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**Experimental infection by a microparasite affects oxidative balance in its avian
reservoir host the blackbird *Turdus merula***

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65 23 **Running head:** Microparasite impacts oxidative balance in natural reservoir
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121 25 **Abstract**
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124 26 By draining resources, microparasites can negatively affect the host fitness, which in
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126 27 turn can result in reduced transmission when virulence leads to reductions in host
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128 28 population size. Therefore, for a microparasite to persist in nature, the level of harm it
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130 29 can do to its host is expected to be limited.
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133 30 We tested this hypothesis for tick-borne *Borrelia* infections in the blackbird *Turdus*
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135 31 *merula*, the most important avian reservoir host in Europe for this zoonotic bacterium.
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137 32 Experimental and observational data were combined to examine the physiological
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139 33 effects caused by *Borrelia* infection in blackbirds. Pathogen-free blackbirds were
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141 34 exposed to *Borrelia*-infected *Ixodes* sp. ticks, and compared with a control group. Their
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143 35 physiological status was evaluated before and after infection with *Borrelia*, through a
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145 36 set of immunological, oxidative stress and general body condition variables.
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149 37 Infected males showed higher levels of oxidative damage to proteins (increased levels
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151 38 of protein carbonyls), decreased glutathione peroxidase activity and increased body
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153 39 mass. Infected females had higher levels of glutathione peroxidase activity after
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155 40 infection by *Borrelia* than the control group. This study shows that *Borrelia* may inflict
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157 41 non-negligible physiological costs on their avian reservoir hosts. We speculate that
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159 42 during energetically demanding periods, these physiological costs may reduce host
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161 43 fitness and affect pathogen transmission.
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168 45 Keywords: oxidative stress; reservoir; host-parasite interactions; zoonosis; tick;
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170 46 *Borrelia burgdorferi* s.l.
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Introduction

Parasites can modulate the physiology, behaviour, and life-history traits of their hosts. Parasites are costly to their hosts because they consume their hosts' metabolites and tissues, and redirect energy from other vital functions and activities of the host to the host immune system (Sheldon and Verhulst, 1996). From an evolutionary, ecological and conservational point of view it is relevant to understand the pathogenicity of a microparasite, and the physiological harm it may impose on its host and the consequent impacts on the host population. The magnitude of the impacts of a parasite on its host may depend on the degree of co-evolution between them (Loye and Zuk 1991), but also on environmental factors such as stressors, multiple effects of different co-occurring parasites (co-infections), phylogenetic group of the parasite and its degree of specialization and mode of transmission, among others (Hawley and Altizer, 2011). For a microparasite to persist and be successfully transmitted in nature, limitations are expected in the level of harm it can inflict on its host, and the mode of transmission is one important factor that affects virulence (i.e. extent of damage, pathology and consequent fitness costs to the hosts). Although it was initially believed that vector-borne parasites would be more virulent than directly transmitted parasites, because mobile arthropod vectors can transmit the former effectively among immobilized hosts, there are other factors in vector-borne transmission that also affect virulence (Ewald, 1993; Ewald, 2004). Transmission success will depend on the ability of the vector to feed on the host, and the blood meal can last from seconds (in the case of mosquitoes) to several days (in the case of hard ticks). In tick species that use an ambushing strategy to find hosts, the tick-borne parasites depend on the host movement for efficient

transmission (Randolph, 1998). Thus, tick-borne parasites that are relatively avirulent may benefit from enhanced host-to-tick transmission success.

Nevertheless, tick-borne parasites (e.g. tick-borne encephalitis virus, *Babesia* spp., *Borrelia burgdorferi* s.l.) can have important effects on their vertebrate hosts including cellular and humoral immune responses (Chauvin et al., 2009; Mansfield et al., 2009; Norte et al., 2013a; Heylen et al., 2015b), oxidative stress (Esmailnejad et al., 2012) and altered body mass (Heylen et al. 2015). *Borrelia burgdorferi* s.l., the etiologic agent of Lyme borreliosis, hereafter *Borrelia*, is a tick-borne zoonotic parasite, comprising over 20 genospecies with different host-specificities (Stanek and Reiter, 2011), whose main vector in Europe is the tick *Ixodes ricinus*. This bacterium infects several vertebrate species of woodlands and gardens and in some species of birds, small and medium-sized mammals, and lizards, some *Borrelia* genospecies are able to establish a widespread (systemic) infection. These often abundant vertebrates species are significantly involved in the maintenance and transmission of *Borrelia* in nature because they are highly infective to the omnipresent vector ticks they are often infested with - those are considered *Borrelia*-competent reservoir hosts (Gern and Humair, 2002; Kurtenbach et al., 2002; Ashford, 2003; Mannelli et al., 2012). In contrast, other vertebrate species such as humans, dogs and large mammals (e.g. ungulates) are considered as incidental or dead-end hosts. These hosts may be infected, and some may show symptoms of disease, but they are not considered competent reservoirs because they are unable to transmit the bacterium to ticks (Gern et al., 1998; Humair and Gern, 2000; Margos et al., 2011). At present, little is known about the host-parasite relationships between *Borrelia* and its avian reservoir hosts. It is expected that this tick-borne microparasite induces limited virulence in its avian reservoir hosts (Olsen, 2007),

given the negative consequences high virulence may have on its transmission success and persistence in nature.

Recently, Heylen et al. (2015b) reported that great tits (*Parus major*) infected with *Borrelia*, through exposure to infected *I. ricinus* collected in an enzootic area, increased their nitric oxide production from immune cells and their body mass with increasing levels of *Borrelia* infection. This study showed that in reservoir hosts (Heylen et al., 2014; Heylen et al., 2015b) some immune components are activated in response to *Borrelia* infection, but there was no uninfected control group in the study design. A previous field study showed that one-year old *Borrelia*-infected blackbirds had higher levels of circulating immunoglobulins (Norte et al., 2013a). Infected blackbirds also had higher white blood cell counts, and one infected-individual had an exceptionally high value of blood glucose, which is indicative of hyperglycaemia in acute stress situations. Studies on the white footed mouse (*Peromyscus leucopus*), a highly competent reservoir host, found no effects of *Borrelia* infection apart from immunological responses (e.g. antibody production (Schwanz et al., 2011). Despite these reported physiological impacts, no fitness effects on its avian and rodent reservoir hosts have been found (Moody et al., 1994; Hofmeister et al., 1999; Schwanz et al., 2011; Chambert et al., 2012; Voordouw et al., 2015).

In this study we experimentally investigated for the first time the effects of *Borrelia*-infected ixodid ticks on the health status of a passerine bird using a variety of physiological variables. We used the blackbird *Turdus merula*, which is one of the most abundant and important avian reservoirs for *Borrelia* (Humair et al., 1998; Taragel'ová et al., 2008; Norte et al., 2013b). We exposed juvenile pathogen-free blackbirds to *Ixodes* sp. nymphs, which had previously fed as larvae on *Borrelia*-infected wild blackbirds. Our study also contained a control group, where juvenile pathogen-free

blackbirds were exposed to uninfected ticks. The effects of *Borrelia* infection, were evaluated using a set of physiological variables reflecting different but interrelated biological systems and functions.

We tested whether *Borrelia* infection reduced the health status of blackbirds, through increased levels of inflammation indicators and increased oxidative stress, compared to controls. Inflammatory responses can lead to an imbalance in the oxidative status of the organism (Costantini, 2014), and, therefore, we measured two metrics of oxidative stress in the blood. Oxidative stress is a biochemical mechanism that has been shown to modulate some life-history traits, including reproduction and ageing (Finkel and Holbrook, 2000; Costantini, 2014), therefore, it may indicate potential impacts of *Borrelia* infection at higher ecological processes involving this reservoir host species (e.g. population dynamics). Finally, we measured physiological variables related to the body and nutritional condition of the birds. Due to potential sex differences in endocrine-immune interactions, and consequent susceptibility to infection, we evaluated if the effects of *Borrelia* infection differed between sexes (Zuk and McKean, 1996; Klein, 2000). Therefore, we expect that by using such a panel of sensitive physiological and clinical biochemistry assays we will be able to detect even subtle detrimental physiological effects of *Borrelia* on its reservoir hosts, which may be expected to be of higher magnitude for non-reservoirs hosts. Furthermore, the assessment of host-parasite relationships and the factors that promote and limit the amplification of *Borrelia* in its reservoir hosts are central to the understanding of the maintenance and emergence of Lyme borreliosis and the impact of this zoonosis on wildlife, and may help to establish mitigating actions to prevent increases in its incidence.

Materials and Methods

Study areas and natural infection data

Wild blackbirds were captured in *Borrelia*-enzootic foci from 10 to 27 February 2015 in Portugal (Mata do Choupal, Coimbra; 40°13'N, 8°27'W) and Belgium (51°19'N, 4°58'E). This period was chosen before the birds' reproductive season began and to increase the probability of contact of the birds with the bacterium, because *Ixodes* nymphs are then most actively searching for hosts (Doby, 1998; Gray, 2008; Norte et al., 2012). Birds were captured in these two enzootic foci to increase the diversity of *Borrelia* genospecies they could be exposed to and the spectrum of genospecies in the experiment. The birds were weighed (in g), their tarsus length (in mm) was measured, were inspected for attached ticks (these were removed), and then taken into captive conditions. They were maintained indoors in individual cages with *ad libitum* food and water.

In Portugal, 5 - 7 days after capture, a skin biopsy and a blood sample were collected from the wild blackbirds. The blood sample (300 µl) was collected into an EDTA tube and was separated into aliquots for molecular screening of *Borrelia* infection and physiological measurements. Additionally, a small volume of blood (50 - 75 µl) was collected into a heparinised capillary tube to make two blood smears, measure blood glucose, erythrocyte sedimentation rate (see below) and hematocrit. The infection status of wild blackbirds in Portugal was assessed through molecular analysis of feeding ticks, skin biopsies and blood (whole blood and buffy coat fraction). In Belgium, a blood sample was collected 16 - 25 days after capture into EDTA tubes and a heparinised capillary tube for physiological measurements, and *Borrelia* infection

status was assessed through xenodiagnoses. Ten *I. ricinus* larvae from a laboratory colony (IS Insect Services GmbH, Berlin) were placed on each bird. The birds were then immobilised inside an air-permeable cotton bag for 2 h (size: 20 cm × 15 cm) inside a darkened cage which kept them inactive. After tick exposure, the birds were placed back in their individual cages with a wire-mesh floor (40 cm × 80 cm). Below each wire-mesh we placed a plastic tray containing damp filter paper and edges streaked with vaseline to prevent larvae from escaping. The engorged larvae that dropped through the mesh cage were collected to be analysed for *Borrelia* infection.

176

177 **Experimental data**

178 **Experimental infection of ticks with *Borrelia burgdorferi* s.l. to obtain challenge** 179 **nymphs**

We created *Borrelia*-infected *I. ricinus* and *I. frontalis* nymphs by feeding pathogen-free larvae of these two tick species on the wild blackbirds and allowed them to moult into nymphs. These nymphs, hereafter referred to as the 'challenge' nymphs, were subsequently used to infect naive experimental blackbirds with *Borrelia* (see below). The challenge nymphs were created as follows: seven to fourteen days after capture, wild blackbirds captured in enzootic foci in Portugal (N = 10) and Belgium (N= 13) were exposed to 50 *I. ricinus* and 50 *I. frontalis* larvae each. Infested birds were immobilized in the dark for 2h for larval attachment and were placed back in their cages, with a wire-mesh floor over a plastic tray containing damp filter paper with edges streaked with vaseline. Both *I. frontalis* and *I. ricinus* larvae were derived from laboratory colonies and were free from *Borrelia* infection. After feeding, the larvae were collected from the trays and maintained at 16h:8h light:dark photoperiod,

25°C:15°C temperature cycle and 85% humidity until moult. On average 2 – 12 individuals from 20-64 nymphs obtained per bird was tested for *Borrelia* infection, before the experimental infestation of the pathogen free (naïve) blackbirds (see below). *Borrelia* prevalence in moulted challenge nymphs was 18.2% (25/137; *I. ricinus* = 17/58; *I. frontalis* = 8/77), which is within the expected range of infection prevalence for *I. ricinus* nymphs fed as larvae on this bird species (8 - 50%; (Humair et al., 1998; Norte et al., 2013b).

Experimental infection of naïve blackbirds

Twenty six naïve juvenile blackbirds (40-50 days old) were obtained from a local breeder (Houthalen, Belgium) in mid-September 2015. These experimental birds were maintained in individual cages with *ad libitum* food and water and a 12:12 light: dark photoperiod. To confirm that the birds had no previous contact with *Borrelia*, we performed a xenodiagnosis (Ginsberg et al., 2005) before the experimental infection by *Borrelia* (Xenodiagnosis 1; Fig.1). Prevalence of *Borrelia* infection in xenodiagnostic ticks was zero, supporting the assumption that those birds had no previous exposure to the bacterium.

After Xenodiagnosis 1, and after an acclimatization period 8 days, we collected a blood sample to assess physiological condition at time 1 (T1) - before *Borrelia* infection. The blood sample (approximately 300 µl) was collected from the brachial vein into heparinised capillary tubes. The birds were weighted (in g) and measured (minimum tarsus length in mm).

Three days after the collection of the blood sample, we infested the experimental birds with *Ixodes* nymphs in a sampling design that was stratified by sex. The

experimental birds were randomly divided in two groups: “control” (N = 7: 5 females and 2 males) and “exposed to challenge nymphs” (N = 19: 10 females and 9 males). The control birds were infested with *I. ricinus* nymphs from a laboratory colony. The exposed birds were infested randomly with either *I. ricinus* or *I. frontalis* nymphs (challenge nymphs) that had fed as larvae on wild infected blackbirds from Portugal or Belgium. Nymphs of *I. ricinus* and *I. frontalis* have similar engorgement weights and rates (mean $\pm$ SE engorged weight *I. ricinus* nymphs = 4.206 ± 0.174 mg, N = 39; *I. frontalis* = 4.141 ± 0.093 mg, N = 49; median number of hours for 50% of nymphs to detach: *I. ricinus* = 84, IQR = 78-84, N = 180; *I. frontalis* = 96, IQR = 81-96, N = 200) and, therefore, it is reasonable to assume that these two tick species have a similar impact on the host.

The blackbirds’ experimental infestation with nymphs was performed in two consecutive episodes (1st and 2nd nymph infestation – Fig. 1) of 20 plus 20 nymphs, placed on the birds 12 days apart. The reason for this infestation procedure was not to excessively burden the birds and in order to be within the natural infestation range for this bird species (Kocianová et al., 2017). The infestation procedure was similar as for the xenodiagnostic larvae, and fed nymphs were collected after feeding from the damp trays placed below the birds. Six days after exposure to the second batch of infected nymphs, and after all the nymphs had completed feeding and dropped off, 60-70 xenodiagnostic *I. ricinus* larvae were placed on the birds (Xenodiagnosis 2) to determine whether the birds were systemically infected with *Borrelia*. Those larvae were collected from the trays placed beneath the birds. Seven days after the exposure to the xenodiagnostic larvae, we took a second blood sample (into both heparinised and EDTA coated tubes) from the birds corresponding to time 2 (T2), i.e. after *Borrelia* infection. This blood sample was taken 13-25 days after the exposure to infected

241 nymphs (in the infected group), a period expected to be of maximum spirochetaemia, as
242 this was the period of maximum infectivity for xenodiagnostic ticks in American robins
243 (Richter et al., 2000). It was shown that hosts develop a strong antibody response 15-30
244 days post-infection (Barthold et al., 1991), but because we challenged the birds on two
245 separate occasions (spaced 12 days apart), this could have introduced some variation in
246 the physiological measurements due to potential variation in the age of infection. At T2
247 the birds were also measured (minimum tarsus length) and weighed.

248

249 **Physiological measurements**

250 The blood samples were used to measure a variety of physiological variables. Blood
251 glucose (mg/dL) was measured using a portable device (ACCU-CHECK Aviva, Roche
252 Diagnostics). Glucose is usually maintained within narrow ranges but may in some
253 instances increase in acute stress situations due to the release of glucocorticoids
254 (Campbell, 2004). One heparinised capillary tube was placed vertically at 4°C for four
255 hours to measure erythrocyte sedimentation rate (ESR; %), as the ratio between the part
256 of the capillary tube occupied by plasma in relation to total blood volume. ESR
257 increases during infection and is an indicator of inflammatory status because the
258 movement of erythrocytes through plasma is enhanced by increased levels of major
259 acute-phase proteins and immunoglobulins (Saadeh, 1998). Hematocrit (%) was
260 measured as the percentage of the capillary tube occupied by red blood cells in relation
261 to total blood volume after centrifuging the blood at 3200 rpm for 10 min. It is an
262 indicator of nutritional status, anabolic capacity, and oxygen-demanding metabolic
263 processes, decreasing under anaemic conditions (Campbell, 1995; Fair et al., 2007).
264 After separating plasma from red blood cells, both fractions were frozen at -80°C. Two

265 blood smears were made with a small drop of freshly collected blood, and were air dried
266 and fixed in 100% methanol and later stained with Giemsa for white blood cell count
267 (WBC) estimation (number of white blood cells per approximately 10000 red blood
268 cells), and heterophil to lymphocyte ratio (H/L; in 100 white blood cells) by scanning
269 the blood smears at 1000x magnification. WBC is an integrative measure of the
270 immunological status reflecting current infection status, and H/L is a generalist stress
271 indicator in birds (Ots et al., 1998).

272 Haptoglobin (mg/ mL) increases during acute infection, inflammation or trauma,
273 but plays also a role of antioxidant (Matson et al., 2012). This protein was measured
274 using a colorimetric assay (Tri-Delta Development, Ireland) based on hemoglobin-
275 binding reaction. A prescan at 405 and 450nm was performed to control for
276 haemolysis in some of the samples (see Vermeulen et al., 2015). Nitric oxide
277 participates in the control of the acute phase of infection, and is an indicator of the
278 magnitude of the innate immune response. Nitric oxide (mmol/L) was measured using
279 the ABEL® Cell Activation test kit with Pholasin® and Adjuvant-K™ (Knight
280 Scientific, Plymouth, UK), which is based on the reduction of nitrate to nitrite by
281 copper-coated cadmium granules, followed by colour development with a Griess
282 Reagent System. Glutathione peroxidase (GPx) is an antioxidant enzyme that detoxifies
283 cells from peroxides and organic hydroperoxides (Halliwell and Gutteridge, 2007). GPx
284 was measured in red blood cells' haemolysate (preparation of lysed red blood cells)
285 using the Ransel assay (Randox Laboratories, Crumlin, UK). This assay is based on the
286 method of Paglia and Valentine (1967). The activity of GPx has been expressed as
287 Units/mg proteins/l. Protein carbonyls are a marker of oxidative damage to proteins
288 (Halliwell & Gutteridge 2007) and were measured in red blood cells using the Protein
289 Carbonyl Colorimetric assay (Cayman Chemical Company, Ann Arbor, MI, USA). The

290 assay is based on the protocol of Levine et al. (1990). The concentration of protein
 291 carbonyls has been expressed as nmoles/mg proteins. Protein concentration in the
 292 haemolysate was estimated using the Bradford protein assay (Bio-Rad Laboratories,
 293 Hercules, CA, USA).
 294
 295 **Detection of *Borrelia burgdorferi* s.l. in ticks and blackbirds' tissues**
 296 DNA was extracted from whole blood and skin biopsies that had been collected from
 297 wild blackbirds using Qiagen DNAeasy blood and tissue kit (Qiagen). Additionally, a
 298 small aliquot of blood (ca 30µl) was collected from the EDTA tube containing the blood
 299 sample into a capillary tube with no anticoagulant. This was centrifuged at 3200 rpm for
 300 10 min and the buffy coat fraction plus the plasma was used for DNA extraction using
 301 DNAeasy tissue kit because *Borrelia* may concentrate in the buffy coat layer during
 302 centrifugation. DNA of ticks collected from wild birds at capture, xenodiagnostic ticks
 303 from wild blackbirds and naïve blackbirds before exposure to infected nymphs, and
 304 moulted *I. frontalis* and *I. ricinus* nymphs after experimental feeding in wild blackbirds,
 305 was extracted by alkaline lysis (Schouls et al., 1999). DNA was extracted from
 306 xenodiagnostic larvae fed on experimentally infected naïve blackbirds using DNAeasy
 307 blood and tissue kit (Qiagen). *Borrelia* infection status of Portuguese wild blackbirds
 308 (assessed by analysis of whole blood, buffy coat layer and attached ticks) was assessed
 309 through a nested PCR that targeted the *flagellin* gene (Johnson et al., 1992). *B.*
 310 *lusitaniae* strain PoTiB2 was used as a positive control and UltraPure DNase/RNase-
 311 Free Distilled Water (Gibco) as a negative control in PCR reactions. *Borrelia* infection
 312 status of wild blackbirds captured in Belgium and of experimental birds (before and
 313 after exposure to nymphs) was assessed though duplex qPCR directed to fragments of

the *ospA* (Gooskens et al., 2006) and *flagellin* (Schwaiger et al., 2001) genes. For a detailed description of primers, probes and qPCR protocol we refer to (Heylen et al., 2013). For each PCR and multiplex qPCR, positive (*B. burgdorferi* s.s. B31 strain), negative controls and blank samples were included.

Statistical analyses

Body condition index of birds was calculated as the residuals of linear regressions of body mass on tarsus length. For experimental birds, we used the linear regression computed for T1 (T1: $b = 2.33 \pm 0.76$; $F_{1,23} = 9.45$, $P = 0.005$), to calculate the residuals for both T1 and T2. Health variables between wild and experimental birds were compared for uninfected/control and infected birds separately, with either one-way ANOVA or Mann-Witney test. For these comparisons we used data collected at T2 from the experimental birds, because an aliquot of the blood sample was collected in EDTA, and, therefore, was comparable with data from wild birds. A statistical comparison between ‘infected’ and ‘uninfected’ wild blackbirds was not possible because of the imbalanced structure among sexes and infection status, and also due to a small sample size.

We assessed the relationship among physiological variables measured in experimental blackbirds using Spearman correlations at T1 and T2. Experimental data were analysed using Linear Mixed Models with repeated measures structure to assess the effects of *Borrelia* infection on blackbirds’ physiology. In these models the fixed effects structure contained the categorical factors of *Borrelia* infection, time, sex and interactions among these terms, as well as three covariates: sampling time of day (time in minutes from sunrise/ “lights on”), total larval tick and nymphal tick engorged

weight. The categorical factor bird ID entered the model as a random effect. The mass of fed nymphs and the mass of fed larvae were included as co-variables to control for any potential physiological effects of tick feeding on the birds' physiology from blood depletion or immuno-modulatory effects. In all models, a step by step selection procedure was used in which the model was interactively refitted after exclusion of the least significant effect, until only significant categorical factors and their lower order interaction terms were left. When modelling ESR, we included haematocrit as a covariable in the model because the movement of red blood cells through plasma depends on haematocrit (Baskurt et al., 2012). Because haptoglobin measurements were significantly affected by the redness of the sample (haemolysis), the 405 and 450 nm readings were included as co-variables in the model of haptoglobin (Matson et al., 2012). H/L and WBC were ln and sqrt transformed, respectively for normality. The residuals of all models were normally distributed. Predicted means obtained from the models in which one of the fixed effects or their interactions was significant were compared with Tukey tests. Statistical analyses were performed in SPSS 20 (IBM Corp. 2011).

Results

Observational data – *Borrelia* prevalence and physiological variables in wild blackbirds

In Portugal, 13 wild blackbirds were captured, and five of these birds were found to be infected by *Borrelia*. Evidence for infection included *Borrelia*-positive skin biopsies and/or *Borrelia*-positive ticks (naturally attached and/or xenodiagnostic ticks). In

Belgium, 13 blackbirds were captured, and 12 of these birds were infected with *Borrelia*, assessed by xenodiagnosis. Prevalence of *Borrelia* infection in xenodiagnostic larval ticks derived from the 13 birds was 79%. This high infection rate in engorged xenodiagnostic larvae is likely to reflect *Borrelia* systemic infection in the birds. Means $\pm$ SE and minimum and maximum values of physiological variables for infected and uninfected blackbirds caught in the wild are presented in Table S1.

Experimental data – *Turdus merula* infection after exposure to challenge nymphs

Two birds died during the experiment: a control and an exposed female blackbird. From these birds we only have physiological measurements from T1, and, therefore, those were both included in the control group. After exposure to *Ixodes* challenge nymphs, experimental blackbirds produced xenodiagnostic ticks with a *Borrelia* prevalence varying between 0 and 97.1% per bird. We defined a bird as *Borrelia*-infected if it produced at least one infected xenodiagnostic tick (N = 13); and for most birds in this group (10 out of 13 birds) prevalence in xenodiagnostic ticks per bird was higher than 60%. As expected, the control birds did not produce any infected xenodiagnostic tick. There were 5 blackbirds (3 females and 2 males) exposed to challenge nymphs which did not produce infected xenodiagnostic ticks – those were considered to be uninfected and were also included in the control group (for both T1 and T2). They could have been exposed to nymphs that were unable to transmit infection, but, because there is also a chance that the birds were exposed to *Borrelia* but did not acquire infection due to an effective immune response towards the bacterium, we performed the statistical analyses with and without those birds. Because the results from both analyses were similar, we present the results including those birds.

386

387 **Experimental data – physiological variables in *Borrelia*-experimentally infected**
 388 **and control blackbirds**

389 Final sample sizes, means and SE of physiological variables for infected and control
 390 birds at T1 and T2 of the experiment are presented in Table S1. Physiological values of
 391 experimental (laboratory bred) birds fell within the natural variation of wild birds.
 392 However, there were significant differences in some of the physiological traits:
 393 uninfected birds differed in H/L ($F_{1,18} = 6.32, P = 0.02$), glucose ($\chi^2_{1,18} = 8.56, P =$
 394 0.003), haematocrit ($\chi^2_{1,18} = 8.56, P = 0.003$) and ESR ($F_{1,17} = 5.53, P = 0.03$), and
 395 infected birds differed in H/L ($\chi^2_{1,27} = 6.03, P = 0.014$), ESR ($F_{1,23.53} = 13.09, P =$
 396 0.0015), haematocrit ($\chi^2_{1,28} = 21.4, P < 0.0001$), GPx ($F_{1,27} = 23.02, P < 0.0001$) and
 397 haptoglobin ($\chi^2_{1,28} = 8.03, P = 0.005$). H/L was higher and haematocrit was lower for
 398 wild birds in both groups, and ESR and haptoglobin were higher for infected wild birds
 399 than experimental birds, but GPx was lower (Table S1).

400 Glutathione peroxidase was negatively correlated with protein carbonyls at both
 401 time points of the experiment ($r_s = -0.48, P = 0.013$ and $r_s = -0.60, P = 0.002$,
 402 respectively), and it was positively correlated with haptoglobin at T2 ($r_s = 0.42, P =$
 403 0.04 ; Table S2). At T1, haptoglobin was positively correlated with nitric oxide and
 404 negatively correlated with ESR ($r_s = 0.65, P = 0.0004$ and $r_s = -0.46, P = 0.020$). At T1,
 405 protein carbonyls were negatively correlated with glucose ($r_s = -0.45, P = 0.019$). There
 406 was a significant interaction between *Borrelia* infection*time affecting WBC ($F_{1,22.95} =$
 407 $5.01, P = 0.035$; Table 1, Fig. 2a). Because our randomization protocol produced
 408 treatment groups that differed in their initial WBC levels, this interaction reflects the
 409 increase in WBC levels in the infected group, which, after infection, showed similar

levels to the control group. There was a significant interaction between *Borrelia* infection*time*sex affecting glutathione peroxidase ($F_{1, 19.57} = 4.70$, $P = 0.043$; Table 1, Fig 2b) and protein carbonyls ($F_{1, 19.57} = 9.95$, $P = 0.005$; Table 1, Fig. 2c). Compared with the control birds, infected males increased the levels of protein carbonyls and decreased glutathione peroxidase activity after *Borrelia* infection, while infected females showed increased glutathione peroxidase activity. The interaction *Borrelia* infection*time*sex significantly affected body mass variation ($F_{1, 24.21} = 5.34$, $P = 0.03$; Table 1, Fig. 2d) because *Borrelia*- infected males increased body mass, compared to control birds. For all the other models the fixed effects and their interactions were non-significant, and the P -values for *Borrelia* infection*time were > 0.2 . Because we tested several response variables, this increases the probability of type I error. If we used Bonferroni corrections to overcome this issue only the effect *Borrelia* infection*time*sex on protein carbonyls would be significant ($P = 0.005$).

Discussion

In this study we showed that this tick-transmitted microparasite induced physiological responses on one of its main natural reservoir hosts with potential fitness costs. The mode of transmission likely modulates the virulence of *Borrelia* but a thorough physiological characterization was able to detect sub-pathological impacts of this microparasite. Our results are particularly important because we mimicked the natural *Borrelia* transmission in an important European avian reservoir host, the blackbird, by exposing pathogen-free birds to endemic *Borrelia* spirochetes via infestations with their endemic ixodid ticks. Because we included a control group that

was exposed to equal numbers of ticks, we were able to separate the effects of *Borrelia* infection from tick infestation. Therefore, our experimental design simulated the *Borrelia* transmission in the wild, and allowed us to investigate for the first time the virulence towards avian hosts of one of the currently most important vector-borne zoonotic pathogenic agents in all its complexity by measuring a large variety of physiological variables.

The infection of naïve blackbirds by *Borrelia* elicited physiological responses that were mostly related with changes in the oxidative status of the birds, but also with some general indicators of body condition, such as body mass. Oxidative stress is known to be a physiological cost associated with the immune response to infection but the majority of experimental avian studies performed to date used either captive species and/or the immunostimulation has been achieved through exposure to non-naturally occurring antigens (e.g. phytohemagglutinin, reviewed in Costantini and Møller, 2009). However it was reported that natural *Plasmodium* sp. infection increases susceptibility to oxidative stress of the avian hosts (van de Crommenacker et al., 2011; Isaksson et al., 2013), and that *Babesia ovis* infection is related with increased red blood cell oxidative stress levels and damage in goats (Esmaeilnejad et al., 2012), but nematode parasite infections do not seem to affect oxidative balance in the red grouse *Lagopus lagopus scoticus* (Mougeot et al., 2010). To the best of our knowledge, this study shows for the first time that infections by naturally occurring bacterial parasites can impact the oxidative balance of their host in an experimental set-up that mirrors the natural transmission cycle of this parasite.

Most of the observed physiological responses differed between females and males. In response to experimental infection with *Borrelia*, glutathione peroxidase levels (GPx) increased in females but decreased in males. Bacterial infection may

induce an unspecific (cell-mediated) immune response which is generally characterized
 by inflammation and the release of reactive oxygen species by leukocytes (Costantini,
 2014). As a defence mechanism against oxidative stress, the activity of GPx, an
 antioxidant enzyme, may have increased, as observed in females. Unexpectedly, we
 observed the opposite trend in GPx activity for males. During acute responses to
 infection, H₂O₂, an oxidant removed by GPx, is used by immune cells to produce HOCl
 to kill bacteria, and this lack of substrate might have been the reason for the decrease in
 GPx activity observed in males. This result suggests that females and males may use
 different physiological strategies to cope with *Borrelia* infection; the reasons for this
 difference are currently unknown. In infected males there was an increase in the levels
 of protein carbonyls, a measure of oxidative damage to tissues. This suggests that
 infected males were probably exposed to high levels of oxidative stress, possibly a
 consequence of a large imbalance in the oxidative status, in favour of pro-oxidants. If
 this was the case, and there was a depletion of antioxidants, including NAPH and,
 consequently, glutathione, needed for the activity of GPx, this may explain the observed
 decrease of GPx activity in infected males. We had expected that infection with
Borrelia would increase the levels of nitric oxide, but we observed no effect of *Borrelia*
 infection on nitric oxide levels in this study. In great tits experimentally infected with
Borrelia (Heylen et al. 2015), nitic oxide increased in response to infection, possibly
 due to its role in the processes responsible for eliminating *Borrelia* spirochetes via
 macrophages (Modolell et al., 1994). We assessed the physiological impacts of *Borrelia*
 infection 13 - 25 days post- infection (depending if infection was acquired in the 1st or
 2nd exposure to challenge nymphs), a period expected to be of maximum
 spirochetaemia, as this was the period of maximum infectivity for xenodiagnostic ticks
 in American robins (Richter et al., 2000). The increase in pro-oxidants (nitric oxide)

may have occurred in an earlier phase of infection, as Heylen et al. (2015) detected an increase in nitric oxide at five days after exposure to *Borrelia*. Different responses to infection between males and females could be due to hormonal differences. The influence of sex hormones has been recognised to account for different susceptibilities and outcomes of infectious diseases between sexes (McClelland and Smith, 2011), including in humans (Fischer et al., 2015), but this deserves further study.

Another explanation for the high levels of oxidative stress in infected males in this study was their increase in body mass. Heylen et al. (2015b) showed that great tits experimentally infected with *Borrelia* also increased their weight, possibly as a strategy to anticipate energetic costs to deal with infection, or, even, induced by the parasite itself, so that it could proliferate better. *Borrelia* infection may have enhanced feeding activity in the male birds causing the observed increase in male body mass. Differences in hormones might explain why males but not females increased their body mass following infection. In the present experiment, the birds were given ad libitum access to a high caloric diet. If *Borrelia*-infected male blackbirds increased their feeding rate, the metabolism of this high caloric diet could have resulted in oxidative stress. It is known that consuming high caloric and protein rich diets may increase oxidative damage and lead to ageing (Ames et al., 1993).

We hypothesize that sexual maturation of the birds and the establishment of hierarchies within the captive facilities might also have influenced the variation in the physiological responses of the males. During sexual development, increases in testosterone levels and social stress associated with establishing hierarchies (or territories in the wild) could increase corticosterone levels in the males, and consequently oxidative stress (Costantini et al., 2011). If males were directing antioxidants such as carotenoids to secondary sexual characteristics (e.g. bill coloration;

(Faivre et al., 2003), infected males could have suffered more heavily from antioxidant depletion (directed both to secondary sexual traits and to counteract ROS produced to fight infection by *Borrelia*) and, as a result, show signs of severe oxidative stress (as observed in the high levels of protein carbonyls and GPx inhibition).

None of the immune status variables and inflammation indicators except WBC (see below) were significantly affected by infection, which was unexpected given previous evidence from the wild in which variation in immune status indicators was found to be correlated with infection, especially in young birds (Norte et al., 2013a). We have to mention that the increase in WBC during the course of experiment was observed in the infected group, which had low values of WBC compared with controls before infection, despite being randomly assigned to the treatment groups (i.e. irrespective of physiological status). The rise in WBC in these birds may have been caused by infection by *Borrelia* or the values were simply returning to normal after a decrease due to an unknown cause. Because this is an exploratory study and very little is known on the physiology of these host-parasite interactions, we presented the results without adjusting the significance levels of the statistical models. However, if those were adjusted, the effect of *Borrelia* infection on protein carbonyls in males would remain significant, and that can be regarded as a downstream consequence of *Borrelia* impact on oxidative balance and potentially reflects other altered physiological functions (e.g. immune response) of the host.

It is still uncertain if *T. merula* acts as competent reservoir for tick-transmitted pathogens other than *B. burgdorferi* s.l., as it was shown for another passerine species, the great tit (*Parus major*), acting as reservoir for *Rickettsia helvetica* in a transmission experiment (Hornok et al., 2013; Jahfari et al., 2014; Heylen et al., 2015a). However, we cannot exclude the possibility that the challenge nymphs could be infected with

other (unknown) co-occurring microparasites that could proliferate in blackbirds, and the effects of *Borrelia* observed in the experimental birds could be conditional on their presence. Because wild birds are exposed to several ecological pressures besides disease (e.g. food availability, competition, predation), with a relatively low sample size, such as the one in our study (wild bird data), it is difficult to test host-parasite interactions in a non-controlled environment without such set up as the experiment performed in captivity. However, data collected from wild birds was useful to confirm that the values of the physiological variables of experimental (laboratory bred) birds fell within the natural variation of wild birds, rendering our result ecologically meaningful.

The observed effects of *Borrelia* infection on the oxidative balance of one of its most abundant avian reservoir hosts, reveals that this bacterium has measurable physiological effects. The direct fitness costs are questionable, but indirectly they can accumulate with other physiologic responses that eventually reduce the host's survival and reproduction. Because oxidative stress is one key factor responsible for cell senescence and ageing (Finkel and Holbrook, 2000), the clinical effects of infections may become important during demanding periods of the birds' life cycle (e.g. establishment of territories, brood rearing, migration) and ultimately result in reduced long-term survival and reproductive success of the birds in the wild, which requires further study. Reported fitness correlates of increased oxidative stress include reduced breeding probability in the wandering albatross (*Diomedea exulans* (Costantini et al., 2015)) and Bize et al. (2008) found that resistance to oxidative damage co-varied with survival and fecundity in another long-lived bird, the alpine swift *Apus melba*. However, field and experimental studies have so far failed to show evidence that *Borrelia* reduces the survival and/or reproduction of their rodent and avian reservoir

hosts (Moody et al., 1994; Hofmeister et al., 1999; Schwanz et al., 2011; Chambert et al., 2012; Voordouw et al., 2015).

Because even in reservoir hosts physiological effects of *Borrelia* infection are detectable as observed in our study, this may affect the *Borrelia* enzootic cycle two-fold: at the organism level, defence mechanisms such as the immune system may limit the amplification of the bacteria in the host, and second, at the population level, if the fitness of the host is affected (e.g. through oxidative stress-mediated mechanisms) it may reduce the success of transmission and infection rate of vector ticks and prevalence of the bacterium in nature through a reduction in the population of hosts. These findings may be partially explained by the fact that *Borrelia* is not a specialist parasite, and it is able to persist in other bird- host species.

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Table 1. Linear Mixed Models with repeated measures structure on the effects of *Borrelia* infection, through exposure to infected ticks, on blackbirds' *Turdus merula* physiology. Fixed effects included 'Borrelia infection (levels: infected/uninfected)', 'time', 'sex' and their higher terms' interactions. Individuals' ID included as random effect. Sampling time of day (time in min from sunrise/ "lights on"), total larval and nymphal tick engorged weight were included as co-variables. WBC was sqrt transformed for normality. Only models in which one of the fixed effects or their interactions was significant are presented.

Effect	Body mass (g)			WBC			Glutathione peroxidase (U/mg prot/l)			Protein carbonyls (nmol/mg proteins)		
	F	df	P	F	df	P	F	df	P	F	df	P
<i>Borrelia</i> infection	0.06	1, 70.77	0.81	1.22	1, 21.98	0.28	5.45	1, 18.03	0.031	0.43	1, 19.84	0.84
Time	3.58	1, 25.76	0.07	0.05	1, 23.95	0.82	0.30	1, 21.36	0.59	0.57	1, 19.84	0.46
<i>Borrelia</i> infection x time	0.49	1, 24.61	0.49	5.01	1, 22.95	0.035	0.10	1, 19.10	0.76	1.75	1, 19.62	0.20
Sex	0.14	1, 71.32	0.71	5.88	1, 21.40	0.024	2.60	1, 17.92	0.12	0.28	1, 19.77	0.60
Sex* time	0.63	1, 24.01	0.44				2.22	1, 19.26	0.15	0.02	1, 19.86	0.89
Sex* <i>Borrelia</i> infection	1.56	1, 70.93	0.22				16.49	1, 18.08	0.001	1.33	1, 19.94	0.26
Sex* <i>Borrelia</i> infection*	5.34	1, 24.21	0.03				4.70	1, 19.57	0.043	9.95	1, 20.04	0.005

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Co-variables

Weight larvae	0.30	1, 24.90	0.59	1.70	1,19.53	0.21	0.53	1, 15.35	0.48	0.07	1, 15.67	0.80
Weight nymphs	3.80	1, 20.57	0.07	1.70	1, 19.14	0.68	0.57	1, 15.44	0.46	0.26	1, 15.82	0.62
Time of day	0.009	1, 36.86	0.92	0.15	1, 22.71	0.70	14.05	1, 18.38	0.001	15.35	1, 20.38	0.001

1971
1972
1973 778 **Figure captions**
1974

1975
1976 779 Fig 1. Diagram representing the timeline of the actions taken during the study of the
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1978 780 physiological effects of the experimental infection of naïve blackbirds *Turdus merula*
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1980 781 with *Borrelia burgdorferi* s.l., via exposure to infected *Ixodes* sp. nymphs.
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1982
1983 782 Fig 2. Relationships between *Borrelia* infection status and WBC (a), glutathione
1984
1985 783 peroxidase (b), protein carbonyls (c) and body mass (d) of experimental blackbirds
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1987 784 *Turdus merula* (N infected birds = 13; N control birds = 11-13, because two birds died
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1989 785 during the experiment). Results (mean predicted values $\pm$ SE) as derived from LMM
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1991 786 (see Table 1 for further details). Post-hoc Tukey tests were performed on the mean
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1993 787 predicted values derived from LMM: groups sharing the same letter are not significantly
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1995 788 different. T1 and T2 are the time points of blood collection during the experiment -
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1997 789 before and after *Borrelia* infection, respectively (see timeline in Fig.1 for further
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1999 790 details).
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Effect of *Borrelia* infection*time*sex, $P = 0.030$

1 **Table S1.** Physiological variables (for explanation of variables see methods) of *Borrelia burgdorferi* s.l. infected and non-infected/control wild
2 and experimental blackbirds *Turdus merula* before (T1) and after (T2) infection by *Borrelia burgdorferi* s.l. Values are presented as means $\pm$ SE.

	Non-infected		Control		Infected		Infected	
	(wild)		(experiment)		(wild)		(experiment)	
	N: 8-9**		N: T1 = 12 - 13 birds (9F; 4M)**; T2 = 11 birds (7F; 4M)*		N: 16-17**		N: T1 = 11-13 birds (6F; 7M)**; T2 = 13 birds (6F; 7M)	
	Mean $\pm$ SE	Min-Max	T1	T2	Mean $\pm$ SE	Min-Max	T1	T2
Body mass (g)	85.44 $\pm$ 1.40	81 - 93	83.00 $\pm$ 1.45	84.82 $\pm$ 1.37	86.91 $\pm$ 2.20	68.5 - 110	82.69 $\pm$ 1.17	85.36 $\pm$ 1.20
Body condition	0.585 $\pm$ 1.94	-8.838 -9.206	0.350 $\pm$ 0.97	-0.308 $\pm$ 1.33	-0.310 $\pm$ 1.48	-8.82 - 14.19	-0.072 $\pm$ 1.22	0.080 $\pm$ 1.15
Glucose (mg/dL)	291.4 $\pm$ 15.99	211 - 395	252.4 $\pm$ 4.35	252.91 $\pm$ 3.93	311.8 $\pm$ 9.21	248 - 388	255.21 $\pm$ 4.33	259.92 $\pm$ 5.24
H/L	0.784 $\pm$ 0.20	0.122 - 1.839	0.294 $\pm$ 0.070	0.305 $\pm$ 0.104	0.67 $\pm$ 0.12	0.116 - 1.917	0.393 $\pm$ 0.142	0.328 $\pm$ 0.079
WBC	25.25 $\pm$ 3.95	13 - 48	20.92 $\pm$ 2.93	19.64 $\pm$ 2.39	18.88 $\pm$ 3.97	4 - 73	13.93 $\pm$ 2.46	19.54 $\pm$ 2.27
Haematocrit (%)	46.58 $\pm$ 0.03	20.0 - 54.0	45.02 $\pm$ 1.39	47.03 $\pm$ 0.85	39.86 $\pm$ 0.02	25.8 - 55.2	46.06 $\pm$ 1.03	48.33 $\pm$ 0.56
ESR (%)	0.093 $\pm$ 0.02	0.023 - 0.254	0.0905 $\pm$ 0.017	0.0502 $\pm$ 0.010	0.116 $\pm$ 0.02	0.037 - 0.337	0.0687 $\pm$ 0.011	0.0350 $\pm$ 0.003
Haptoglobin (mg/mL)	0.357 $\pm$ 0.088	0.095 - 0.828	0.417 $\pm$ 0.050	0.423 $\pm$ 0.040	0.403 $\pm$ 0.085	0.109 - 1.616	0.415 $\pm$ 0.019	0.350 $\pm$ 0.035
Nitric oxide (mmol/L)	0.0042 $\pm$ 0.0005	0.003 - 0.008	0.0046 $\pm$ 0.0004	0.0038 $\pm$ 0.0005	0.0056 $\pm$ 0.0006	0.002 - 0.012	0.0048 $\pm$ 0.0003	0.0049 $\pm$ 0.0004
GPx (U/mg prot/l)	0.691 $\pm$ 0.078	0.31 - 1.00	0.911 $\pm$ 0.060	0.906 $\pm$ 0.069	0.442 $\pm$ 0.051	0.11 - 0.8	0.844 $\pm$ 0.033	0.850 $\pm$ 0.079

Protein carbonyls (nmol/mg proteins)	1.37 ± 0.13	1.00 - 2.14	1.17 ± 0.07	1.12 ± 0.08	1.60 ± 0.12	0.8 - 2.49	1.05 ± 0.06	1.10± 0.09
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3 F – female; M – male; *Two birds died; ** Volume too low

Table S2. Spearman correlations among physiological variables (for explanation of variables see methods) measured in experimental blackbirds at T1 (N=26) and T2 (N=24). Significant correlations are in bold. ESR was used as the residuals from linear regressions of ESR on haematocrit at T1 and T2. The LS residuals of GPx, protein carbonyls and nitric oxide on time from sunrise/ “lights on” (in min) were used. Haptoglobin stands for its LS residuals on 405 and 450 nm readings. Upper part of the table corresponds to T1 and lower part of the table (in grey) corresponds to T2.

	Body mass (g)	Body condition	Glucose (mg/ dL)	H/L	WBC	ESR (%)	Haematocrit (%)	Haptoglobin (mg/ mL)	GPx (U/mg prot/l)	Protein carbonyls (nmol/mg proteins)	Nitric oxide (mmol/L)
Body mass (g)		0.557	-0.009	-0.097	0.124	-0.344	-0.103	0.161	-0.113	0.112	0.282
Body condition	0.964		0.328	-0.125	0.024	-0.245	0.132	0.335	0.057	-0.128	0.076
Glucose (mg/ dL)	-0.127	-0.056		0.277	0.046	0.030	-0.181	-0.107	0.180	-0.454	-0.218
H/L	-0.080	-0.078	-0.301		-0.157	0.175	-0.245	-0.288	0.002	-0.024	-0.218
WBC	0.166	0.125	-0.047	-0.210		-0.270	-0.171	0.181	-0.088	0.093	0.130
ESR (%)	0.225	0.176	0.037	0.287	-0.321		0.204	-0.463	0.098	0.182	-0.102
Haematocrit (%)	-0.038	-0.081	0.082	0.178	-0.120	0.127		0.244	0.109	-0.111	0.223

Haptoglobin (mg/mL)	-0.381	-0.321	0.159	-0.041	0.128	-0.095	-0.072		0.122	-0.285	0.646
GPx (U/mg prot/l)	-0.306	-0.359	0.110	-0.171	0.210	0.244	0.283	0.424		-0.480	0.131
Protein carbonyls (nmol/mg proteins)	0.197	0.129	-0.348	0.053	0.085	-0.319	-0.337	-0.068	-0.597		-0.209
Nitric oxide (mmol/L)	0.391	0.405	0.180	-0.272	-0.029	-0.119	-0.218	-0.314	-0.165	0.104	