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

Keywords Antibodies • Birds • Immunity • Oxidative damage • Song
Introduction

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

The costs of immune response can also impinge on the expression of secondary sexual traits. Bird song is one renowned behavioural trait that is under sexual selection. Hamilton and Zuk (1982) proposed that bird song may reflect attributes of male quality, such as the capacity to cope with parasites. Hence, females can use the song to discriminate between males in good or bad health status. For example, it has been found that
immunization can reduce song rate in collared flycatchers *Ficedula albicollis* (Garamszegi et al. 2004) or rattle duration in barn swallows *Hirundo rustica* (Dreiss et al. 2008). Duffy and Ball (2002) also found that male starlings (*Sturnus vulgaris*) having a higher song rate or a longer song-bout length (which females prefer, Eens et al. 1991; Gentner and Hulse 2000) exhibited higher cell-mediated and humoral immunity as compared to those with a lower song rate or a shorter song-bout length.

Although song rate can convey some attributes of the individual immunocompetence, an important question then is which mechanisms link song rate to immunocompetence. The immune response certainly demands various kinds of resources, such as nutrients (Klasing 2007) and energy (Cutrera et al. 2010) that are also needed to sustain singing (Chappell et al. 1995; Garamszegi et al. 2006). However, energetic costs of song production are generally small (Oberweger and Goller 2001; Ward et al. 2003, 2004; Zollinger et al. 2011). The immune response can also have more subtle costs. One putative mechanism that has received considerable recent attention is the role of oxidative stress, which occurs when there is an increase in oxidative molecular damage and oxidation of non-protein and protein thiols that regulate the cell oxidative balance (Jones 2006; Halliwell and Gutteridge 2007; Sohal and Orr 2012). Production of reactive oxygen and nitrogen species by immune cells is an important component of the immune response (Sorci and Faivre 2009). The cytotoxic effects of reactive molecules produced by leukocytes are exploited by the organism when coping with pathogens during the innate immune response.

Given the unspecificity in the action of reactive molecules, generation of oxidative damage upon biomolecules can also occur. A core idea of the so-called immuno-oxidative ecology is that oxidative stress may provide a currency to quantify costs resulting from the impact
of immune activation on traits like sexual traits, growth, reproduction or senescence (Costantini 2014). Although many experimental reports have shown that the innate immune response may result in oxidative stress (e.g., Bertrand et al. 2006; Torres and Velando 2007; van de Crommenacker et al. 2010), comparatively little is known about the mechanisms underlying the costs of acquired immunity (Costantini and Møller 2009).

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

Material and Methods

Housing conditions and experimental setup
The European starling is a seasonally breeding songbird in which song during the reproductive season is used as a mating signal (Mountjoy and Lemon 1991; Eens et al. 1991a; Eens 1997; Gentner and Hulse 2000; Gentner et al. 2001). All 60 male starlings used in this study had been captured previously in the Antwerp district and were then housed in large single sex outdoor aviaries on the grounds of Campus Drie Eiken of the University of Antwerp (Wilrijk, Belgium). The duration of the housing in captivity before the start of the experiment varied and was respectively: 4 months (n=18), 2 years (n=22), 3 years (n=9), 7 years (n=8), 8 years (n=2) and 10 years (n=1). On February 27, 2014, the sixty male starlings were moved to four experimental outdoor aviaries (L x W x H: 27.0 x 7.0 x 2.75 m), with 15 birds in each aviary (duration of previous housing in captivity did not differ significantly between aviaries; $F_{3,51}=0.47$, $P = 0.70$). Each aviary had 15 nest-boxes and each nest-box had a perch in front of it. In each cage, we had both control and immunized birds (2 cages with 7 control and 8 immunized birds and 2 cages with 8 control and 7 immunized birds). In total, we had 30 control birds and 30 immunized birds. However, nine individuals were excluded from the analyses because one control individual died before the first immunization, two immunized birds died over the course of the experiment and six control birds had high initial antibody levels against Newcastle disease (see later), possibly because they had been previously exposed to the virus in the wild. Therefore, the final sample size was 23 control and 28 immunized starlings (11, 13, 13 and 14 starlings in each cage, respectively). All starlings were marked with a unique combination of coloured bands and a metal ring, which allowed individual recognition. Food (Orlux, Deinze, Belgium; Nifra Van Camp, Boechout, Belgium) and water were provided ad libitum. All males used in this study were adults (see below).
The experiment was performed according to the timescale illustrated in Fig. 1. The times of blood sampling were chosen in order to have blood samples at peaks of lymphocyte activity (Kapczynski et al. 2013; Scott et al. 2013). A sample of blood (ca. 500µl) was collected by venipuncture using a heparinised microvette (Sarstedt, Nümbrecht, Germany) just before each immunization. Blood samples were maintained cool and were then centrifuged to separate plasma from red blood cells. After centrifugation, the plasma of each sample was pipetted out from the tube and divided in 4 different tubes (in order to have one tube allocated to each assay plus one extra tube), while red blood cells were divided in 2 different tubes (one for the assay plus one extra tube). We did so in order to avoid to defrost repeatedly the same aliquot. Samples were stored at -80°C.

Quantification of song rate

Behavioural observations of song rate were made for four consecutive days by SC and GC at three different periods of the experiment: prior to the first immunization; prior to the second immunization; and prior to the collection of the last blood sample (Fig. 1). During each observation day, the song rate of all the starlings within each aviary was recorded during a session of 60 minutes between 09h00 and 13h00 (when singing activity of starlings during the day is highest; Eens 1997). We alternated the order of the aviaries between subsequent days in order to have a balanced distribution of the timing of observations. All song observations were made (using a binocular) from behind a shelter located ca. 5 m from each aviary, using a one-zero sampling technique (Martin and Bateson 2007) with an interval of one minute. Hence, every minute we scanned all males inside an
aviary and recorded which males were singing. We also scored whether males produced
nest-box oriented song (i.e., song produced from inside the nest-box with the head sticking
out from the entrance hole, on the top of it or on the perch in front of the nest-box) or
undirected song (i.e., song produced away from the nest-box). Given that European
starlings, while singing, adopt a characteristic upright stance, upturned bill, and the throat
feathers and beak can be seen moving (Feare 1984), singing behaviour can be easily
quantified. Furthermore, during the breeding season, male starlings sing the majority of
their song (90 % or more) in long and complex song bouts (uninterrupted singing) that last
more than 30 seconds (Eens et al. 1991b). Nest-box oriented song rate and undirected song
rate were quantified as the percentage of one-minute intervals during which a male was
observed singing nest-box oriented and undirected song, respectively (Pinxten et al. 2002).

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

Previous studies in European starlings showed that the nest-box oriented song is
important in mate choice: males singing more have higher mating success (Eens et al.
1991a; Wright and Cuthill 1992; Eens 1997; Pinxten et al. 2002; Ball et al. 2006). In
contrast, the undirected song is used for flock maintenance (Hausberger et al. 1995) and for
establishing dominance hierarchy (Eens 1997), although its precise function is less clear
than the nest-box oriented song (Kelm-Nelson and Ritters 2013). Song rate quantified using
this protocol is significantly repeatable within individuals across different sessions (Van Hout et al. 2009, 2011).

Immunization

We assessed the response of starlings to immunization with a Newcastle disease virus (NDV) inactivated vaccine (Nobilis Paramyxo P201, MSD Animal Health, Brussels, Belgium). NDV is a globally distributed and highly virulent avian paramyxovirus (Seal et al. 2000; Al-Garib et al. 2003). Previous studies showed that immunization of individual birds with an inactivated vaccine of NDV elicits a significant antibody response, but does not significantly induce an inflammatory response (Al-Garib et al. 2003; Broggi et al. 2013). Doses for the immunized and control birds were chosen according to previous studies (Nordling et al. 1998; Saino et al. 2002). Immunized birds were subcutaneously injected in the breast with 100 µl of vaccine and control birds were injected with 100 µl of phosphate buffered saline. The antigen strain concentration (expressed as hemagglutination inhibition score) was ≥6.8 and ≤10.2 log₂ units. Vaccination was repeated twice in order to stimulate antibody response over a long period of the reproductive time.

Analysis of antibody concentration

The Newcastle Disease Antibody Elisa kit (BioCheck, Reeuwijk, Holland) was used to measure the amount of antibodies to NDV in plasma. It is important to quantify the antibody concentration because antibody response can be suppressed in low quality
individuals or under certain life-history stages (e.g., Deerenberg et al. 1997). Plasma samples were diluted 1:10 with a phosphate buffer with protein stabilisers and sodium azide preservative (0.1% w/v) provided with the kit. Test procedure and calculation of antibody status were done following manufacturer’s instructions. The antibody status is expressed as positive (the bird produced antibodies to NDV) or negative (the bird did not produce antibodies to NDV).

Analyses of blood oxidative status

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

The d-ROMs assay (Reactive Oxygen Metabolites; Diacron International, Grosseto, Italy) was used to measure plasma oxidative damage metabolites (mostly hydroperoxides) that are generated early in the oxidative cascade. The small interference of the enzyme ceruloplasmin that was found in humans (Alberti et al. 2000) did not occur in starling plasma. Inhibition of ceruloplasmin activity with 50 µM or 1 mM of sodium azide (inhibitor of ceruloplasmin activity; Sigma-Aldrich, code 08591) did not cause any decrease in absorbance (paired t-test, \( t_{14} \geq 0.76, P \geq 0.14 \), coefficient of variation (mean±SD) = 4.81±1.34%). Moreover, the reaction of a dilution series of cumene hydroperoxide with the d-ROMs reagents was highly linear (range: 0 to 4.5 µM, \( R^2=0.9996 \); physiological values in vertebrates) at the incubation temperature of 37 ºC, required by the manufacturer’s instructions. Incubation at lower temperatures (4 and 24ºC)
reduced the efficiency of the Fenton reaction (i.e., chemical reaction of the d-ROMs assay), as testified by a strong and similar reduction in absorbance of both plasma samples and cumene hydroperoxide (data not shown). Analyses of reactive oxygen metabolites were therefore done according to manufacturer’s instructions as in previous studies. Quality controls (Diacron International) were also assessed in each assay. Values of reactive oxygen metabolites have been expressed as mM of H$_2$O$_2$ equivalents. Analyses were run in duplicate; the intra- and inter-assay coefficients of variation were 4.81 and 5.32%, respectively.

The Protein Carbonyl Colorimetric assay (Cayman Chemical Company, Ann Arbor, MI, USA) was used to measure the plasma concentration of protein carbonyls. The assay is based on the protocol of Levine et al. (1990). Protein carbonyls indicate oxidative damage to proteins caused by free radicals or lipid peroxidation products (malondialdehyde and hydroxynonenal; Halliwell and Gutteridge 2007). All plasma samples were first diluted with distilled water in order to have a concentration of 2 mg proteins ml$^{-1}$, as measured using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using albumin as a reference standard. Nucleic acids were removed by adding 1 volume of a 10% solution of streptomycin sulphonate (Sigma-Aldrich, code S6501) to 9 volumes of sample. Then, analyses were done according to the protocol of Levine et al. (1990). A control plasma was also assessed in each assay. The concentration of protein carbonyls has been expressed as nmol mg$^{-1}$ proteins or as total amount by multiplying the concentration per mg of proteins for the total concentration of proteins in the plasma. Analyses were run in duplicate; the intra- and inter-assay coefficients of variation were 6.44 and 8.68%, respectively.
High-performance liquid chromatography with electrochemical detection was applied for simultaneous determination of reduced (GSH) and oxidized (GSSG) glutathione in red blood cells by a Reversed-Phase HPLC of Shimadzu (Hai Zhonglu, Shanghai). We applied the protocol as described in Sinha et al. (2014). Concentrations of GSH and GSSG were expressed as µmol g⁻¹ fresh weight of red blood cells. We calculated the GSH/GSSG ratio that was used as an index of redox state (higher values indicate lower oxidative stress; Jones 2006).

Statistical analyses

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

To test whether song rate variables (nest-box oriented or undirected) were associated with blood oxidative status in both controls and immunized birds, linear mixed
models with a repeated measures design (as described above) were performed by adding reactive oxygen metabolites, protein carbonyls (or total protein carbonyls) and GSH/GSSG altogether as covariates. Outcomes of all models were unchanged if each oxidative status biomarker was included alone. Preliminary analyses also showed that the interaction between treatment group and covariate was never significant for each oxidative status biomarker.

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

Outcomes of all models described above were unchanged if individual age, duration of housing in captivity prior to the experiment, body mass, tarsus length, blood sampling order or ownership of a nest-box were included as covariates (data not shown). For each model, post-hoc comparisons were performed using both the t-test and the Tukey test when we found a statistically significant interaction. We opted to run both tests in order to have a comparison between a less (t-test) and a more (Tukey test) conservative approach.
Linear mixed models with cage as a random factor showed that at the time of the first immunization (i.e., 4 April) controls and immunized birds did not differ in age (mean±SE: controls, 3.3±0.4 years; immunized, 3.4±0.5 years; $P = 0.94$), in body mass (mean±SE: controls, 93.2±2.0 grams; immunized, 92.5±1.6 grams; $P = 0.76$) nor in tarsus length (mean±SE: controls, 29.6±0.1 mm; immunized, 29.9±0.1 mm; $P = 0.15$).

**Results**

Antibody response

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

Control versus immunized birds

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

The total song rate and both the nest-box oriented song rate and the undirected song rate did not differ significantly between control and immunized birds at the time of the first immunization (linear mixed model with cage as random factor, $P \geq 0.08$). Both the total song rate and the nest-box oriented song rate did not show any significant changes in both
groups over the experimental period (Fig. 2, Table 1). Overall, immunized birds had a lower nest-box oriented song rate than control birds (estimate±SE: 18.12±8.34, \(P = 0.0346\); Fig. 2, Table 1). There was a significant interaction between treatment group and sampling period for the undirected song rate (Fig. 2, Table 1). The undirected song rate increased in control birds during the first part of the experiment (beginning vs. interim sample, \(P = 0.024\)) and decreased during the second part of the experiment (interim vs. end sample, \(P = 0.016\)). In contrast, the undirected song rate of immunized birds did not vary during the first and second part (beginning vs. interim sample, \(P = 0.083\); interim vs. end sample, \(P = 0.61\)), but song rate recorded before the first immunization was significantly higher than that recorded after the second immunization (beginning vs. end sample, \(P = 0.026\)). A separate LMM with nest-box oriented song rate as dependent variable and undirected song rate as a covariate showed that these two types of song were positively correlated (coefficient estimate±SE: 11.1±2.1, \(P < 0.001\)).

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

Irrespective of treatment group, starlings with a higher nest-box oriented song rate had significantly lower levels of total protein carbonyls (coefficient estimate±SE: -490.7±223.6, \(P = 0.029\)) and marginally significantly lower levels of protein carbonyls...
expressed per mg of proteins (coefficient estimate±SE: -675.3±353.7, \( P = 0.058 \)).

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

Responsive versus unresponsive birds

At the time of the first immunization, responsive and unresponsive birds did not differ in any of the variables considered in the present experiment (\( P \geq 0.12 \)). Although immunization resulted in a decrease in undirected song rate, responsive starlings had overall a higher undirected song rate than unresponsive starlings during the whole experiment (least square mean±SE: responsive, 10.7±1.4; unresponsive, 6.0±1.7; \( P = 0.049 \)). The interaction between group and sampling period was never significant (\( P \geq 0.19 \)).

Discussion

Song rate

Although reduced song rate and increased oxidative stress have been associated with a number of immune responses, our study provides support only for a moderate effect of antibody response against Newcastle virus on one particular mode of singing (undirected song rate). We also found that immunized birds had a lower nest-box oriented song rate than control birds; however, the interaction between treatment and sampling period was not significant. A visual examination of Fig. 2 suggests that the nest-box oriented song rate decreased in immunized birds as compared to control birds. However, the lack of difference between groups at the end of the experiment might have reduced the power of our model to
detect an effect of immunization. Overall, these results suggest that the effect of
immunization on the nest-box oriented song rate was small.

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

Blood oxidative status

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

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

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

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

Seasonal variation in oxidative status

We found that the blood oxidative damage decreased during the season, independently
from the treatment. Previous studies also found seasonal variation in individual oxidative
status, for example, in relation to the breeding stage (e.g., incubation vs. nestling rearing,
Casagrande et al. 2011) or the individual hormonal status (Alonso-Alvarez et al. 2007;
Casagrande et al. 2012a). One explanation for our results might lie with the
photorefractoriness, a status characterised by a decline in the production of the luteinizing
hormone (which promotes synthesis of sexual steroids), which occurs between the end of
April and the start of May in starlings (Dawson and Sharp 2010). Since sexual steroids can influence the blood oxidative status (e.g., increased basal production of damage; Alvarez et al. 2007; Casagrande et al. 2012a), a seasonal decline in their synthesis might explain, at least partly, the decline in oxidative damage.

Skipping the antibody response

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

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

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

Acknowledgments

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Ethical standards

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

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circulating antioxidants (albumin and cholesterol) and a decrease in oxidative damage.


Costantini D (2014) Oxidative stress and hormesis in evolutionary ecology and physiology. Springer-Verlag, Berlin


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Table captions
Table 1 Linear mixed models of factors affecting song rate variables and oxidative status parameters of male European starlings.
Outcomes of all models were unchanged if non-significant interactions were excluded

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Fig. 1 Timeline of the experiment. Dark grey arrow indicates the removal of birds to the experimental cages; white arrow indicates when the song performance was recorded; black arrow indicates when the immunization was done; light grey arrow indicates the days of collection of blood samples and body mass (BM)
Fig. 2 Beginning (before the first immunization, 4 April), interim (before the second immunization, 30 April) and end (after the second immunization, 26 May) values of song rate variables (expressed as percentage of minutes with song during one hour, averaged for 4 consecutive days), and oxidative status parameters levels of starlings in relation to treatment (controls vs. immunized). Results of post-hoc tests are shown when there was a significant effect of sampling period or a significant interaction between treatment group and sampling period. Post-hoc tests for the undirected song rate refer to all possible pairwise comparisons. Post-hoc tests for reactive oxygen metabolites and for both protein carbonyls variables refer to the effect of sampling period irrespective of treatment group. Means that are not sharing a same superscript (i.e., letters a, b or c) are significantly different from each other (t-test, $P<0.05$). Note that post-hoc comparisons tend to be more conservative if a more restrictive test (Tukey test) is used. Note that significant results were also obtained using the Tukey test for reactive oxygen metabolites and total protein carbonyls. For protein carbonyls, the differences between beginning and interim samples and between interim and end samples were close to significance with the Tukey test (both $P$-values=0.07). Values are shown as least square means + SE. GSH=reduced glutathione; GSSG=oxidized glutathione