Resveratrol supplementation reduces oxidative stress and modulates the immune response in free-living animals during a viral infection

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Manrico Sebastiano[1*], Marcel Eens[1], Simone Messina[1], Hamada AbdElgawad[2], Kévin Pineau[3], Gerrit T.S. Beemster[2], Olivier Chastel[4], David Costantini[1,5,6]

1. Behavioural Ecology and Ecophysiology group, Department of Biology, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium

2. Integrated Molecular Plant Physiology Research (IMPRES), Department of Biology, University of Antwerp, Groenenborgerlaan 171, 2020 Antwerp, Belgium

3. Groupe d'Etude et de Protection des Oiseaux en Guyane (GEPOG), 15 Avenue Pasteur 97300 Cayenne, French Guiana

4. Centre d'Etudes Biologiques de Chizé (CEBC), UMR7372- CNRS/University of La Rochelle, F-79360, France

5. UMR 7221 CNRS/MNHN, Muséum National d'Histoire Naturelle, Sorbonne Universités, 7 rue Cuvier 75005 Paris, France

6. Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Graham Kerr Building, Glasgow G12 8QQ, UK

*** corresponding author.** E-mail address: Manrico.Sebastiano@uantwerpen.be
phone number: +32484566385

Abstract

1. Diet quality may have an important effect on the regulation of oxidative status and the immune system during an infectious disease. However, the relationship among intake of specific dietary molecules, an individual's oxidative status and the occurrence and progress of a viral disease remains almost unexplored in free-living organisms.

2. Here, we study a wild, long-lived animal, the Magnificent frigatebird *Fregata magnificens* to investigate: *i)* the differences in a number of physiological traits (biomarkers of blood oxidative status, corticosterone (CORT), immunity, and inflammation) between sick and healthy nestlings; and *ii)* whether experimentally increased intake of resveratrol (a polyphenol with antioxidant and antiviral properties) affects these physiological markers during the progress of a severe viral disease.

3. Birds with visible clinical signs showed higher oxidative damage, hemolysis and hemagglutination scores, and lower antioxidant defenses in comparison to birds without clinical signs. At the end of the experiment, supplemented birds showed: *i)* increased plasma haptoglobin levels and circulating antioxidant defenses; *ii)* reduced generation of lipid oxidative damage; and *iii)* negligible to no influence on immune markers, baseline CORT levels, and activity of antioxidant enzymes.

4. Our work illustrates how the availability of specific organic molecules in the diet may constrain the individuals' capacity to cope with viral infections in free-living animals.

Keywords: antioxidant defenses of birds; avian glucocorticoid; avian infectious diseases; Frigatebird; immune response; oxidative stress; stress hormones; wild animals

Introduction

Infectious agents are an important selective force, potentially reducing survival and reproduction of the host and, eventually, resulting in population declines (Smith, Sax & Lafferty 2006; Preece *et al.* 2017). A well-documented infectious disease is represented by the West Nile virus (WNV), a virus that is mostly found in birds because they are suspected to be the most important amplifying hosts**.** WNV has been found in 63 bird species one year after its first appearance in 1999 (Kramer & Bernard 2001), and has caused massive mortality in many corvid species (McLean 2006). Similarly, avian influenza virus (AIV) has caused worldwide severe outbreaks in poultry, wild birds, and humans (Chatziprodromidou *et al.* 2018).

The association between diseases and immune defenses (Savage *et al.* 2016) or the selection of immune traits during outbreaks (Legagneux *et al.* 2014) have been of great interest in evolutionary ecology and physiology, yet the patho-physiological mechanisms underlying the impact of infectious diseases on wildlife remain poorly documented. An approach that simultaneously quantifies physiological stress and immune status would be ideal to determine how an organism is affected by a given pathogen (Hawley & Altizer 2011). For instance, estimating stress hormone corticosterone (CORT) levels during infection may be relevant because exposure to stressors stimulates CORT release (Sapolsky, Romero & Munck 2000). Although CORT coordinates the stress-response (Sapolsky, Romero & Munck 2000), high levels and/or chronic release of CORT are known to suppress the immune response (Bourgeon & Raclot 2006; Gao, Sanchez & Deviche 2017) and to increase the impact of virus infection in birds (Owen *et al.* 2012).

Similarly, oxidative stress is known to limit immune function in birds (Catoni, Schaefer & Peters 2008). Thus, oxidative stress may contribute to the spread of infectious diseases (Keles *et al.* 2010; van de Crommenacker *et al.* 2012). For instance, Marek's disease increases damage to DNA, lipids, and proteins in chickens (Keles *et al.* 2010), suggesting that oxidative stress might partially explain an

organism's vulnerability to viral diseases (Li, Feng & Sun 2011), and viral replication (Li, Feng & Sun 2011; Costantini *et al.* 2018b). Measuring both antioxidant defenses and oxidative damage, which respectively reflect the ability to mount a protective response to an adverse condition and the deleterious effects the animals undergo (Beaulieu & Costantini 2014), might therefore prove valuable to infer the individual's capacity to cope with a pathogen.

Importantly, dietary antioxidants might impact the host's capacity to cope with an infectious disease because antioxidants reduce immunopathology associated with the immune/inflammatory response (Dhinaut *et al.* 2017); they occur in limited supply for free-ranging animals, potentially constraining their capacity to cope with oxidative stress (Catoni, Schaefer & Peters 2008; Costantini *et al.* 2018a); and may limit steroid synthesis (Ozdemir *et al.* 2011). Accordingly, antioxidants can inhibit the replication of several viruses (i.e. feline immunodeficiency virus Mortola *et al.* 1998; influenza virus Han *et al.* 2000; duck enteritis virus Xu *et al.* 2013; herpes simplex virus Civitelli *et al.* 2014), whereas dietary antioxidants exhibit antiviral effects that are apparently not directly connected to their antioxidant properties (e.g., Abba et al. 2015). However, to the best of our knowledge, experimental supplementation of molecules with antioxidant and antiviral properties to study the impact on oxidative status, CORT levels, and immunity during a viral infection has never been carried out in free-living animals.

Here, we studied nestlings of Magnificent frigatebird (*Fregata magnificens*) coping with a severe viral disease. We investigated whether birds with visible clinical signs (i.e. skin crusts) showed an alteration of their physiological traits (blood oxidative balance, immune status, CORT levels, and inflammation) in comparison with birds without clinical signs. We then tested whether supplementation of resveratrol, a polyphenol with both antioxidant and antiviral activity (Abba *et al.* 2015), improves the physiological traits, short-term progress of the disease and survival perspectives of supplemented birds. Our experiment was carried out on a protected island in French Guiana, where outbreaks of viral

infections occur yearly, causing 85 to 95% nestling mortality (field observations). Bacterial cultures, viral screening and microscopic evaluation of skin samples excluded the presence of ectoparasites, avian poxvirus (de Thoisy *et al.* 2009), and avian influenza (unpublished results), but detected herpesvirus DNA in body crusts (de Thoisy *et al.* 2009). Recent work found up to 10 million copies of herpesviral DNA in nestlings with clinical signs of the disease, suggesting that herpesvirus replication is involved in the appearance of clinical signs (Sebastiano *et al.* 2017b). This population offers an unprecedented opportunity to investigate the potential connection between viral disease progression and diet quality employing an experimental approach, because clinical signs are associated with several physiological biomarkers of oxidative stress and inflammation (Sebastiano *et al.* 2017b; Sebastiano *et al.* 2017c), and we can experimentally feed frigatebirds in the wild (Dearborn 2001)

Material and methods

Sample collection

The fieldwork was carried out in 2016 on Grand Connétable Island, a protected area off the Northern Atlantic coast of South America (French Guiana, 4°49'30N; 51°56'00W), which hosts approximately 1,300 reproductive pairs of frigatebirds (GEPOG field observations). Most frigatebird pairs in this colony start breeding between the end of November and the beginning of December. Consequently, all nestlings were approximately of the same age (~4 months old) when captured (see also statistical analysis section). A total of 26 nestlings without visible clinical signs and 34 sick nestlings showing visible clinical signs were randomly chosen at different sites of the island (Figure 1). Visible clinical signs of the disease include crusts on the head and the body, hyperkeratosis on eyes and the consequent thickening of the cornea (de Thoisy *et al.* 2009). Our field observations suggest that the disease does not spread among neighbouring nestlings. All 60 nestlings were captured at the nest by hand on June 7th (D1). Within 3 minutes after capture, two mL of blood were collected from the brachial vein using a heparinized

118 syringe and a 25G needle. Immediately afterwards, each bird was ringed with an aluminum ring for
119 individual recognition and the beak-head distance was measured to control for the age of nestlings. This
120 first sample of blood was used to test any pre-treatment difference among groups.
121 The experiment started on D2. We administered pills of *trans*-Resveratrol (see supplementary
122 material) to the experimental groups (12 out of 26 healthy nestlings and 18 out of 34 sick nestlings,
123 respectively), while the remaining individuals (14 healthy and 16 sick nestlings, respectively; i.e., control
124 groups) were administered an empty pill as a placebo to account for the effect of handling. We chose
125 resveratrol because of its strong antiviral activity against herpesvirus (Abba *et al.* 2015; Sebastiano *et al.*
126 2016b), which actively replicates in sick nestling frigatebirds (Sebastiano *et al.* 2017c). Pills were dipped
127 in fish oil (Crafty catcher, Ipswich, UK) to facilitate swallowing. The administration of pills was carried out
128 nine times (D2-4, 8^{th}–10^{th} of June; D12-14, 18^{th}–20^{th} of June; D21-23, 27^{th}–29^{th} of June). On D24 the
129 experiment ended and a second sample of two mL of blood was taken from the same individuals. Blood
130 samples were kept cold while in the field and centrifuged within less than two hours to separate plasma
131 (used for CORT, oxidative stress biomarkers, inflammatory and immune markers) and red blood cells.
132 Both samples of plasma and red blood cells were then kept in dry ice until the end of the fieldwork and,
133 upon arrival in the laboratory, were kept in a -80°C freezer. Two pictures of each bird were taken from
134 the same distance and same position at the start and the end of the experiment (pre- and post-
135 treatment), to score clinical signs of the disease and assess if visible clinical signs increased or decreased
136 during the experiment (see the specific section below).
137
138 **Molecular analyses**
139 All analyses were performed using established protocols for vertebrates. The determination of non-
140 enzymatic antioxidants was performed using reduced (GSH) and oxidized (GSSG) glutathione in red
141 blood cells. High performance liquid chromatography (HPLC) with electrochemical detection (Reversed-

142 Phase HPLC of Shimadzu, Hai Zhonglu, Shanghai) was applied following Sinha *et al.* (2014) and
143 concentrations were expressed as µmol/g of fresh weight. We also calculated the GSH/GSSG ratio as a
144 metric of oxidative balance. Furthermore, the non-enzymatic antioxidant power of erythrocytes (TAC, an
145 index of circulating non-enzymatic antioxidants) was estimated following Benzie and Strain (1996) and
146 expressed as µmol Trolox/g of fresh weight. The enzymatic antioxidant capacity was measured using
147 three different biomarkers in red blood cells. Superoxide dismutase (SOD) activity was estimated by
148 measuring the inhibition of nitroblue tetrazolium reduction at 560 nm and was expressed as U/mg
149 protein per minute. Catalase activity (CAT) was measured by monitoring the rate of decomposition of
150 hydrogen peroxide (H_2O_2) at 240 nm and was expressed as µmol H_2O_2/mg protein per minute.
151 Glutathione peroxidase (GPX) activity was determined by measuring the decrease in NADPH absorbance
152 at 340 nm and was expressed as µmol NADPH/mg protein per minute. Damage to biomolecules was
153 assessed by quantifying the plasma level of Thiobarbituric Acid Reactive Substances (TBARS), which
154 reflect lipid peroxidation, and the level of protein carbonyls in red blood cells as a measure of oxidative
155 damage to proteins. Results are expressed, respectively, as nmol of Malondialdeyde (MDA)
156 equivalents/mL (Hodges *et al.* 1999) and nmol/mg protein (Levine *et al.* 1994).
157 Plasma haptoglobin concentrations were quantified using a commercially available assay (PHASE
158 Haptoglobin assay; Tridelta Development Ltd), and concentrations were expressed as mg/mL. The
159 plasma concentration of nitric oxide (NO) was estimated from the concentration of the stable end
160 products of nitric oxide oxidation (i.e. nitrate and nitrite), and expressed in µmol/L. Innate humoral
161 immunity was determined by the hemolysis-hemagglutination assay as described in Matson, Ricklefs
162 and Klasing (2005). Finally, the plasma concentration of corticosterone was measured by
163 radioimmunoassay following Lormée *et al.* (2003), and expressed as ng/mL.
164 Detailed protocols are provided in the supplementary material.
165

Bird classification based on clinical signs

To classify the sampled birds based on the severity of visible clinical signs, pictures were analyzed and blindly scored twice by the same person (one week apart). Scores ranged from 0 (absolute absence of clinical signs) to 10 (bird fully covered by crusts), including half scores. Reproducibility between the two scores (calculated from variance components derived from a one-way analysis of variance, accordingly to Lessells & Boag 1987) of each individual was significantly high both before (r=0.96, F=49.67, P<0.0001) and after (r=0.93, F=31.64, P<0.0001) the treatment. An average score was, therefore, calculated and used to further divide nestlings into three study groups to be used for statistical comparisons: healthy group (hereafter "*no signs*", average score < 1), nestlings with few crusts on the neck and around the eyes (hereafter "*mild*", average score ≥ 1 or < 4), and very sick nestlings showing more widespread and thicker crusts (hereafter "*severe*", average score ≥ 4; for reference see Figure 1). This classification enabled us to detect birds that changed group over the progress of the disease (which matches a change in the visible clinical signs), thus to identify: *i)* birds that never showed the appearance of clinical signs, hereafter "*always healthy*"; *ii)* birds that showed the appearance of clinical signs, hereafter "*new sick*"; *iii)* birds that had an improvement of visible clinical signs, hereafter "*better condition*"; *iv)* birds that did not change their status, hereafter "*same severity*". Finally, none of the sick birds showed an increased severity of clinical signs, probably because the worsening of an already critical condition coincided with the death of the bird (hereafter called "*did not survive*").

Statistical analyses

Two general linear models were used to analyse pre-treatment group differences: *i)* among birds classified on the severity of clinical signs (no signs, mild, and severe); and *ii)* among groups based on the progress of the disease (always healthy, new sick, better condition, same severity, and did not survive*)*. A third linear model (which included all 34 sick nestling as independent observations, thus not divided in

groups) was used to investigate whether the scores assigned to the severity of clinical signs of each nestling (included as a continuous variable) were associated with the analyzed biomarkers.

Two linear mixed models with a repeated measures design were used to assess the effects of the treatment: *i)* between the two groups based on the presence or absence of visible clinical signs of the disease at the first sampling period (MODEL 1); and *ii)* among groups based on the progress of the disease (4 groups: "*always healthy*", "*new sick*", "*better condition*", and "*same severity*"; MODEL 2). Mixed models only included nestlings for which we had two measurements (pre- and post-treatment), implying that nestlings that did not survive or that were not found at the second sampling period were not included. Since preliminary analysis showed that the head-beak distance (used as a proxy of the age) was similar between healthy and sick (t-test; $t = -1.85$; $p = 0.07$) and between supplemented and un-supplemented nestlings (t-test; $t = 1.85$; $p = 0.07$), this variable was not further included in statistical analyses. The reduced model was obtained by sequentially removing non-significant interactions from the full model starting from the three-way interaction. Post-hoc Tukey tests were used to explore further significant interactions.

Finally, a generalized linear model using a binomial error variance and a logit link function was used to assess whether the treatment influenced the probability of the occurrence of clinical signs, of a decrease in visible clinical signs of the disease, or death. Detailed information on data transformation and setup of the linear mixed models can be found in the supplementary material.

All analyses were performed using R (v. 3.3.1, R Core Team 2013).

Results

Bird classification based on clinical signs

Before the experiment, we had a total of 26 birds without clinical signs and 34 sick birds (13 mild and 21 severely affected). Over the course of the experiment, out of the 26 birds classified as healthy

214 before the experiment, we had a total of 11 birds (4 supplemented) that never showed the appearance
215 of clinical signs, hereafter "*always healthy*"; 11 birds (4 supplemented) that showed the appearance of
216 clinical signs, hereafter "*new sick*". Out of the 34 birds classified as sick before the experiment, 8 birds (4
217 supplemented) had an improvement of visible clinical signs, hereafter "*better condition*"; 18 birds (9
218 supplemented) did not change their status, hereafter "*same severity*"; 7 birds (4 supplemented) died,
219 hereafter called "*did not survive*". Four healthy and one sick bird (all supplemented) were not found at
220 the end of the experiment.
221
222 *Pre-treatment basal differences among groups*
223 Birds with *no clinical signs* showed *i)* significantly lower reduced glutathione, oxidized
224 glutathione, and oxidative damage to lipids than birds with *severe* ($t>2.76$, $P<0.02$; Figure 2a,b,c) clinical
225 signs, and *ii)* significantly lower reduced glutathione and oxidative damage to lipids than birds with *mild*
226 ($t>2.59$, $P<0.03$, Figure 2a,c) clinical signs. Oxidative damage to lipids was also significantly higher in
227 birds with *severe* clinical signs than in birds with *mild* clinical signs ($t=2.58$, $P=0.03$, Figure 2c). *New sick*
228 birds had a tendency to have pre-treatment lower oxidized glutathione than birds that were in a *better*
229 *condition* at the end of the experiment ($t=2.80$, $P=0.052$; Figure 3a). Finally, pre-treatment oxidative
230 damage to lipids was significantly lower both in birds that were *always healthy* and in *new sick* birds
231 than in birds that *i)* that were in a *better condition* at the end of the experiment, *ii)* showed the *same*
232 *severity* of clinical signs at the end of the experiment, or *iii) did not survive* ($t>2.90$, $P<0.04$; Figure 3b).
233 Among markers of immunity, the inflammation protein haptoglobin was significantly higher in
234 birds with *severe* clinical signs than birds with *mild* clinical signs ($t=3.75$, $P<0.01$, Figure 2d) or with *no*
235 *clinical signs* ($t=-3.93$, $P<0.01$, Figure 2d). Furthermore, higher haptoglobin levels were found in birds
236 that *did not survive* ($t>2.95$, $P<0.04$, Figure 3c). Birds with *no clinical signs* showed *i)* significantly lower
237 hemagglutination than birds with *mild* ($z=-3.88$, $P<0.01$, Figure 2e) and *severe* ($z=-3.19$, $P<0.01$; Figure

2e) clinical signs, and *ii)* lower hemolysis than birds with *severe* clinical signs (z=-2.72, P=0.02; Figure 2f). Hemagglutination was also higher in birds that *did not survive* and in birds with the *same severity* of clinical signs than *new sick* birds (z>3.49, P<0.01; Figure 3d), or birds that were *always healthy* (z>2.98, P<0.02; Figure 3d) over the course of the experiment.

None of the other biomarkers of oxidative status, immunity, and basal plasma CORT showed a significant association with the disease status (Table 1). Finally, none of the biomarkers showed a significant relationship when clinical signs were used as a continuous variable, with the exception of haptoglobin (i.e. haptoglobin increases with increasing clinical signs; F=30.47, P<0.01; Figure S1).

Effect of resveratrol administration

We then examined whether administration of resveratrol would affect the oxidative state of the birds. Circulating non-enzymatic antioxidants increased in supplemented birds with *no clinical signs* (t=-3.94, P<0.01; Figure 4, Table S1). Specifically, this increase of non-enzymatic antioxidants only occurred in supplemented birds that were *always healthy* during the experiment (t=-3.70, P=0.03; Figure 5). Oxidative damage to lipids did not change in supplemented birds (Figure 4), while it strongly increased in un-supplemented sick birds (t=-6.19, P<0.01; Figure 4, Table S1), indicating that resveratrol prevented increased production of oxidative damage. This increase in oxidative damage to lipids occurred mostly in birds that showed the *same severity* of clinical signs during the experiment (t=-4.91, P<0.01; Figure 5, Table S2).

Among immune markers, the plasma concentration of haptoglobin increased in supplemented *new sick* birds (birds that showed the appearance of clinical signs at the end of the experiment, t=-3.86, P=0.03; Figure 5, Table S2). The concentration of nitric oxide did not change in supplemented birds, while it significantly decreased in un-supplemented birds (t=2.96, P<0.01, Table S1, S2). Other biomarkers of immunity and stress were not significantly influenced by the treatment considering both

262 birds divided based on the presence/absence of clinical signs (MODEL 1) and birds divided on the
263 progress of the disease (MODEL 2; Table S1 and S2).
264
265 *Effects of resveratrol administration on the progress of the disease*
266 When we compared supplemented and un-supplemented birds, no significant difference in the progress
267 of the disease was detected. Resveratrol supplementation did not influence the probability of
268 developing clinical signs (z=0, P=1; exact same number of individuals in each group), nor did it influence
269 the probability of mortality and/or reduction of visible clinical signs (z=0.19, P=0.85).
270
271 **Discussion**
272 Our study is the first to measure the effect of resveratrol administration on oxidative status,
273 inflammation, immunity, and CORT levels in a wild vertebrate facing a severe virus outbreak. Before the
274 start of the experiment, there were significant differences in the oxidative and immune statuses
275 between sick and healthy birds in our study population. There was also a strong increase of lipid
276 oxidative damage during the progress of the disease. The experimental part of our study demonstrated
277 that resveratrol supplementation increased antioxidant defenses and limited the generation of lipid
278 oxidative damage during the progress of the disease.
279 The analysis of several biomarkers prior to the antioxidant treatment enabled us to discover
280 that a viral disease can affect diverse functional pathways. Viral diseases are known to affect the
281 oxidative status (Keles *et al.* 2010; Durgut *et al.* 2013) and immunity (Staley & Bonneaud 2015) of
282 exposed animals. Accordingly, we found that nestlings with visible clinical signs showed a pronounced
283 alteration of their immune status and cellular oxidative balance in comparison with birds without clinical
284 signs. Contrary to our expectation, baseline CORT levels did not differ among the different groups. CORT
285 also did not increase during the progress of the disease, which is in agreement with our previous results

(Sebastiano *et al.* 2017c). By dividing birds according to the severity of clinical signs and the progress of the disease over the course of the experiment, instead, we found novel findings. Birds without clinical signs showed lower hemolysis and hemagglutination scores, results that that had not previously emerged (Sebastiano *et al.* 2017c).

Supplementation with resveratrol did not affect hemolysis and hemagglutination scores, which might suggest that these particular aspects of the immune system play a minor role in coping with a viral infection in frigatebirds (Sebastiano *et al.* 2017c). In contrast, nestlings supplemented with resveratrol showed an elevated production of nitric oxide during the progress of the disease, while nitric oxide production drastically decreased in un-supplemented birds. Previous studies have found that cells from knockout mice lacking nitric oxide production showed a lower antimicrobial activity during *Salmonella* infection (Vazquez-Torres *et al.* 2000), indicating that nitric oxide is important to defeat pathogens. Previous studies have shown that dietary antioxidants can act as immunostimulants in birds (Hooda *et al.* 2005; Catoni, Schaefer & Peters 2008). Thus, the stimulating effect of resveratrol on the production of nitric oxide might underlie its antiviral activity because nitric oxide controls the function of natural killer cells and cytokines that are essential to combat pathogens (Bogdan, Rollinghoff & Diefenbach 2000). Although our results support a potential role of resveratrol in promoting nitric oxide production in a wild bird (Gülçin 2010), it is unclear why there was a decrease in nitric oxide in un-supplemented birds that never showed the occurrence of clinical signs. Similarly to nitric oxide, synthesis of haptoglobin increases during infections to protect from oxidative damage (MacKellar & Vigerust 2016). In nestlings supplemented with resveratrol, haptoglobin was up-regulated during the early stage of the infection. In contrast, in those nestlings that were not given resveratrol, haptoglobin was higher in birds at an advanced stage of the disease. This is in agreement with previous results (Asasi *et al.* 2013; Sebastiano *et al.* 2017c), and suggests that haptoglobin production is up-regulated at an advanced stage of the disease, but resveratrol stimulated the production of haptoglobin at an earlier phase of the

disease. The consequences of this change in haptoglobin production for individual survival are unclear and need further investigation.

Supplementation of organic molecules with antioxidant properties (e.g., vitamins, polyphenols) may reduce oxidative stress (Costantini 2014). However, an increased intake of these compounds may also interfere with the endogenous antioxidant systems, leading to a decrease of enzymatic antioxidant activity (Wang, Chien & Pan 2006) and expression of antioxidant genes (Selman *et al.* 2006). In contrast, we found an increase of non-enzymatic antioxidant capacity in erythrocytes, which occurred in birds that never showed the occurrence of clinical signs, suggesting that resveratrol had antioxidant effects. To date, the high antioxidant activity of resveratrol has been linked with its capacity to induce glutathione synthesis (Kode *et al.* 2008; Bellaver *et al.* 2014), but our results do not show such relationship, implying that this mechanism needs further assessment.

Resveratrol is also known to have a very strong inhibition power against lipid peroxidation (Gülçin 2010), and its administration prevented an increase in oxidative damage to lipids. This result might have fundamental implications for the progress of the disease because: *i)* high levels of oxidative damage are associated with reduced short-term survival probability (Sebastiano *et al.* 2017b); *ii)* birds that were naturally recovering from clinical signs did not show an increase in oxidative damage; and *iii)* a cell condition of oxidative stress appears to facilitate virus replication, while cells that are able to up-regulate antioxidant defenses and limit damage can survive viral infections (Qiang *et al.* 2006). This result, however, does not enable us to assess whether the protection from oxidative damage was due to the antioxidant or the antiviral activity of resveratrol, and further studies are warranted to clarify this mechanism.

Increased production of CORT is another mechanism activated in animals facing an infection because it induces a number of physiological changes (e.g. mobilization of stored energy, stimulation of immune function) that sustain the body function and maintain cellular homeostasis (Sapolsky, Romero &

Munck 2000). However, our results provide no support for this relationship between virus-induced stress and CORT release, but the high variation in CORT levels among individuals could arise from exposure to strong environmental stressors (Martinet & Blanchard 2009; Sebastiano *et al.* 2016a; Sebastiano *et al.* 2017a), and clearly deserves further investigations.

The antiviral and antioxidant effects of resveratrol might also result in life extension as previously suggested by a meta-analytic study (Hector, Lagisz & Nakagawa 2012). However, we could not detect the effect of resveratrol supplementation on the survival probability of birds. This might have been due to the relatively short treatment period compared to the rapid progress of the disease. A longer-term treatment would prove useful to understand whether resveratrol increases survival probabilities of sick birds and whether it can prevent the occurrence of clinical signs. This might have fundamental implications for the long-term viability of this population, which is now at risk due to the massive mortality events of nestlings. In long-lived species with low fecundity, as the Magnificent frigatebird, even a small rate of nestling mortality can indeed have important negative demographic effects (Finkelstein *et al.* 2010).

Conclusions

We have provided experimental support to the hypothesis that dietary compounds may constrain the capacity of organisms to cope with a viral disease. Our work shows that the effects of these dietary organic molecules may come through both antioxidant protection and antiviral properties. It will be important to expand our study to other species and environmental conditions to further assess the conditions under which the quality of diet may affect the capability of animals to cope with a viral disease. Although our work focused on the effects of a polyphenol (i.e., resveratrol), naturally-occurring diets may be rich in many other compounds that show similar properties (e.g., carotenoids, vitamins) to

those of polyphenols, but whose effects on viral diseases in free-ranging animals have not been explored so far.

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Authors' contributions

D.C., O.C., M.S., designed the study. M.E., D.C., O.C., G.B., and K.P. coordinated different phases of the study. M.S., S.M., and K.P. collected the samples. M.S., S.M., and H.A. performed laboratory analyses. M.S. analysed the data and wrote the manuscript with the contribution of all authors.

Data accessibility

Data are deposited in the Dryad Digital Repository doi:10.5061/dryad.r75251m (Sebastiano et al. 2018).

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Table 1. Linear models explaining whether the pre-treatment level of a specific biomarker differed among groups classified on the severity of clinical signs (no signs, mild and severe) or the progress of the disease (always healthy, new sick, same severity, better condition, and did not survive). The linear model on the progress of the disease included all nestlings excluding the 5 individuals that were not found at the second sampling period. Significant *P*-values are in bold.

	Grouping factor			
	severity of clinical signs		**progress of the disease**	
Biomarker	***F*-value**	***P*-value**	***F*-value**	***P*-value**
Catalase	0.48	0.62	0.80	0.53
Carbonyls	2.07	0.14	0.74	0.57
Corticosterone	0.32	0.73	0.28	0.89
Glutathione peroxidase (GPX)	1.84	0.17	0.88	0.48
Reduced glutathione (GSH)	5.18	**<0.01**	2.45	**<0.06**
Oxidized glutathione (GSSG)	4.33	**0.02**	2.71	0.04
GSH/GSSG ratio	0.30	0.74	0.78	0.54
Haptoglobin	10.1	**<0.01**	5.16	**<0.01**
Hemagglutination	9.78	**<0.01**	6.18	**<0.01**
Hemolysis	3.05	<0.06	1.84	0.14
Nitric oxide (NOX)	0.20	0.82	1.55	0.20
Superoxide dismutase (SOD)	0.89	0.41	0.72	0.58
Non-enzymatic antioxidants (TAC)	1.07	0.35	0.93	0.45
Oxidative damage (TBARS)	22.2	**<0.01**	8.83	**<0.01**

Figure legends:

Figure 1: Nestlings' classification based on visible clinical signs of the disease: *a)* "no signs", *b)* "mild", and *c)* "severe".

Figure 2: Pre-treatment differences among birds classified on the severity of visible clinical signs (no signs, n=26; mild, n=13; severe, n=21) of: *a)* reduced glutathione (µmol/g of fresh weight); *b)* oxidized glutathione (µmol/g of fresh weight); *c)* oxidative damage (nmol MDA equivalents/mL); *d)* haptoglobin (mg/mL); *e)* hemagglutination score; and *f)* hemolysis score. Data are shown as mean ± standard error. Values that do not share the same letter are significantly different from each other.

Figure 3: Pre-treatment differences among birds classified based on the progress of the disease (always healthy, n=11; new sick, n=11; better condition, n=8; same severity, n=18; did not survive, n=7) of: *a)* oxidized glutathione (µmol/g of fresh weight); *b)* oxidative damage (nmol MDA equivalents/mL); *c)* haptoglobin (mg/mL); and *d)* hemagglutination score. Data are shown as mean ± standard error. Values that do not share the same letter are significantly different from each other.

Figure 4: Effect of resveratrol administration on the levels of *left)* circulating non-enzymatic antioxidants (µmol Trolox/g of fresh weight), and *right)* oxidative damage to lipids (nmol of MDA equivalents/mL) levels. Asterisks indicate significant differences: * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Data are shown as mean ± standard error.

Figure 5: Effect of resveratrol administration on the levels of *a)* Haptoglobin levels (mg/mL), *b)* circulating non-enzymatic antioxidants (µmol Trolox/g of fresh weight), and *c)* oxidative damage to lipids (nmol of MDA equivalents/mL). Asterisks indicate significant differences: * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Data are shown as mean ± standard error.

Figure 1

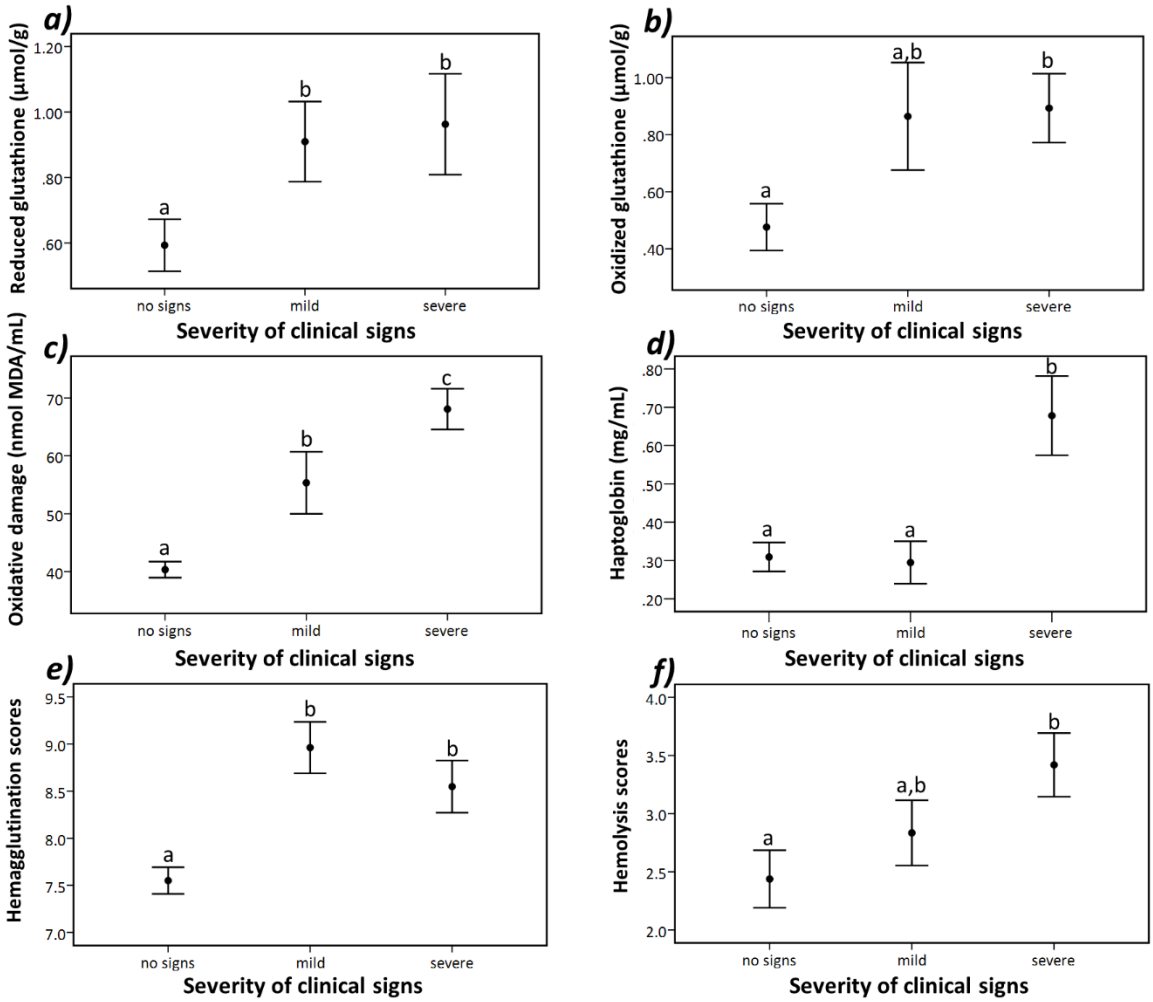
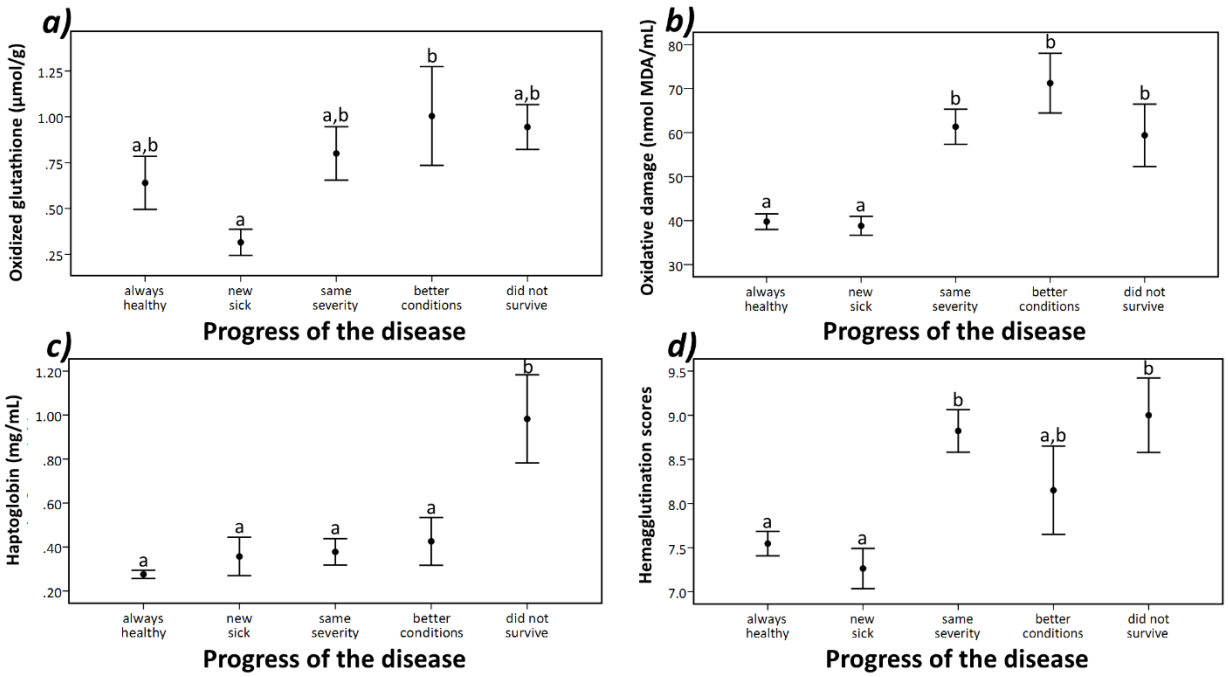

a)
Reduced glutathione (µmol/g)
1.20
1.00
.80
.60
a
b
b
no signs
mild
severe
Severity of clinical signs
b)
Oxidized glutathione (µmol/g)
1.00
.80
.60
.40
a
a,b
b
no signs
mild
severe
Severity of clinical signs
c)
Oxidative damage (nmol MDA/mL)
70
60
50
40
a
b
c
no signs
mild
severe
Severity of clinical signs
d)
Haptoglobin (mg/mL)
.80
.70
.60
.50
.40
.30
.20
a
a
b
no signs
mild
severe
Severity of clinical signs
e)
Hemagglutination scores
9.5
9.0
8.5
8.0
7.5
7.0
a
b
b
no signs
mild
severe
Severity of clinical signs
f)
Hemolysis scores
4.0
3.5
3.0
2.5
2.0
a
a,b
b
no signs
mild
severe
Severity of clinical signs

Figure 2



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599 **Figure 3**

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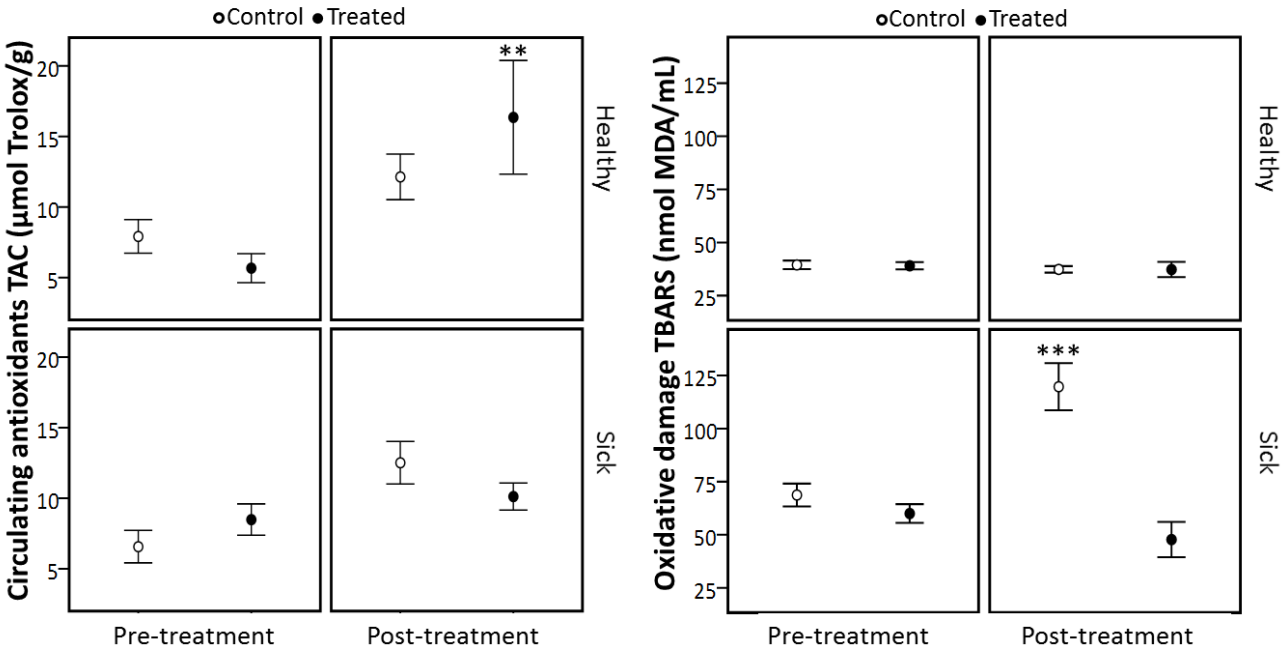
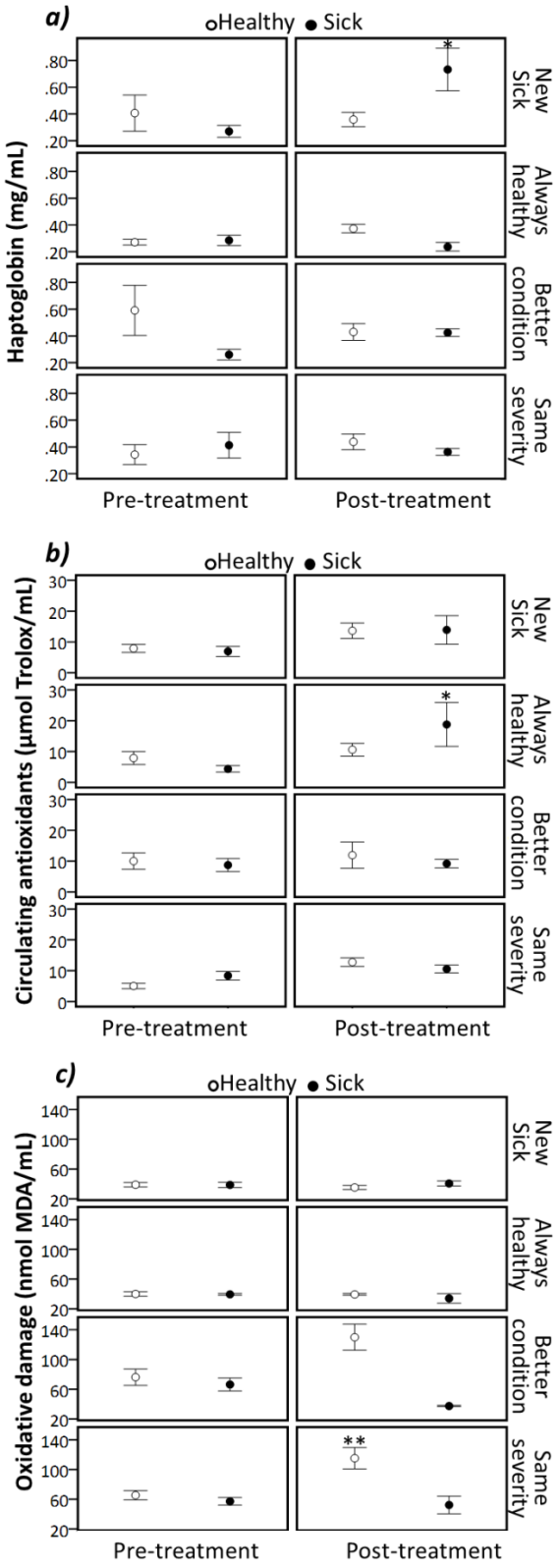

Control Treated
Circulating antioxidants TAC (µmol Trolox/g)
Oxidative damage TBARS (nmol MDA/mL)
Healthy
Sick
Pre-treatment
Post-treatment
**
***

Figure 4



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620 **Figure 5**