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RESEARCH ARTICLE

Low-dose immune challenges result in detectable levels of oxidative damage

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ABSTRACT

Infection can result in substantial costs to animals, so they frequently respond by removing infectious agents with an immune response. However, immune responses entail their own costs, including upregulation of processes that destroy pathogens (e.g. the production of reactive oxygen species) and processes that limit the extent of self-damage during the immune response (e.g. production of anti-inflammatory proteins such as haptoglobin). Here, we simulated bacterial infection across a 1000-fold range using lipopolysaccharide (LPS) administered to northern bobwhite quail (*Colinus virginianus*), and quantified metrics related to pro-inflammatory conditions [i.e. generation of oxidative damage (d-ROMs), depletion of antioxidant capacity], anti-inflammatory mechanisms (i.e. production of haptoglobin, expression of the enzyme heme oxygenase, production of the organic molecule biliverdin) and nutritional physiology (e.g. circulating triglyceride levels, maintenance of body mass). We detected increases in levels of haptoglobin and d-ROMs even at LPS doses that are 1/1000th the concentration of doses frequently used in ecoimmunological studies, while loss of body mass and decreases in circulating triglycerides manifested only in individuals receiving the highest dose of LPS (1 mg LPS kg^{-1} body mass), highlighting variation among dose-dependent responses. Additionally, individuals that lost body mass during the course of the experiment had lower levels of circulating triglycerides, and those with more oxidative damage had greater levels of heme oxygenase expression, which highlights the complex interplay between pro- and anti-inflammatory processes. Because low doses of LPS may simulate natural infection levels, variation in dose-dependent physiological responses may be particularly important in modeling how free-living animals navigate immune challenges.

KEY WORDS: Dose–response, d-ROMs, Heme oxygenase, Lipopolysaccharide, Oxidative stress, Triglyceride

INTRODUCTION

As animals navigate their environment, they address challenges such as food acquisition, predator avoidance and mating success. Although these forces are critical in shaping an individual's fitness, it has become apparent that the role of pathogens and parasites is also substantial (Burgan et al., 2019). Infection status and the subsequent immune response explains variation in host reproductive success (Bonneaud et al., 2003) and ability to escape from predators (Eraud et al., 2009; Stephenson et al., 2016), and response to parasitism may even foster the spread of non-native host species (Brown et al., 2018; Céspedes et al., 2019; Martin et al., 2017; White and Perkins, 2012). The development of ecoimmunology, which has undergone dramatic growth in the past 25 years (Brock et al., 2014), has examined the ways in which the successful elimination of pathogens can increase fitness (Langeloh et al., 2017; Paczesniak et al., 2019), despite the costs of an activated immune system (Hasselquist and Nilsson, 2012).

The costs of an activated immune system in general, and pro-inflammatory conditions in particular, are apparent in multiple contexts. Animals engaged in an immune response consume less food (Ramirez-Otarola et al., 2019), exhibit reductions in circulating triglyceride levels (Frisard et al., 2010; Shini et al., 2008), lose body mass (Owen-Ashley et al., 2006), alter their reproductive investment (Sköld-Chiriac et al., 2019), modify their body temperature (Coon et al., 2011), increase energy expenditure (Lochmiller and Deerenberg, 2000; Demas et al., 2003) and generally experience reductions in fitness-associated traits (Hasselquist and Nilsson, 2012). Some of these costs may be linked to pro-inflammatory mechanisms, which are valuable for destroying pathogens, but can also cause damage to the host. For example, immune system activation increases production of reactive oxygen species (Paardekooper et al., 2019), which induce oxidative damage in pathogens, resulting in their destruction. However, reactive oxygen species also interact with the host's own tissues (Hoffmann and Griffiths, 2018), resulting in increased levels of oxidative damage in the host as well (Hasselquist and Nilsson, 2012; Arulselvan et al., 2016; Baylor and Butler, 2019).

Owing to such costs of immune activation, an attenuated immune response may be adaptive (Smith and French, 2017). As such, immunological responses have seemingly counter-productive pathways initiated concurrently (e.g. upregulation of both pro- and anti-inflammatory cytokines; Burgan et al., 2018), thus avoiding a hyper-responsive, damaging outcome to the host. During an acute phase response, which is characteristic of inflammation, there is an increase in pro-inflammatory cytokine expression, which is useful for elimination of pathogens (Guivier et al., 2016). However, the acute phase response also encompasses the activation of anti-inflammatory mechanisms, including an increase in the production of haptoglobin (Dobryszycka, 1997). Haptoglobin is associated with elimination of the pro-oxidant heme (Quaye, 2008) and can mitigate inflammatory effects (Arredouani et al., 2005). Haptoglobin may also interface with other anti-inflammatory pathways, as it stimulates the expression of heme oxygenase (Belcher et al., 2018), the enzyme responsible for converting heme from its pro-oxidant form to a molecule called biliverdin (Medzhitov et al., 2012; McDonagh, 2001). Biliverdin has

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anti-inflammatory (Tian et al., 2017) and anti-viral (Zhu et al., 2010) properties, and also has putative antioxidant properties (Baylor and Butler, 2019; Mancuso et al., 2012). Thus, inflammation may simultaneously result in an increase in oxidative damage, while also promoting the production of molecules and enzymes associated with anti-inflammatory and antioxidant functions.

To quantify such physiological changes in response to inflammation, many studies involve the administration of lipopolysaccharide (LPS), a molecule found in gram-negative bacteria cell walls that targets immune receptors and induces inflammation (Batista et al., 2019). However, it is unclear what dose represents a physiologically relevant immune challenge. Many eco-immunologists use doses such as 0.1 (Sköld-Chiriac et al., 2014), 0.5 (Baylor and Butler, 2019), 1.0 (Matson et al., 2005; Coon et al., 2011) and 2.5 mg LPS kg^{-1} body mass (Hegemann et al., 2013). These doses are frequently selected either because pilot work indicates that there is a physiological response (Palacios et al., 2011), or because there is precedent in the literature (Ben-Hamo et al., 2017). However, birds have the ability to maintain physiological and behavioral function at doses of LPS that induce severe illness or death in mammals (Fink, 2014), so the use of comparatively high doses in birds may be driven by their ability to resist bacterial infection, rather than the utility of these doses to model a naturalistic challenge. Thus, to investigate how metrics related to nutritional and oxidative physiology respond across a range of simulated immune challenges that encompass a variety of potentially ecologically relevant scenarios, we administered northern bobwhite quail (*Colinus virginianus*) injections of LPS across three orders of magnitude and collected data related to nutrient physiology, oxidative physiology, the immune system and heme oxygenase.

MATERIALS AND METHODS

Husbandry and sample collection

In November 2017, we purchased 48 adult female northern bobwhite quail [*Colinus virginianus* (Linnaeus 1758)] from Red Fox Farm (Mohrsville, PA, USA), and placed birds in cages (A&E Double Flight Bird Cages, 1.86×0.81×0.53 m) with five birds per cage. We elected to use females to eliminate the possibility of a sex effect, and each cage included one bird from each treatment group. The cages were in a greenhouse that had been converted to an aviary, providing birds with access to natural light cycles (approximately 10.5 h:13.5 h light:dark) through frosted glass walls and a glass ceiling outfitted with translucent corrugated polycarbonate sheets (SUNTUF Solar Gray, Palram, Kutztown, PA, USA) to limit head load. The temperatures in the aviary cycled with overnight lows of 15.2°C and daily highs of 20°C throughout the study. We provided birds with Home Fresh Multi-Flock Chick N Game Starter/Grower Crumble (Kent Nutrition Group, Inc., Muscatine, IA, USA) and water *ad libitum*. We banded individuals with one colored spiral band per leg for identification, and measured each bird's initial body mass to the nearest 0.1 g and the length of the right tarsus to the nearest 0.1 mm.

After a 12-day acclimation period, we initiated our study by measuring body mass and injecting each individual intra-abdominally with 100 µl of either 0 (N=10), 0.001 (N=9), 0.01 (N=10), 0.1 (N=9) or 1 mg LPS kg^{-1} body mass (N=10) (L7261, Sigma-Aldrich, St Louis, MO, USA) to simulate different intensities of an immune challenge. We returned all birds to their cages following injections (administered between 14:01 and 16:31 h), and injection order was random with respect to treatment. We did not collect a baseline blood sample to eliminate the risk of a hematoma, which would have resulted in the release of heme, thereby affecting metrics related to heme oxygenase and its products. Approximately 18 h (18 h 12±27 min) later, we again measured body mass and collected approximately 600 µl of whole blood from the alar vein of each bird with needles (25G, Becton Dickinson, Franklin Lakes, NJ, USA) and heparinized capillary tubes (02-668-25, Fisher Scientific, Waltham, MA, USA), with all sample collection occurring between 07:28 and 11:28 h. We opted for an 18-h duration because this time span is shortly after the peak expression increase of heme oxygenase-1 (HO-1; Malaguarnera et al., 2005) and shortly before the peak production of haptoglobin (Coon et al., 2011) in response to LPS. We then centrifuged the blood sample for 3 min at 12,000 ***g*** to separate plasma from erythrocytes, and placed the plasma sample on ice. We then euthanized individuals with CO_2 (all birds were euthanized within 18 min of blood collection), and placed carcasses on ice for dissection later that day. Upon dissection, the spleen was removed, weighed to the nearest 0.1 mg and placed in a microcentrifuge tube, and a portion of the posterior section of the central lobe of the liver was removed. Plasma, erythrocyte pellets, liver samples and spleen samples were stored at −80°C until future analysis. All procedures were conducted in accordance with Lafayette College's Institutional Animal Care and Use Committee (approval date: 16 November 2017).

Measurement of glycerol, triglyceride and haptoglobin

To quantify glycerol and triglyceride concentration in the plasma, we pipetted 240 µl glycerol reagent (Sigma-Aldrich, F6428) into a clear 96-well plate (Greiner, Sigma-Aldrich) according to Butler et al. (2020). We then added 5 µl of each plasma sample in duplicate wells, with water as the control blank and glycerol standard (Sigma-Aldrich, G7793) as the calibrator. We scanned the plate using an Infinite M200Pro (Tecan US, Inc., Morrisville, NC, USA) at 540 nm before adding 60 µl triglyceride reagent (T2449, Sigma-Aldrich) to each well, incubating for 10 min at 37°C and scanning again at 540 nm. We calculated glycerol and total triglyceride concentration by subtracting absorbance of the blank from the samples and the calibrator, then dividing absorbance of the samples by that of the calibrator. Free triglycerides were calculated by subtracting glycerol from total triglycerides (Butler et al., 2020, 2016).

We measured haptoglobin using a colorimetric assay kit from Tridelta DD (TP-801, Kildare, Ireland). In accordance with kit directions, we added 100 µl of the provided reagent to each well of a clear 96-well plate along with 7.5 µl of plasma sample or standard in duplicate. We then scanned the plate at 630 nm to correct for initial differences in plasma color (Matson et al., 2012) using an Infinite M200Pro. Next, we added 140 µl of the chromogen reagent to each well and incubated the plate for 5 min at room temperature before scanning absorbance immediately at 630 nm. We calculated the average of duplicate values, and subtracted initial absorbance from final absorbance. We then used a standard curve generated from the kit's components to calculate haptoglobin concentration for each sample.

Markers of oxidative physiology

We measured total antioxidant capacity using the OXY-Adsorbent test (hereafter, OXY; Diacron International, Grosetto, Italy) according to the directions, with several modifications (Costantini and Dell'Omo, 2006). We first pipetted 200 µl aliquots of HClO into each well of a clear 96-well plate and 5 µl of diluted plasma (1:100 plasma:ddH_2O), a blank (ddH_2O) or calibrator in duplicate. We then incubated the plate for 10 min at 37°C before adding 5 µl

chromogen and measured absorbance at 505 nm in an Infinite M200Pro. We measured the concentration of reactive oxygen metabolites (ROMs) in the plasma using the d-ROMs test (Diacron International) according to the directions, with a few modifications (Costantini and Dell'Omo, 2006). We pipetted 200 µl aliquots of reagent mixture (1:100 ratio of chromogen:buffer) to each well in a clear 96-well plate, and added 20 µl plasma and calibrator in duplicate, and blanks (ddH_2O) in quadruplicate. We then incubated the plate for 75 min at 37°C and scanned absorbance at 485 nm. To incorporate both antioxidant and pro-oxidant markers into a single metric, we calculated oxidative index (Isaksson et al., 2011; Vassalle et al., 2008; Stahlschmidt et al., 2017), which subtracts the standardized OXY value from the standardized d-ROMs value. Therefore, positive oxidative index values are associated with more oxidative damage, and negative values are associated with greater antioxidant capacity.

Measurement of biliverdin in the liver and spleen

We quantified biliverdin according to Butler et al. (2017) using a fluorophore developed by Berlec and Štrukelj (2014). First, we weighed the whole spleen or approximately 0.1 g of liver and placed the tissue into a homogenization tube with four zirconium oxide grinding balls (OPS Diagnostics, Lebanon, NJ, USA) and 1 ml DMSO:ddH_2O (80:20). We homogenized the tissue for 30 s at 4000 rpm using a BeadBug Microtube Homogenizer (Benchmark Scientific, Edison, NJ) and let it sit for 5 min to allow precipitate to settle. We then pipetted 270 µl aliquots of homogenate into two tubes in order to calculate sample-specific recovery, which we achieved by adding 30 µl of 2 µmol l^{-1} biliverdin dissolved in 80:20 DMSO:ddH_2O to one aliquot, and 30 µl of 80:20 DMSO:ddH_2O to the other. We then vortexed each sample for 3 s and centrifuged for 4 min at 12,000 ***g***. Aliquots of 50 µl supernatant were added in duplicate to a black 96-well plate along with a standard curve. Lastly, we added 50 µl biliverdin determination reagent (Berlec and Štrukelj, 2014; Butler et al., 2017) containing a fluorescent protein that binds with high affinity to biliverdin molecules, thus allowing for the quantification of biliverdin in each sample. After a 75 min incubation at room temperature in the dark, we measured fluorescence (excitation: 680 nm; emission: 714 nm) using an Infinite M200Pro. We then calculated biliverdin concentration in each sample using the standard curve, correcting for recovery, and dividing by sample mass to get biliverdin concentration per tissue wet mass.

Immunoblot analyses

We quantified heme oxygenase expression in both liver and erythrocyte pellets. To do so, we homogenized 0.1 g of frozen liver in 1 ml ddH_2O for 30 s at 4000 rpm using a BeadBug Microtube Homogenizer, and exposed the red blood cell pellet to a freeze–thaw cycle. Crude homogenate was clarified by centrifugation at 8°C for 10 min at 14,100 ***g*** and the clarified lysate was stored again at −80°C. We then added 5 µl of clarified cell lysate to 20 µl of loading buffer (12 mmol l^{-1} Tris-Cl pH 6.8, 0.4% SDS, 0.2% Bromophenol Blue, 5% glycerol, 2.9 mmol l^{-1} 2-mercaptoethanol), and used 0.3 µg of human HMOX-1 (ProSpec) as a positive control for visualization. Lysates were boiled for 5 min, then resolved in 12.5% polyacrylamide gels. The protein was transferred to a nitrocellulose membrane at 1 A for 1 h at 25°C in a tris-glycine/methanol buffer. The nitrocellulose membrane was first probed with polyclonal rabbit antibody raised against full-length human GAPDH (Abcam; antibody: ab9485). We reprobed the membrane with polyclonal rabbit antibody raised against a C-terminal fragment of actin attached to the Multiple Antigen Peptide backbone (Sigma Life Sciences; antibody: A2066). Immunoblot analyses were carried out using ECL Anti-Rabbit IgG (GE Healthcare; antibody: NA930V) and the Lumigen ECL Ultra (Lumigen) chemi-luminescence system according to the manufacturer's suggestions. Densitometry measurements used to estimate relative protein expression levels were made using ImageJ software. As an internal control, heme oxygenase expression was normalized to actin expression within each sample.

Statistical analyses

We used general linear models (GLM) to test for treatment effects on spleen mass, spleen-somatic index (i.e. spleen mass divided by body mass; Butler and Ligon, 2015), biliverdin concentration in the spleen and liver, amount of biliverdin in the spleen (i.e. concentration of biliverdin in the spleen multiplied by spleen mass), heme oxygenase expression in the liver and blood, haptoglobin, OXY, glycerol and free triglycerides. We also used GLM to test for treatment effects on change in body mass during the experimental period both with and without the covariate of average body mass (i.e. the average of pre-injection and blood collection body mass). Because free triglyceride levels may contribute to amount of lipid-associated oxidative damage, we produced models for d-ROMs and oxidative index both alone and with free triglyceride as a covariate, as recommended by Pérez-Rodríguez et al. (2015). Because both haptoglobin and heme oxygenase are part of a single pathway that limits heme-induced toxicity (Thomsen et al., 2013), we used linear regression to test for associations between haptoglobin and heme oxygenase expression in both liver and blood. Because oxidative damage can damage erythrocytes, resulting in lysis or macrophage-associated clearance (Oishi et al., 1999), which can induce heme oxygenase expression (Ryter et al., 2006), we used linear regressions to test whether d-ROMs or oxidative index predicted heme oxygenase expression in liver and blood. We analyzed all data using SAS (Version 9.4, Cary, NC, USA); data were transformed to meet the assumptions of normality, and *post hoc* tests were used to compare least-squares means.

RESULTS

Dose-dependent effects of LPS on physiology and morphology

Several physiological and size-related metrics changed in response to administration of different doses of LPS, with a loss of body mass (approximately 5 g, or 2.5% of body mass) over the 18-h experimental period (Fig. 1A) and a decrease of approximately 38% in free triglyceride concentration (Fig. 1B) manifesting at the highest doses. An increasing dose of LPS was generally associated with a nearly 5-fold increase in d-ROMs levels (Fig. 1C), a 2.5-fold increase in haptoglobin concentration (Fig. 1D) and a shift in oxidative index from antioxidant capacity to oxidative damage (Fig. 1E, Table 1). Treatment effects on change in body mass remained if average body mass was included as a covariate (treatment: $F_{4,42}$=3.82, P=0.0098; average body mass: $F_{1,42}$=4.10, P=0.0492), and treatment effects persisted with the inclusion of free triglyceride concentration as a covariate for both d-ROMs (treatment: $F_{4,42}$=5.99, P=0.0007; free triglyceride: $F_{1,42}$=3.45, P=0.0704) and oxidative index (treatment: $F_{4,42}$=3.37, P=0.0177; free triglyceride: $F_{1,42}$=7.35, P=0.0097). There were no statistically significant treatment effects on the remaining metrics (Table 1).

Associations between body mass and lipid levels

Circulating glycerol levels were negatively related to change in body mass over the 18-h experimental period ($F_{1,45}$=4.12, P=0.0478), but not to average body mass ($F_{1,45}$=0.08, P=0.78). Free triglyceride

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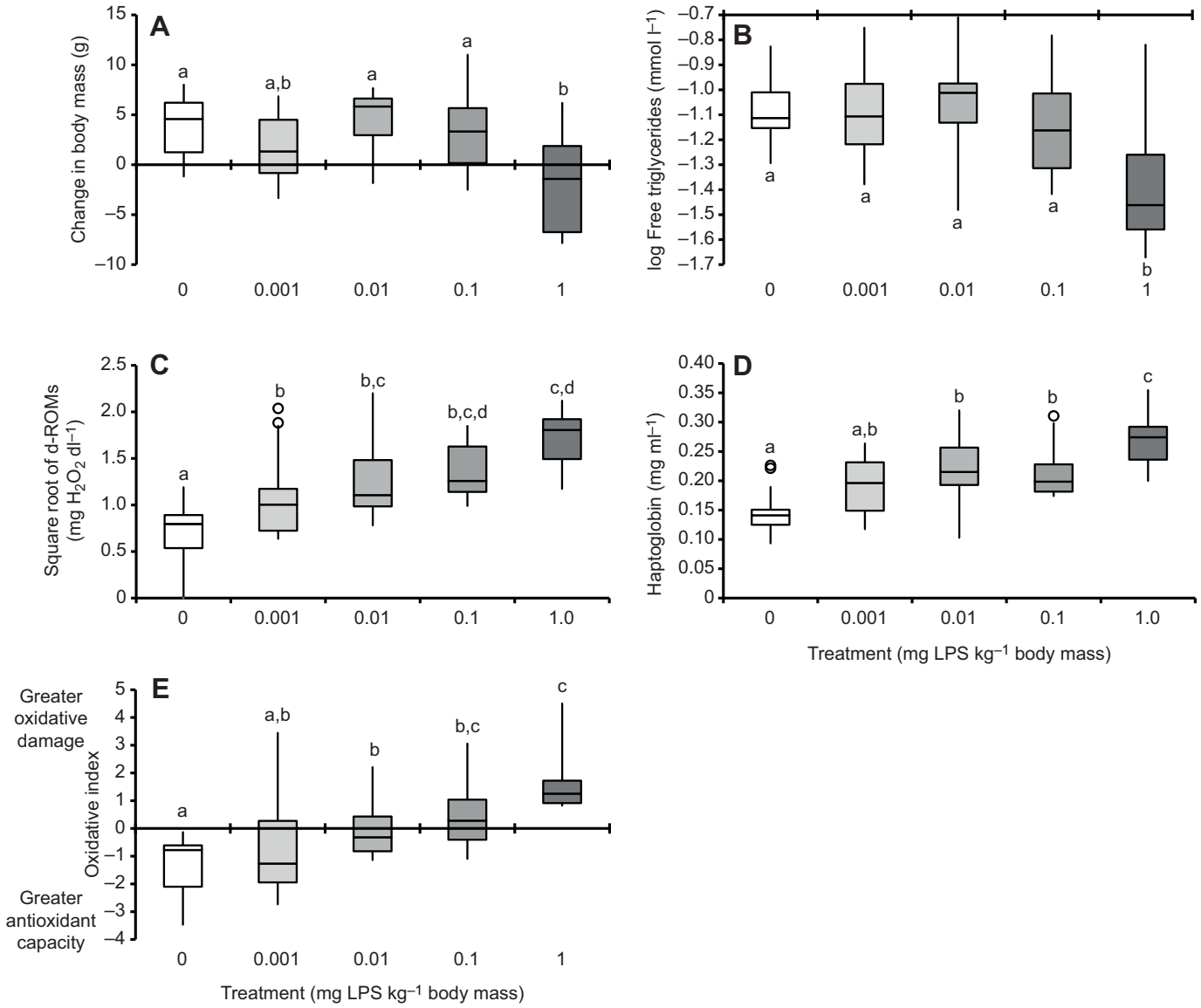

Fig. 1. Administration of lipopolysaccharide (LPS) affected physiological metrics in a dose-dependent manner. Northern bobwhite quail injected with the highest dose of LPS lost body mass (A) and circulated lower levels of triglycerides (B) relative to most other treatment groups, while individuals injected with higher doses of LPS also had greater levels of d-ROMs (C; a measure of oxidative damage), increased concentrations of circulating haptoglobin (D) and increased levels of oxidative damage relative to antioxidant capacity (E; oxidative index, which is a metric calculated using standardized values of both d-ROMs and OXY). Box plots depict the median (central horizontal line), the interquartile range (box) and the range of observed values falling within 1.5× the interquartile range (whiskers). Statistically significant differences (least-squares means, $P<0.05$) among treatment levels are denoted with different letters. Circulating triglyceride levels were log-transformed and levels of d-ROMs were square-root-transformed to achieve normality.

concentration was positively related to change in body mass over the 18-h experimental period ($F_{1,45}=20.61$, $P<0.0001$; Fig. 2), and was also positively related to average body mass ($F_{1,45}=6.86$, $P=0.012$).

Associations between the heme oxygenase system and other physiological metrics

Both haptoglobin concentration and d-ROMs levels (Fig. 3) were positively associated with an increase in heme oxygenase expression in the liver, but not in the blood, whereas oxidative index was not associated with heme oxygenase expression in either tissue (Table 2). These relationships remained statistically similar when free triglyceride concentration was included as a covariate in models including either d-ROMs or oxidative index. Additional correlations among variables are listed in Table S1.

DISCUSSION

We uncovered evidence that administration of LPS at doses between 100- and 1000-fold lower than those frequently used in ecoimmunology studies affected physiological metrics related to oxidative physiology and the acute phase response. This pattern is analogous to changes in thermoregulatory patterns that are also detectable at similarly low doses of LPS (Skold-Chiriac et al., 2015), highlighting that these low doses of LPS have physiologically relevant effects on homeostasis. However, we did not detect any effects on nutrient physiology or body mass at these low doses of LPS, with a loss in body mass and decrease in circulating triglyceride levels apparent only at a dose of 1 mg LPS kg^{-1} body mass. Thus, although high doses of LPS may be useful in modeling relatively intense levels of simulated

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Table 1. Multiple physiological metrics changed in response to administration of lipopolysaccharide (LPS) at doses of 0, 0.001, 0.01, 0.1 or 1.0 mg LPS kg^{-1} body mass in northern bobwhite quail

Dependent variable	*F*	d.f.	*P*
Change in body mass	**3.90**	**4, 43**	**0.0087**
Spleen mass	0.66	4, 43	0.62
Spleen-somatic index	0.95	4, 43	0.44
Biliverdin concentration in spleen	0.60	4, 43	0.66
Biliverdin amount in spleen	1.18	4, 43	0.33
Biliverdin concentration in liver	0.08	4, 43	0.99
Haptoglobin concentration	**7.61**	**4, 43**	**<0.0001**
d-ROMs	**8.48**	**4, 43**	**<0.0001**
OXY	1.14	4, 43	0.35
Oxidative index	**5.67**	**4, 43**	**0.0009**
Glycerol	1.59	4, 43	0.19
Free triglycerides	**3.37**	**4, 43**	**0.017**
Liver HO expression	2.26	4, 42	0.078
Blood HO expression	0.75	4, 41	0.57

Spleen mass, spleen-somatic index, amount of biliverdin in the spleen, and biliverdin concentration in the liver were inverse-transformed; biliverdin concentration in the spleen, glycerol and free triglyceride concentration, and heme oxygenase (HO) expression in the liver and blood were log-transformed; d-ROMs was square-root transformed; and OXY was cubed to achieve normality. Residuals from all analyses were normally distributed. Statistically significant treatment effects are in bold.

immune function, the frequent use of such high doses (Matson et al., 2012; Coon et al., 2011; Koutsos and Klasing, 2001; Merrill et al., 2016; Baylor and Butler, 2019; Toomey et al., 2010) could result in a reduced understanding of the physiological patterns associated with milder, but potentially ecologically relevant, immune challenges.

Administration of doses of 0.001 mg LPS kg^{-1} body mass and higher resulted in a statistically significant increase in oxidative damage. This pattern was mirrored by oxidative index, a metric that incorporates relative levels of oxidative damage with antioxidant capacity. Oxidative damage increased in a dose-dependent nature, reaching its peak at the highest LPS dose. Increases in oxidative damage are common in response to a variety of physiological perturbations, including immune challenges (Baylor and Butler, 2019), heat stress (Costantini et al., 2012), exercise (Cooper-Mullin

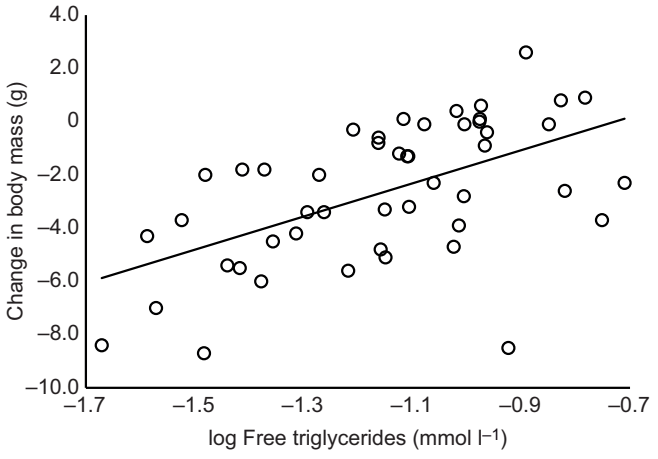

Fig. 2. Northern bobwhite quail circulating lower concentrations of triglyceride lost more body mass during the 18-h experimental period. In contrast, those circulating higher levels of triglyceride generally maintained body mass. Raw data are plotted, and average body mass (average of body mass at time of injection and body mass at end of the experimental period) was a statistically significant covariate ($F_{1,45}$=6.86, P=0.012).

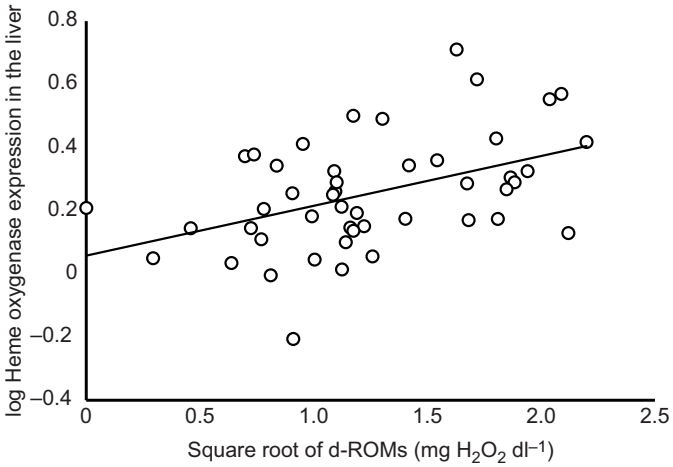

Fig. 3. Northern bobwhite quail with higher levels of oxidative damage expressed higher levels of heme oxygenase in the liver. As d-ROMs, a measure of oxidative damage, increases there is a corresponding increase in heme oxygenase, which is consistent with a role for the heme oxygenase system in oxidative physiological processes. Raw data are plotted, and inclusion of free triglyceride concentration as a covariate did not affect statistical findings (d-ROMs: $F_{1,44}$=11.49, P=0.0015; free triglyceride: $F_{1,44}$=0.69, P=0.41).

and McWilliams, 2016) and even consumption of a meal (Butler et al., 2016). Such increases in oxidative damage may have important fitness-related consequences (Costantini, 2016), including being associated with early- and late-life survival rates (Herborn et al., 2016). However, although we did detect increases in oxidative damage in response to low-dose LPS administration, it is less clear the extent to which these more modest increases in oxidative damage would affect survival probabilities. Future work that manipulates either degree of simulated infection status or levels of oxidative damage directly, and then quantifies variation in survivorship, would provide a useful context for this pattern.

Low levels of LPS (e.g. 0.01 mg LPS kg^{-1} body mass) resulted in detectable increases in haptoglobin, as did higher doses of LPS. This dose-dependent pattern is consistent with the ability of LPS to elicit an increase in acute phase proteins such as haptoglobin (Dobryszycka, 1997; Lee et al., 2012). Haptoglobin is an acute phase protein that has been associated with increases of immune challenge-induced oxidative damage (Costantini et al., 2015; Sebastiano et al., 2018), although this pattern is not ubiquitous (Fritze et al., 2019). Despite the positive correlation between oxidative damage and haptoglobin (present study; Costantini et al., 2015), haptoglobin is frequently viewed as a mechanism for limiting the extent of oxidative damage rather than promoting it

Table 2. Heme oxygenase (HO) expression correlates with both haptoglobin and oxidative physiology in northern bobwhite quail

Independent variable	Dependent variable	*F*	d.f.	*P*
Haptoglobin	**Liver HO expression**	**5.26**	**1,45**	**0.027**
	Blood HO expression	0.04	1,44	0.85
d-ROMs	**Liver HO expression**	**11.31**	**1,45**	**0.0016**
	Blood HO expression	0.97	1,44	0.33
Oxidative Index	Liver HO expression	1.29	1,45	0.26
	Blood HO expression	0.16	1,44	0.69

Heme oxygenase expression in the liver and blood was log-transformed and d-ROMs was square-root transformed to achieve normality. Statistically significant associations are in bold, and variables in both associations were positively related.

(Fritze et al., 2019). This interpretation is favored because haptoglobin is generally associated with anti-inflammatory pathways (Arredouani et al., 2005) that limit the extent of damage that an individual incurs during an inflammatory response, although the severity of disease is an important factor in driving these associations (Sebastiano et al., 2018). One mechanism driving the reduction of damage to the host is the removal of the pro-oxidant heme from circulation (Thomsen et al., 2013), and increasing expression of heme oxygenase (Belcher et al., 2018), which is generally associated with anti-inflammatory (Thomsen et al., 2013) and antioxidant (Otterbein and Choi, 2000; Surai et al., 2019) processes. The positive correlation we detected between haptoglobin concentration and heme oxygenase expression in the liver supports a role for haptoglobin in promoting antioxidant processes, thus limiting self-damage.

Initially, the positive correlation between heme oxygenase expression and oxidative damage seems to refute an antioxidant role of heme oxygenase and its products, despite robust support in the mammalian literature (Wang and Chau, 2010; Mishra and Ndisang, 2014; Mancuso et al., 2012). However, the temporal relationships between upregulation of heme oxygenase in the liver, a subsequent increase in circulating biliverdin and a commensurate reduction of oxidative damage in the blood are poorly studied outside of mammals (Butler et al., 2020). Thus, here we may have collected data after heme oxygenase expression increased, but prior to any downstream alterations to antioxidant activity in the plasma. Additionally, different doses of LPS have varying effects on the timing of changes to physiological parameters in mammals (Vedder et al., 1999; Bison et al., 2008), suggesting that peak responses for each variable may even vary by dose. Thus, the temporal dynamics of the interplay between LPS, haptoglobin, heme oxygenase expression, biliverdin and oxidative physiology are a critical area for future research.

Traits related to body mass and triglyceride levels were not affected by administration of LPS, except in those individuals receiving the highest dose, which lost more body mass during the experiment and also had lower levels of circulating triglycerides. This pattern suggests that rather than a proportional dose-dependent response, there is a threshold at which activation of the immune system affects food consumption and body mass maintenance. Loss of body mass is common in animals that are engaged in an immune response (Owen-Ashley et al., 2006; Koch et al., 2018; Palacios et al., 2011), and is frequently associated with a reduction in food consumption (Ben-Hamo et al., 2017; Skold-Chiriac et al., 2015) or an increase in energy expenditure (Lochmiller and Deerenberg, 2000; Demas et al., 2003; Burness et al., 2010; Hegemann et al., 2012). Relatedly, we found that individuals who lost the most body mass also circulated higher levels of glycerol and lower levels of triglycerides, a state associated with reliance on energetic reserves (Fokidis et al., 2012; Neuman-Lee et al., 2015). Therefore, individuals receiving the highest dose of LPS were likely relying on energetic reserves during the experimental period, even in the presence of *ad libitum* access to food. Sickness-induced anorexia is a common response to infection (Kyriazakis et al., 1998), and while we could not track individual-level food consumption because of group-housing conditions, our data are consistent with either a decrease in food consumption or an increase in metabolic rate that resulted in higher energy consumption in response to a threshold-dependent effect of LPS.

In conclusion, we found that LPS elicits changes in metrics related to an immune response (e.g. haptoglobin concentration, levels of oxidative damage) in a dose-dependent manner, even at very low concentrations. However, metrics more indirectly related to an immune response (e.g. circulating triglyceride levels, maintenance of body mass) were affected 18 h post-exposure at only the highest dose, although the possibility remains that different patterns existed at different time points. Further research should focus on establishing the relationship between LPS dose and natural variation in infection intensity, allowing for a more targeted approach to simulating infection. For example, quantifying transcriptomic, physiological and behavioral data in response to various doses of LPS, as well as various levels of actual infection, could provide a valuable context for the ability of LPS to simulate infection in free-living populations. Additionally, research that disentangles the costs of different components of the immune response (e.g. various elements of constitutive or induced immunity) would provide valuable information for modeling the costs and benefits of different immune processes as a function of infection intensity.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: E.M.A., M.W.B.; Methodology: E.M.A., T.L.B., J.K.H., M.W.B.; Validation: M.W.B.; Formal analysis: J.K.H., M.W.B.; Investigation: E.M.A., T.L.B., J.K.H., M.W.B.; Resources: J.K.H., M.W.B.; Data curation: M.W.B.; Writing - original draft: E.M.A., M.W.B.; Writing - review & editing: E.M.A., T.L.B., J.K.H., M.W.B.; Visualization: M.W.B.; Supervision: J.K.H., M.W.B.; Project administration: M.W.B.

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Table S1. Correlation analysis of all dependent variables. Spleen mass, spleen-somatic index, amount of biliverdin in the spleen, and biliverdin concentration in the liver were inverse-transformed; biliverdin concentration in the spleen, glycerol and free triglyceride concentration, heme oxygenase expression in the liver and blood were log-transformed; d-ROMs was square-root transformed; and OXY was cubed to achieve normality.

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