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5 **David Costantini • Stefania Casagrande • Giulia Casasole**

6 **Hamada AbdElgawad • Han Asard • Rianne Pinxten • Marcel Eens**

7
8
9 David Costantini

10 Department of Biology, Ethology group, University of Antwerp, Universiteitsplein 1, 2610

11 Wilrijk, Belgium

12 IBAHCM, University of Glasgow, Glasgow G12 8QQ, UK

13 Email: david.costantini@uantwerpen.be

14
15 Stefania Casagrande

16 Department of Biology, Ethology group, University of Antwerp, Universiteitsplein 1, 2610

17 Wilrijk, Belgium

18 Present address: Max Planck Institute for Ornithology, Evolutionary Physiology Group,

19 Eberhard-Gwinner-Str. 82319 Seewiesen, Germany

20
21 Giulia Casasole

22 Institute of Environmental Sciences, Jagiellonian University, Ulica Gronostajowa 7, 30-387

23 Kraków, Poland

24

25 Hamada AbdElgawad • Han Asard

26 Molecular Plant Physiology and Biotechnology group, Department of Biology, University

27 of Antwerp, Antwerp, Belgium

28

29 Hamada AbdElgawad

30 Department of Botany, Faculty of Science, University of Beni-Sueif, Beni-Sueif 62511,

31 Egypt.

32

33 Rianne Pinxten

34 Department of Biology, Ethology group, University of Antwerp, Universiteitsplein 1, 2610

35 Wilrijk, Belgium

36 Institute for Education and Information Sciences, Research Unit Didactica, University of

37 Antwerp, Antwerp, Belgium

38

39 Marcel Eens

40 Department of Biology, Ethology group, University of Antwerp, Universiteitsplein 1, 2610

41 Wilrijk, Belgium

42

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44 **Running header:** Immunization, song and oxidative stress

45 **Abstract** It is hypothesised that variation in immune function between individuals is due to
46 costs incurred to sustain it. Support for this hypothesis mostly comes from short-term
47 studies either on the costs of innate responses or a combination of innate and antibody
48 responses. Key studies on the fitness and physiological costs of acquired immunity, in
49 which the antibody response is specifically stimulated over a long period, are lacking. We
50 specifically stimulated the antibody response against a virus (Newcastle virus) in male
51 European starlings (*Sturnus vulgaris*) for two months to test whether immunization reduces
52 a fitness-related trait (song rate) and increases oxidative stress. Immunization did not affect
53 the total song rate, but it caused a reduction of the undirected song rate (produced away
54 from the nest-box and mostly used for establishing dominance hierarchy). We also found
55 that immunized birds had a lower nest-box oriented song rate (mostly used to attract
56 females) than control birds although the interaction between treatment and sampling period
57 was not significant. Immunization did not cause any changes in the blood oxidative status.
58 Starlings with a higher nest-box oriented song rate had significantly lower levels of
59 oxidative protein damage. Finally, starlings that skipped the antibody response had an
60 oxidative status similar to that of starlings that produced antibodies, but they had overall a
61 lower rate of undirected song. Our results suggest that (i) immunized starlings preserved the
62 song used to attract mates but not that used in social interactions and (ii) the antibody
63 response incurs costs that are reflected in the expression of song, but also that these costs
64 are unlikely to be determined by oxidative stress. Our results also suggest that bird song
65 might convey information about a male's oxidative status.

66

67 **Keywords** Antibodies • Birds • Immunity • Oxidative damage • Song

68 **Introduction**

69 An organism's physiological equilibrium is critically reliant on its immune system, which
70 provides protection against parasites and allows recovery from injuries. Positive selection
71 of individuals having more efficient immune defences should therefore be expected to sift
72 out poorly functioning individuals. Conversely to this prediction, much variation in
73 immunological functions persists in animal populations (Sheldon and Verhulst 1996;
74 Martin et al. 2011). The reason for this high variation might lie with the costs of mounting
75 an immune response. For example, feral Soay sheep (*Ovis aries*) having higher
76 concentrations of autoimmune antinuclear antibodies had higher survival over harsh
77 winters, but they also had diminished fecundity (Graham et al. 2010). Similarly, Bonneaud
78 et al. (2012) found that house finches (*Carpodacus mexicanus*) that lost more body mass
79 during an experimental infection had lower parasite counts and upregulated more
80 immunological genes. Fitness costs also emerge when acquisition of humoral
81 immunocompetence (i.e., immunization) is experimentally induced. For example, it was
82 found that immunization can reduce reproductive success (Marzal et al. 2007) or body
83 condition (Dreiss et al. 2008). Moreover, antibody responsiveness can be reduced or
84 suppressed in order to divert resources toward other functions, such as reproduction, at the
85 cost of decreasing survival (Deerenberg et al. 1997; Nordling et al. 1998).

86 The costs of immune response can also impinge on the expression of secondary
87 sexual traits. Bird song is one renowned behavioural trait that is under sexual selection.
88 Hamilton and Zuk (1982) proposed that bird song may reflect attributes of male quality,
89 such as the capacity to cope with parasites. Hence, females can use the song to discriminate
90 between males in good or bad health status. For example, it has been found that

91 immunization can reduce song rate in collared flycatchers *Ficedula albicollis* (Garamszegi
92 et al. 2004) or rattle duration in barn swallows *Hirundo rustica* (Dreiss et al. 2008). Duffy
93 and Ball (2002) also found that male starlings (*Sturnus vulgaris*) having a higher song rate
94 or a longer song-bout length (which females prefer, Eens et al. 1991; Gentner and Hulse
95 2000) exhibited higher cell-mediated and humoral immunity as compared to those with a
96 lower song rate or a shorter song-bout length.

97 Although song rate can convey some attributes of the individual
98 immunocompetence, an important question then is which mechanisms link song rate to
99 immunocompetence. The immune response certainly demands various kinds of resources,
100 such as nutrients (Klasing 2007) and energy (Cutrera et al. 2010) that are also needed to
101 sustain singing (Chappell et al. 1995; Garamszegi et al. 2006). However, energetic costs of
102 song production are generally small (Oberweger and Goller 2001; Ward et al. 2003, 2004;
103 Zollinger et al. 2011). The immune response can also have more subtle costs. One putative
104 mechanism that has received considerable recent attention is the role of oxidative stress,
105 which occurs when there is an increase in oxidative molecular damage and oxidation of
106 non-protein and protein thiols that regulate the cell oxidative balance (Jones 2006;
107 Halliwell and Gutteridge 2007; Sohal and Orr 2012). Production of reactive oxygen and
108 nitrogen species by immune cells is an important component of the immune response (Sorci
109 and Faivre 2009). The cytotoxic effects of reactive molecules produced by leukocytes are
110 exploited by the organism when coping with pathogens during the innate immune response.
111 Given the unspecificity in the action of reactive molecules, generation of oxidative damage
112 upon biomolecules can also occur. A core idea of the so-called immuno-oxidative ecology
113 is that oxidative stress may provide a currency to quantify costs resulting from the impact

114 of immune activation on traits like sexual traits, growth, reproduction or senescence
115 (Costantini 2014). Although many experimental reports have shown that the innate immune
116 response may result in oxidative stress (e.g., Bertrand et al. 2006; Torres and Velando
117 2007; van de Crommenacker et al. 2010), comparatively little is known about the
118 mechanisms underlying the costs of acquired immunity (Costantini and Møller 2009).

119 Here we tested whether a specific experimental manipulation of the antibody
120 response reduces song rate (a fitness-related trait that is constrained by antioxidant and
121 nutrient availability in starlings; Van Hout et al. 2011; Casagrande et al. 2014) and causes
122 increased levels of oxidative stress. To this end, we vaccinated a group of male European
123 starlings twice over a period of two months of the reproductive season using an inactivated
124 strain of the Newcastle virus and compared their song rate and blood oxidative status to a
125 group of non-vaccinated males. This experimental approach enabled us (i) to assess
126 whether oxidative stress is one constraint of song that guarantees signal reliability (i.e.,
127 honesty of song), (ii) to uniquely examine the costs of acquired immunity against a relevant
128 avian virus without the confounding of strong inflammatory processes that would have
129 occurred exposing birds to a live strain of the virus and (iii) to assess whether the song
130 conveys information about individual oxidative stress.

131

132 **Material and Methods**

133

134 Housing conditions and experimental setup

135

136 The European starling is a seasonally breeding songbird in which song during the
137 reproductive season is used as a mating signal (Mountjoy and Lemon 1991; Eens et al.
138 1991a; Eens 1997; Gentner and Hulse 2000; Gentner et al. 2001). All 60 male starlings
139 used in this study had been captured previously in the Antwerp district and were then
140 housed in large single sex outdoor aviaries on the grounds of Campus Drie Eiken of the
141 University of Antwerp (Wilrijk, Belgium). The duration of the housing in captivity before
142 the start of the experiment varied and was respectively: 4 months (n=18), 2 years (n=22), 3
143 years (n=9), 7 years (n=8), 8 years (n=2) and 10 years (n=1). On February 27, 2014, the
144 sixty male starlings were moved to four experimental outdoor aviaries (L x W x H; 27.0 x
145 7.0 x 2.75 m), with 15 birds in each aviary (duration of previous housing in captivity did
146 not differ significantly between aviaries; $F_{3,51}=0.47$, $P = 0.70$). Each aviary had 15 nest-
147 boxes and each nest-box had a perch in front of it. In each cage, we had both control and
148 immunized birds (2 cages with 7 control and 8 immunized birds and 2 cages with 8 control
149 and 7 immunized birds). In total, we had 30 control birds and 30 immunized birds.
150 However, nine individuals were excluded from the analyses because one control individual
151 died before the first immunization, two immunized birds died over the course of the
152 experiment and six control birds had high initial antibody levels against Newcastle disease
153 (see later), possibly because they had been previously exposed to the virus in the wild.
154 Therefore, the final sample size was 23 control and 28 immunized starlings (11, 13, 13 and
155 14 starlings in each cage, respectively). All starlings were marked with a unique
156 combination of coloured bands and a metal ring, which allowed individual recognition.
157 Food (Orlux, Deinze, Belgium; Nifra Van Camp, Boechout, Belgium) and water were
158 provided ad libitum. All males used in this study were adults (see below).

159 The experiment was performed according to the timescale illustrated in Fig. 1. The
160 times of blood sampling were chosen in order to have blood samples at peaks of
161 lymphocyte activity (Kapczynski et al. 2013; Scott et al. 2013). A sample of blood (ca.
162 500µl) was collected by venipuncture using a heparinised microvette (Sarstedt, Nümbrecht,
163 Germany) just before each immunization. Blood samples were maintained cool and were
164 then centrifuged to separate plasma from red blood cells. After centrifugation, the plasma
165 of each sample was pipetted out from the tube and divided in 4 different tubes (in order to
166 have one tube allocated to each assay plus one extra tube), while red blood cells were
167 divided in 2 different tubes (one for the assay plus one extra tube). We did so in order to
168 avoid to defrost repeatedly the same aliquot. Samples were stored at -80°C.

169

170 Quantification of song rate

171

172 Behavioural observations of song rate were made for four consecutive days by SC and GC
173 at three different periods of the experiment: prior to the first immunization; prior to the
174 second immunization; and prior to the collection of the last blood sample (Fig. 1). During
175 each observation day, the song rate of all the starlings within each aviary was recorded
176 during a session of 60 minutes between 09h00 and 13h00 (when singing activity of
177 starlings during the day is highest; Eens 1997). We alternated the order of the aviaries
178 between subsequent days in order to have a balanced distribution of the timing of
179 observations. All song observations were made (using a binocular) from behind a shelter
180 located ca. 5 m from each aviary, using a one-zero sampling technique (Martin and Bateson
181 2007) with an interval of one minute. Hence, every minute we scanned all males inside an

182 aviary and recorded which males were singing. We also scored whether males produced
183 nest-box oriented song (i.e., song produced from inside the nest-box with the head sticking
184 out from the entrance hole, on the top of it or on the perch in front of the nest-box) or
185 undirected song (i.e., song produced away from the nest-box). Given that European
186 starlings, while singing, adopt a characteristic upright stance, upturned bill, and the throat
187 feathers and beak can be seen moving (Feare 1984), singing behaviour can be easily
188 quantified. Furthermore, during the breeding season, male starlings sing the majority of
189 their song (90 % or more) in long and complex song bouts (uninterrupted singing) that last
190 more than 30 seconds (Eens et al. 1991b). Nest-box oriented song rate and undirected song
191 rate were quantified as the percentage of one-minute intervals during which a male was
192 observed singing nest-box oriented and undirected song, respectively (Pinxten et al. 2002).

193 For each individual and each period of the experiment, we then averaged the two
194 song rates of the four days of observations. We also recorded whether males occupied a
195 nest-box (i.e., repeatedly inspected a particular nest-box, or were seen bringing nest
196 material to this nest-box, or sat and sang in front of or on it). These data were used to
197 identify which individuals were non-owners or owners of a nest-box.

198 Previous studies in European starlings showed that the nest-box oriented song is
199 important in mate choice: males singing more have higher mating success (Eens et al.
200 1991a; Wright and Cuthill 1992; Eens 1997; Pinxten et al. 2002; Ball et al. 2006). In
201 contrast, the undirected song is used for flock maintenance (Hausberger et al. 1995) and for
202 establishing dominance hierarchy (Eens 1997), although its precise function is less clear
203 than the nest-box oriented song (Kelm-Nelson and Ritters 2013). Song rate quantified using

204 this protocol is significantly repeatable within individuals across different sessions (Van
205 Hout et al. 2009, 2011).

206

207 Immunization

208

209 We assessed the response of starlings to immunization with a Newcastle disease virus
210 (NDV) inactivated vaccine (Nobilis Paramyxo P201, MSD Animal Health, Brussels,
211 Belgium). NDV is a globally distributed and highly virulent avian paramyxovirus (Seal et
212 al. 2000; Al-Garib et al. 2003). Previous studies showed that immunization of individual
213 birds with an inactivated vaccine of NDV elicits a significant antibody response, but does
214 not significantly induce an inflammatory response (Al-Garib et al. 2003; Broggi et al.
215 2013). Doses for the immunized and control birds were chosen according to previous
216 studies (Nordling et al. 1998; Saino et al. 2002). Immunized birds were subcutaneously
217 injected in the breast with 100 μ l of vaccine and control birds were injected with 100 μ l of
218 phosphate buffered saline. The antigen strain concentration (expressed as hemagglutination
219 inhibition score) was ≥ 6.8 and ≤ 10.2 \log_2 units. Vaccination was repeated twice in order to
220 stimulate antibody response over a long period of the reproductive time.

221

222 Analysis of antibody concentration

223

224 The Newcastle Disease Antibody Elisa kit (BioCheck, Reeuwijk, Holland) was used to
225 measure the amount of antibodies to NDV in plasma. It is important to quantify the
226 antibody concentration because antibody response can be suppressed in low quality

227 individuals or under certain life-history stages (e.g., Deerenberg et al. 1997). Plasma
228 samples were diluted 1:10 with a phosphate buffer with protein stabilisers and sodium azide
229 preservative (0.1% w/v) provided with the kit. Test procedure and calculation of antibody
230 status were done following manufacturer's instructions. The antibody status is expressed as
231 positive (the bird produced antibodies to NDV) or negative (the bird did not produce
232 antibodies to NDV).

233

234 Analyses of blood oxidative status

235

236 We assessed the blood oxidative status using colorimetric and chromatographic methods
237 commonly applied to vertebrates (Costantini et al. 2006; van de Crommenacker et al. 2010;
238 Montgomery et al. 2011; Sinha et al. 2014).

239 The d-ROMs assay (Reactive Oxygen Metabolites; Diacron International, Grosseto,
240 Italy) was used to measure plasma oxidative damage metabolites (mostly hydroperoxides)
241 that are generated early in the oxidative cascade. The small interference of the enzyme
242 ceruloplasmin that was found in humans (Alberti et al. 2000) did not occur in starling
243 plasma. Inhibition of ceruloplasmin activity with 50 μ M or 1 mM of sodium azide
244 (inhibitor of ceruloplasmin activity; Sigma-Aldrich, code 08591) did not cause any
245 decrease in absorbance (paired t-test, $t_{14} \geq 0.76$, $P \geq 0.14$, coefficient of variation
246 (mean \pm SD) = 4.81 \pm 1.34%). Moreover, the reaction of a dilution series of cumene
247 hydroperoxide with the d-ROMs reagents was highly linear (range: 0 to 4.5 μ M,
248 $R^2=0.9996$; physiological values in vertebrates) at the incubation temperature of 37 $^{\circ}$ C,
249 required by the manufacturer's instructions. Incubation at lower temperatures (4 and 24 $^{\circ}$ C)

250 reduced the efficiency of the Fenton reaction (i.e., chemical reaction of the d-ROMs assay),
251 as testified by a strong and similar reduction in absorbance of both plasma samples and
252 cumene hydroperoxide (data not shown). Analyses of reactive oxygen metabolites were
253 therefore done according to manufacturer's instructions as in previous studies. Quality
254 controls (Diacron International) were also assessed in each assay. Values of reactive
255 oxygen metabolites have been expressed as mM of H₂O₂ equivalents. Analyses were run in
256 duplicate; the intra- and inter-assay coefficients of variation were 4.81 and 5.32%,
257 respectively.

258 The Protein Carbonyl Colorimetric assay (Cayman Chemical Company, Ann Arbor,
259 MI, USA) was used to measure the plasma concentration of protein carbonyls. The assay is
260 based on the protocol of Levine et al. (1990). Protein carbonyls indicate oxidative damage
261 to proteins caused by free radicals or lipid peroxidation products (malondialdehyde and
262 hydroxynonenal; Halliwell and Gutteridge 2007). All plasma samples were first diluted
263 with distilled water in order to have a concentration of 2 mg proteins ml⁻¹, as measured
264 using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using
265 albumin as a reference standard. Nucleic acids were removed by adding 1 volume of a 10%
266 solution of streptomycin sulphate (Sigma-Aldrich, code S6501) to 9 volumes of sample.
267 Then, analyses were done according to the protocol of Levine et al. (1990). A control
268 plasma was also assessed in each assay. The concentration of protein carbonyls has been
269 expressed as nmol mg⁻¹ proteins or as total amount by multiplying the concentration per
270 mg of proteins for the total concentration of proteins in the plasma. Analyses were run in
271 duplicate; the intra- and inter-assay coefficients of variation were 6.44 and 8.68%,
272 respectively.

273 High-performance liquid chromatography with electrochemical detection was
274 applied for simultaneous determination of reduced (GSH) and oxidized (GSSG) glutathione
275 in red blood cells by a Reversed-Phase HPLC of Shimadzu (Hai Zhonglu, Shanghai). We
276 applied the protocol as described in Sinha et al. (2014). Concentrations of GSH and GSSG
277 were expressed as $\mu\text{mol g}^{-1}$ fresh weight of red blood cells. We calculated the GSH/GSSG
278 ratio that was used as an index of redox state (higher values indicate lower oxidative stress;
279 Jones 2006).

280

281 Statistical analyses

282

283 Statistical analyses were carried out using SAS Version 9.3 (Cary, NC, USA). Linear
284 mixed models with a repeated measures design were used to test the effects of
285 immunization on song rate and blood oxidative status. Response variables were as follows:
286 total song rate (sum of nest-box oriented song rate plus undirected song rate), nest-box
287 oriented song rate, undirected song rate, reactive oxygen metabolites, protein carbonyls,
288 total protein carbonyls and GSH/GSSG. In each model, we included treatment group,
289 sampling period and their interaction as fixed factors; individual (nested within cage) and
290 cage were included as random factors to control for the non-independence of multiple
291 measures from a same individual and for non-independence of measures taken from
292 individuals sharing the same cage, respectively. Response variables were transformed
293 where needed to achieve normality of residuals and homogeneity of variance.

294 To test whether song rate variables (nest-box oriented or undirected) were
295 associated with blood oxidative status in both controls and immunized birds, linear mixed

296 models with a repeated measures design (as described above) were performed by adding
297 reactive oxygen metabolites, protein carbonyls (or total protein carbonyls) and GSH/GSSG
298 altogether as covariates. Outcomes of all models were unchanged if each oxidative status
299 biomarker was included alone. Preliminary analyses also showed that the interaction
300 between treatment group and covariate was never significant for each oxidative status
301 biomarker.

302 Analyses of antibodies showed that 11 immunized birds did not mount any humoral
303 response (see results). Linear mixed models were therefore used to test if responsive (i.e.,
304 those that produced antibodies) and non-responsive (i.e., those that did not produce
305 antibodies) starlings differed in pre-treatment values of the following variables: age, total
306 song rate, nest-box oriented song rate, undirected song rate, reactive oxygen metabolites,
307 protein carbonyls, total protein carbonyls and GSH/GSSG. In each model, we included
308 group (responsive vs. non-responsive) as fixed factor and cage as random factor. We then
309 ran additional linear mixed models with a repeated measures design to test whether
310 responsive and unresponsive birds differed at any time of the experiment. In each model,
311 we included treatment group, sampling period and their interaction as fixed factors;
312 individual (nested within cage) and cage were included as random factors.

313 Outcomes of all models described above were unchanged if individual age, duration
314 of housing in captivity prior to the experiment, body mass, tarsus length, blood sampling
315 order or ownership of a nest-box were included as covariates (data not shown). For each
316 model, post-hoc comparisons were performed using both the t-test and the Tukey test when
317 we found a statistically significant interaction. We opted to run both tests in order to have a
318 comparison between a less (t-test) and a more (Tukey test) conservative approach.

319 Linear mixed models with cage as a random factor showed that at the time of the
320 first immunization (i.e., 4 April) controls and immunized birds did not differ in age
321 (mean±SE: controls, 3.3±0.4 years; immunized, 3.4±0.5 years; $P = 0.94$), in body mass
322 (mean±SE: controls, 93.2±2.0 grams; immunized, 92.5±1.6 grams; $P = 0.76$) nor in tarsus
323 length (mean±SE: controls, 29.6±0.1 mm; immunized, 29.9±0.1 mm; $P = 0.15$).

324

325 **Results**

326

327 Antibody response

328

329 Six control starlings were excluded from the analyses because they had initial high
330 antibodies, indicating that they had probably been previously exposed to Newcastle disease
331 in the wild. Of the 28 immunized birds included in the following analyses, 11 individuals
332 did not produce any antibody response.

333

334 Control versus immunized birds

335

336 In the following models, we included all immunized birds irrespective of whether they
337 produced antibodies or did not.

338 The total song rate and both the nest-box oriented song rate and the undirected song
339 rate did not differ significantly between control and immunized birds at the time of the first
340 immunization (linear mixed model with cage as random factor, $P \geq 0.08$). Both the total
341 song rate and the nest-box oriented song rate did not show any significant changes in both

342 groups over the experimental period (Fig. 2, Table 1). Overall, immunized birds had a
343 lower nest-box oriented song rate than control birds (estimate±SE: 18.12±8.34, $P = 0.0346$;
344 Fig. 2, Table 1). There was a significant interaction between treatment group and sampling
345 period for the undirected song rate (Fig. 2, Table 1). The undirected song rate increased in
346 control birds during the first part of the experiment (beginning vs. interim sample, $P =$
347 0.024) and decreased during the second part of the experiment (interim vs. end sample, $P =$
348 0.016). In contrast, the undirected song rate of immunized birds did not vary during the first
349 and second part (beginning vs. interim sample, $P = 0.083$; interim vs. end sample, $P =$
350 0.61), but song rate recorded before the first immunization was significantly higher than
351 that recorded after the second immunization (beginning vs. end sample, $P = 0.026$). A
352 separate LMM with nest-box oriented song rate as dependent variable and undirected song
353 rate as a covariate showed that these two types of song were positively correlated
354 (coefficient estimate±SE: 11.1±2.1, $P < 0.001$).

355 Reactive oxygen metabolites and both protein carbonyls metrics did not differ
356 between control and immunized birds at the time of the first immunization (linear mixed
357 model with cage as random factor, $P \geq 0.42$). However, immunized birds had lower initial
358 values of the GSH/GSSG ratio ($P = 0.02$). Reactive oxygen metabolites and both protein
359 carbonyls metrics significantly decreased during the experimental period irrespective of
360 treatment group (Fig. 2, Table 1). The GSH/GSSG ratio did not change significantly during
361 the experimental period in both experimental groups (Fig. 2, Table 1).

362 Irrespective of treatment group, starlings with a higher nest-box oriented song rate
363 had significantly lower levels of total protein carbonyls (coefficient estimate±SE: -
364 490.7±223.6, $P = 0.029$) and marginally significantly lower levels of protein carbonyls

365 expressed per mg of proteins (coefficient estimate±SE: -675.3 ± 353.7 , $P = 0.058$).

366 Undirected song was not associated with any of the oxidative status variables ($P \geq 0.13$).

367

368 Responsive versus unresponsive birds

369

370 At the time of the first immunization, responsive and unresponsive birds did not differ in

371 any of the variables considered in the present experiment ($P \geq 0.12$). Although

372 immunization resulted in a decrease in undirected song rate, responsive starlings had

373 overall a higher undirected song rate than unresponsive starlings during the whole

374 experiment (least square mean±SE: responsive, 10.7 ± 1.4 ; unresponsive, 6.0 ± 1.7 ; $P =$

375 0.049). The interaction between group and sampling period was never significant ($P \geq$

376 0.19).

377

378 **Discussion**

379 Song rate

380 Although reduced song rate and increased oxidative stress have been associated with a

381 number of immune responses, our study provides support only for a moderate effect of

382 antibody response against Newcastle virus on one particular mode of singing (undirected

383 song rate). We also found that immunized birds had a lower nest-box oriented song rate

384 than control birds; however, the interaction between treatment and sampling period was not

385 significant. A visual examination of Fig. 2 suggests that the nest-box oriented song rate

386 decreased in immunized birds as compared to control birds. However, the lack of difference

387 between groups at the end of the experiment might have reduced the power of our model to

388 detect an effect of immunization. Overall, these results suggest that the effect of
389 immunization on the nest-box oriented song rate was small.

390 Immunization resulted in starlings not increasing their undirected song as did
391 control birds after the first immunization. This suppressive effect of immunization on
392 undirected song has to be considered moderate because it is not significant if a more
393 restrictive post-hoc test is used (Fig. 2). Although the precise functions of undirected song
394 may differ across species, this song trait is used for establishing dominance hierarchy in
395 male European starlings (Eens 1997). Our results suggest that male starlings may have
396 prioritized preservation of the song component that is mostly used to attract mates and to
397 defend the nest boxes (i.e., the nest-box oriented song rate). Our study was done during the
398 reproductive season of the European starling, when singing to attract a mate is likely more
399 relevant than investing in social interactions with other individuals (Wright and Cuthill
400 1992). Conversely, during fall and winter months (i.e., the non-breeding season), male
401 starlings sing at high rates (undirected song; Eens 1997; Ritters et al. 2000; Kelm-Nelson
402 and Ritters 2013). It is possible that the results could have been different if immunization
403 had been done during the non-breeding season. Hence, further studies will be needed to
404 assess how starlings solve the trade-off of investing resources (e.g., energy, nutrients)
405 between antibody production and social interactions (e.g., access to a dominant position) in
406 a period where pressures of mate search are relaxed.

407

408 Blood oxidative status

409 The production of antibodies against Newcastle virus did not affect the blood oxidative
410 status in the long-term. Previous studies found that levels of antibodies can be correlated to

411 blood oxidative status parameters. For example, Casagrande et al. (2012b) found that sheep
412 red blood cell antibodies titres were positively correlated with plasma oxidative damage
413 and negatively correlated with plasma non-enzymatic antioxidant capacity in captive
414 diamond doves *Geopelia cuneata*, respectively. One possible explanation for our results is
415 that exposure of birds to an inactivated strain of Newcastle virus does not result in a strong
416 antibody response as compared to that induced by exposure to a virulent strain (Al-Garib et
417 al. 2003). Conversely to our study, infection of one-day-old male chickens with a
418 moderately virulent Newcastle strain caused increased levels of oxidative damage, reduced
419 levels of antioxidant protection and cell necrosis (Venkata Subbaiah et al. 2011, 2013).
420 However, the studies by Venkata Subbaiah et al. (2011, 2013) used sexually immature and
421 immunological naïve individuals that are known to experience higher mortality than adult
422 individuals when exposed to virus strains that are moderately virulent (Al-Garib et al.
423 2003). On the other hand, in agreement with our work, vaccination of fully mature male
424 Japanese quails with a live strain of Newcastle virus resulted in negligible effects on the
425 oxidative status (Paskova et al. 2011). We do not know whether the oxidative status of
426 starlings would have been affected by vaccination with a live virus strain. Although this
427 cannot be ruled out, as compared to young individuals, adults are equipped with fully
428 matured antioxidant mechanisms that better shield them against oxidative stress (Surai
429 2002; Fontagné et al. 2008; Vázquez-Medina et al. 2011). Replication of different virus
430 strains is facilitated by a status of oxidative stress (e.g., Schwarz 1996; De Luca et al.
431 2012). Hence, protecting cells against oxidation might be vital. Identification of these
432 mechanisms and quantification of individual variation in shielding capabilities are
433 important areas for future research.

434

435 Relationship between song and oxidative stress

436 Our results suggest that the undirected song is unrelated to oxidative stress. In fact, there
437 was no significant covariation between undirected song rate and all markers of blood
438 oxidative stress. Although immunized birds had lower initial values of the GSH/GSSG
439 ratio, this difference does not seem relevant because all markers of damage did not differ
440 between control and immunized birds. We cannot rule out that oxidative stress may have
441 been localized in the brain (i.e., where song production is controlled), but it is unclear why
442 we should not have also detected it in the blood, given the high immune activity that occurs
443 in this tissue. It might also be that immunization activated other physiological mechanisms
444 that affected the song rate. For example, the immune response results in increased
445 consumption of nutrients (Klasing 2007) or energy expenditure (Cutrera et al. 2010; but see
446 Ward et al. 2003, 2004), possibly reducing their investment in song. Moreover, the immune
447 activation may reduce the plasma concentration of testosterone (Boonekamp et al. 2008),
448 which modulates the expression of starling song (Pinxten et al. 2002; Van Hout et al.
449 2009).

450 Conversely to the undirected song rate, the nest-box oriented song rate was
451 negatively associated with oxidative protein damage. These results support the hypothesis
452 that song could convey some information about the individual's oxidative status to
453 prospective mates. Some components of bird song (nest-box oriented song rate in this
454 study; see also Wright and Cuthill 1992) are considered to have evolved via sexual
455 selection and should as such honestly signal aspects of the quality of its bearer (Andersson
456 1994). Hence, the female might acquire information about either the capability of the male

457 to withstand oxidative stress or simply on the male health status as long as the oxidative
458 status is linked to other important physiological functions (Hill 2011). However, both nest-
459 box oriented song rate and undirected song rate were also found to negatively correlate with
460 oxidative damage in another study, but this correlation was found during the non-
461 reproductive season (Casagrande et al. 2014). Hence, the information conveyed by the
462 undirected song rate might be season-dependent. Whether an increase in oxidative stress
463 directly causes a decrease in the nest-box oriented song rate is, however, unclear because
464 immunization did not increase oxidative damage. Although some studies point to oxidative
465 stress as a potential mechanism linking song rate to male quality (Van Hout et al. 2011;
466 Baldo 2012; Casagrande et al. 2014), a clear demonstration of such a link is still missing.
467 Given that the song (both in terms of song bout length and repertoire size) of male
468 European starlings is sexually selected (Eens et al. 1991a; Gentner and Hulse 2000), it
469 would be interesting to examine the relationships between measures of song complexity
470 and oxidative stress.

471

472 Seasonal variation in oxidative status

473 We found that the blood oxidative damage decreased during the season, independently
474 from the treatment. Previous studies also found seasonal variation in individual oxidative
475 status, for example, in relation to the breeding stage (e.g., incubation vs. nestling rearing,
476 Casagrande et al. 2011) or the individual hormonal status (Alonso-Alvarez et al. 2007;
477 Casagrande et al. 2012a). One explanation for our results might lie with the
478 photorefractoriness, a status characterised by a decline in the production of the luteinizing
479 hormone (which promotes synthesis of sexual steroids), which occurs between the end of

480 April and the start of May in starlings (Dawson and Sharp 2010). Since sexual steroids can
481 influence the blood oxidative status (e.g., increased basal production of damage; Alvarez et
482 al. 2007; Casagrande et al. 2012a), a seasonal decline in their synthesis might explain, at
483 least partly, the decline in oxidative damage.

484

485 Skipping the antibody response

486 Another finding of our study is that some individuals skipped the antibody response. We
487 could not find any differences between responsive and unresponsive individuals to
488 vaccination in initial values of nest-box oriented song rate or oxidative status, nor could we
489 detect any differences at any point of the experiment. Previous studies on other songbird
490 species showed that suppression of antibody response against an inactivated strain of the
491 Newcastle virus or against sheep red blood cells can occur in favour of reproduction
492 (Deerenberg et al. 1997; Nordling et al. 1998). Hence, production of antibodies might have
493 been too costly for unresponsive individuals to be afforded. These unresponsive birds also
494 tended to have a lower undirected song rate than responsive starlings. It might be that the
495 undirected song rate reflected some individual qualities of the birds (e.g., immunogenetic
496 architecture, dominance status). Another explanation might be that unresponsive birds kept
497 their undirected song rate low in order to save resources for the nest-box oriented song rate,
498 as we did not detect any differences in this song trait between responsive and unresponsive
499 individuals. This strategy might have allowed birds to increase their chance of defending a
500 nest-box and attracting a mate, but possibly at the cost of decreasing their social position
501 within the hierarchy of the flock.

502

503 **Conclusions**

504 In conclusion, our specific experimental manipulation of the antibody response caused a
505 moderate reduction of the undirected song rate in a songbird species and had a small effect
506 on the nest-box oriented song rate. The antibody response did not cause any changes in the
507 blood oxidative status, suggesting that other mechanisms might have been responsible for
508 the reduction in the undirected song rate. On the other hand, starlings with a higher nest-
509 box oriented song rate had significantly lower levels of oxidative protein damage. The
510 initial oxidative status did not explain why some starlings skipped the antibody response.
511 Starlings unresponsive to vaccine tended to have a lower rate of undirected song,
512 suggesting that this song trait might reflect some attribute of individual quality.

513 Future challenges include determining the consequences of the antibody response
514 for both the song rate and oxidative stress when males have access to females and under
515 harsher environmental conditions than those of this study.

516

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526

527 **Ethical standards**

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

530

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713 **Table captions**

714 Table 1 Linear mixed models of factors affecting song rate variables and oxidative status parameters of male European starlings.

715 Outcomes of all models were unchanged if non-significant interactions were excluded

716

Dependent variable	Effect	Num DF	Den DF	F	P
Total song rate	Treatment group	1	46.5	1.12	0.2955
	Sampling period	2	98	1.52	0.2236
	Treatment group × Sampling period	2	98	1.85	0.1625
Nest-box oriented song rate	Treatment group	1	49	4.72	0.0346
	Sampling period	2	98	1.31	0.2737
	Treatment group × Sampling period	2	98	1.25	0.2920
Undirected song rate	Treatment group	1	46.2	0.02	0.8815
	Sampling period	2	98	2.55	0.0832
	Treatment group × Sampling period	2	98	4.15	0.0186
Reactive oxygen metabolites	Treatment group	1	49	0.08	0.7788
	Sampling period	2	98	13.55	<0.0001
	Treatment group × Sampling period	2	98	0.83	0.4380
Protein carbonyls	Treatment group	1	46.3	0.03	0.8650
	Sampling period	2	98	9.89	0.0001
	Treatment group × Sampling period	2	98	0.62	0.5408
Total protein carbonyls	Treatment group	1	46.3	0.12	0.7300
	Sampling period	2	98	12.88	<0.0001

	Treatment group × Sampling period	2	98	0.79	0.4546
GSH/GSSG	Treatment group	1	46.2	1.82	0.1842
	Sampling period	2	98	0.76	0.4714
	Treatment group × Sampling period	2	98	1.91	0.1532

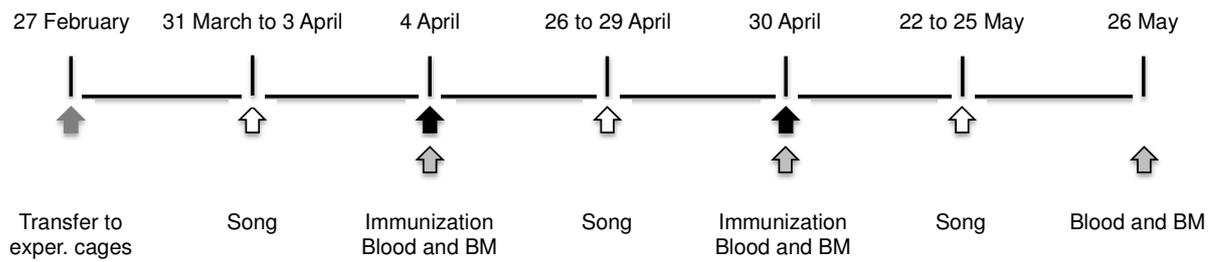
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719 Fig. 1 Timeline of the experiment. Dark grey arrow indicates the removal of birds to the
720 experimental cages; white arrow indicates when the song performance was recorded; black
721 arrow indicates when the immunization was done; light grey arrow indicates the days of
722 collection of blood samples and body mass (BM)

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738 Fig. 2 Beginning (before the first immunization, 4 April), interim (before the second
739 immunization, 30 April) and end (after the second immunization, 26 May) values of song
740 rate variables (expressed as percentage of minutes with song during one hour, averaged for
741 4 consecutive days), and oxidative status parameters levels of starlings in relation to
742 treatment (controls vs. immunized). Results of post-hoc tests are shown when there was a
743 significant effect of sampling period or a significant interaction between treatment group
744 and sampling period. Post-hoc tests for the undirected song rate refer to all possible pair-
745 wise comparisons. Post-hoc tests for reactive oxygen metabolites and for both protein
746 carbonyls variables refer to the effect of sampling period irrespective of treatment group.
747 Means that are not sharing a same superscript (i.e., letters a, b or c) are significantly
748 different from each other (t-test, $P < 0.05$). Note that post-hoc comparisons tend to be more
749 conservative if a more restrictive test (Tukey test) is used. Note that significant results were
750 also obtained using the Tukey test for reactive oxygen metabolites and total protein
751 carbonyls. For protein carbonyls, the differences between beginning and interim samples
752 and between interim and end samples were close to significance with the Tukey test (both
753 P -values=0.07). Values are shown as least square means + SE. GSH=reduced glutathione;
754 GSSG=oxidized glutathione

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