | Sampling day | 1,13 | 1.63 | 0.22 | 1,13 | 1.63 | 0.22 |
| | Treatment group x sampling day | 1,13 | 7.39 | 0.02 | 1,13 | 7.39 | 0.02 |
| Body mass | Treatment group | 1,26 | 0.21 | 0.65 | 1,27 | 0.22 | 0.64 |
| | Ownership | 1,26 | 0.51 | 0.48 | 1,27 | 0.54 | 0.47 |
| | Sampling day | 1,27 | 25.13 | <0.001 | 1,30 | 27.15 | <0.001 |
| | Treatment group x ownership | 1,26 | 0.03 | 0.88 | | | |
| | Treatment group x sampling day | 1,27 | 0.30 | 0.59 | | | |
| | Ownership x sampling day | 1,27 | 0.64 | 0.43 | | | |
| | Treatment group x ownership x sampling day | 1,27 | 0.02 | 0.90 | | | |

591

592

593 Table 2. Outcomes of both full and reduced generalized linear mixed models with a
 594 binomial error distribution showing the factors affecting total song rate, undirected song
 595 rate and nest-box oriented song rate of starlings.

596

| Variable | Effect | Full model | | Reduced model | |
|--|--|------------|--------|---------------|--------|
| | | z | p | z | p |
| Total song rate | Treatment group | -0.61 | 0.54 | -1.07 | 0.29 |
| | Ownership | 0.39 | 0.69 | 2.0 | 0.045 |
| | Sampling day | 2.49 | 0.013 | 4.17 | <0.001 |
| | Treatment group x ownership | 0.46 | 0.64 | | |
| | Treatment group x sampling day | -1.12 | 0.26 | | |
| | Ownership x sampling day | -0.31 | 0.76 | | |
| | Treatment group x ownership x sampling day | 1.03 | 0.31 | | |
| Undirected song rate owners | Treatment group | -1.07 | 0.29 | -0.22 | 0.83 |
| | Sampling day | 2.0 | 0.046 | 4.33 | <0.001 |
| | Treatment group x sampling day | 1.56 | 0.12 | | |
| Nest-box oriented song rate owners | Treatment group | 1.07 | 0.28 | 0.56 | 0.57 |
| | Sampling day | 0.26 | 0.80 | -0.77 | 0.44 |
| | Treatment group x sampling day | -1.12 | 0.26 | | |
| Undirected song rate non-owners | Treatment group | 1.47 | 0.14 | 1.47 | 0.14 |
| | Sampling day | 4.14 | <0.001 | 4.14 | <0.001 |
| | Treatment group x sampling day | -3.56 | <0.001 | -3.56 | <0.001 |
| Nest-box oriented song rate non-owners | Treatment group | -3.35 | <0.001 | -3.35 | <0.001 |
| | Sampling day | -2.9 | 0.004 | -2.9 | 0.004 |
| | Treatment group x sampling day | 3.5 | <0.001 | 3.5 | <0.001 |

597

598

599 Table 3. Outcomes of linear mixed models showing the association between d-ROMs
600 values (oxidative damage) and each metric of song rate. In each model, pre- and post-
601 treatment values of d-ROMs were pooled together. Pre-treatment values of each song
602 rate metric are those measured on day 1, i.e., before the start of the treatment (see Fig.
603 1). As post-treatment values of each song rate metric, we used data collected on day 2,
604 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 13, respectively. Significant associations are shown in
605 bold time. SE = standard error

606

| | Total song rate | | Undirected song rate | | Nest-box oriented song rate | |
|-----|-------------------|--------------|----------------------|--------------|-----------------------------|------------------|
| Day | estimate±SE | p-value | estimate±SE | p-value | estimate±SE | p-value |
| 2 | -0.35±0.16 | 0.027 | 0.25±0.12 | 0.048 | -0.57±0.12 | <0.001 |
| 3 | -0.02±0.13 | 0.90 | 0.29±0.13 | 0.035 | -0.28±0.13 | 0.029 |
| 4 | -0.15±0.13 | 0.24 | 0.36±0.14 | 0.015 | -0.48±0.13 | <0.001 |
| 5 | -0.07±0.12 | 0.54 | 0.31±0.13 | 0.017 | -0.39±0.11 | 0.001 |
| 6 | -0.08±0.16 | 0.62 | 0.38±0.16 | 0.024 | -0.46±0.13 | <0.001 |
| 7 | 0.03±0.17 | 0.86 | 0.51±0.15 | 0.002 | -0.48±0.14 | 0.001 |
| 8 | -0.07±0.15 | 0.64 | 0.23±0.14 | 0.11 | -0.31±0.12 | 0.014 |
| 9 | -0.20±0.15 | 0.18 | 0.18±0.14 | 0.21 | -0.33±0.13 | 0.009 |
| 10 | -0.32±0.17 | 0.068 | 0.23±0.17 | 0.18 | -0.51±0.16 | 0.002 |
| 11 | -0.18±0.19 | 0.34 | 0.15±0.15 | 0.33 | -0.32±0.14 | 0.022 |
| 12 | -0.21±0.15 | 0.18 | 0.17±0.14 | 0.21 | -0.41±0.15 | 0.006 |
| 13 | 0.03±0.17 | 0.85 | 0.39±0.19 | 0.041 | -0.29±0.15 | 0.053 |

607

608

609 **Figure captions**

610 Figure 1. Timeline of the experiment. BM = body mass. The average values of song rate
611 scores recorded from 12 to 21 November were used as pre-treatment values.

612

613 Figure 2. Pre- and post-manipulation levels of reduced (GSH) and oxidized (GSSG)
614 glutathione and the ratio GSH/GSSG in our control (C) and treated (T) male European
615 starlings (*Sturnus vulgaris*). The experimental treatment with sulfoximine (inhibitor of
616 glutathione synthesis) was able to prevent an upregulation of GSH. Means that do not
617 share the same letter are significantly different from each other. Data are shown as mean
618 \pm standard error.

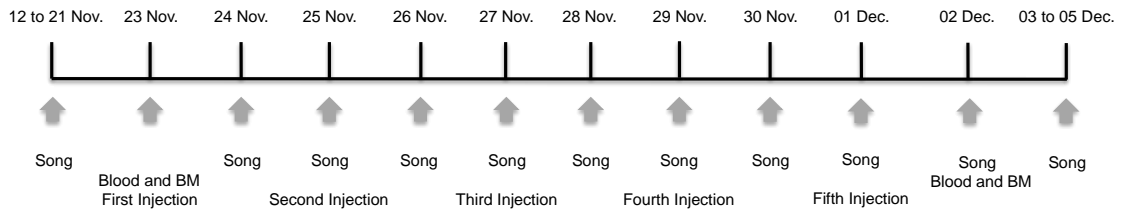
619

620 Figure 3. Pre- and post-manipulation levels of plasma reactive oxygen metabolites
621 (marker of oxidative damage) of male European starlings (*Sturnus vulgaris*) in relation
622 to treatment (C = control, T = treated) and nest-box ownership (N = non-owner of a
623 nest-box, Y = owner of a nest-box). The experimental treatment with sulfoximine
624 (inhibitor of glutathione synthesis) caused an increase of reactive oxygen metabolites in
625 non-owner treated birds only. Means that do not share the same letter are significantly
626 different from each other. Data are shown as mean \pm standard error.

627

628 Figure 4. Temporal trends of song rate metrics throughout the experiment in relation to
629 treatment (C = control, T = treated) and nest-box ownership (N = non-owner, Y =
630 owner) in male European starlings (*Sturnus vulgaris*). Pre-treatment (i.e., sampling day
631 1) values of each song metric are expressed as the average of the values recorded from
632 12 to 21 November. The (*) indicates a difference statistically significant. Lines that

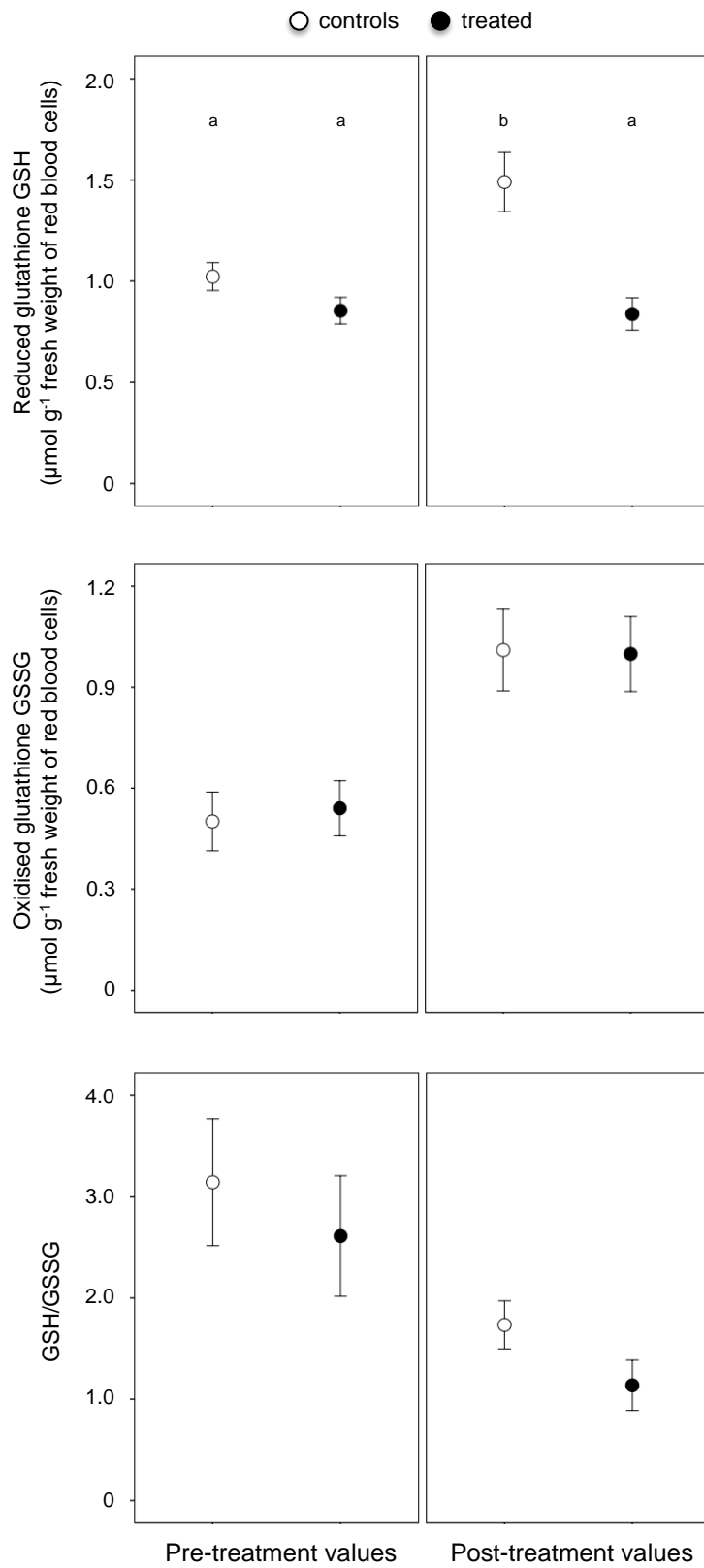
633 join the box-plots are shown when there are differences statistically significant. Data are
634 shown as mean \pm standard error.
635



636

637 Figure 1

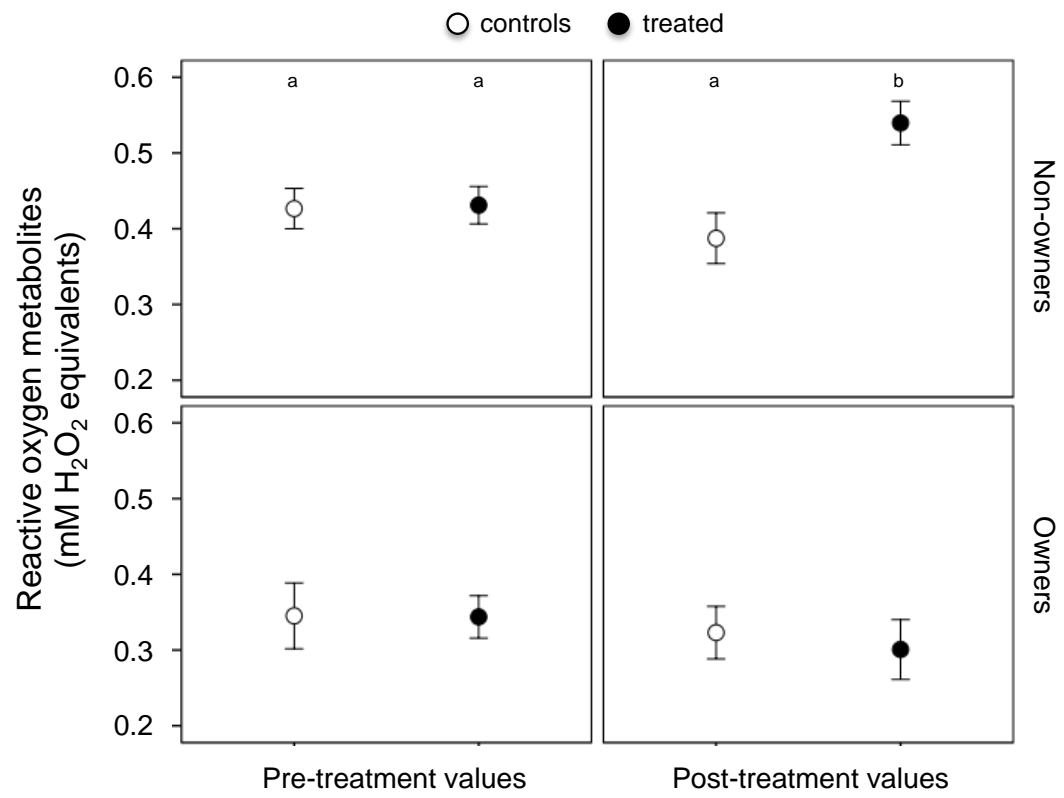
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640 Figure 2

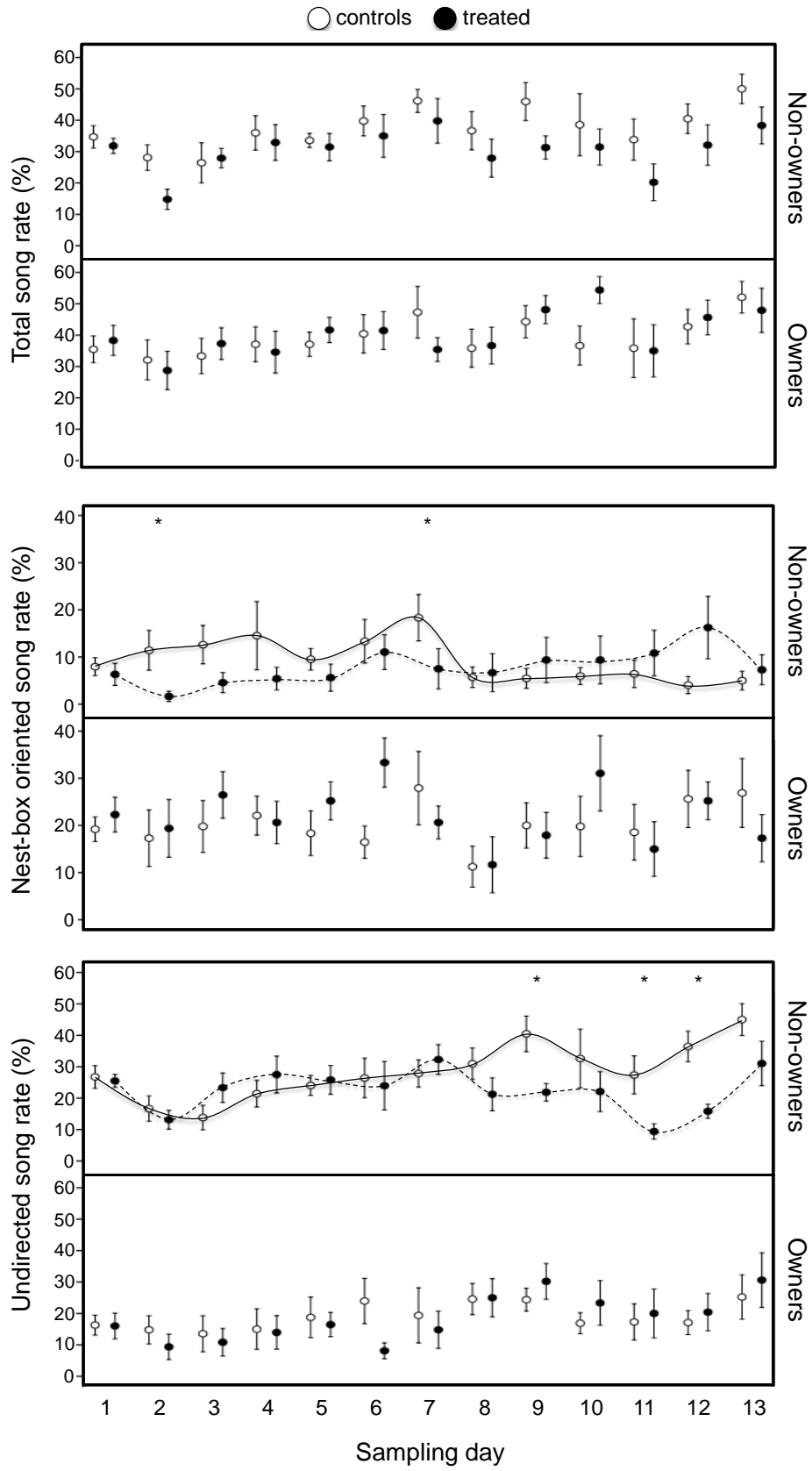
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642

643 Figure 3

644



645

646 Figure 4