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RESEARCH ARTICLE

Oxidative damage increases with degree of simulated bacterial infection, but not ectoparasitism, in tree swallow nestlings

Michael W. Butler[1,§], Emma N. Stierhoff[1,*], Julianna M. Carpenetti[1,‡], Matthew A. Bertone[2], Alyssa M. Addesso[3] and Sarah A. Knutie[3,4]

ABSTRACT

The purpose of mounting an immune response is to destroy pathogens, but this response comes at a physiological cost, including the generation of oxidative damage. However, many studies on the effects of immune challenges employ a single high dose of a simulated infection, meaning that the consequences of more mild immune challenges are poorly understood. We tested whether the degree of immunological challenge in tree swallows (*Tachycineta bicolor*) affects oxidative physiology and body mass, and whether these metrics correlate with parasitic nest mite load. We injected 14 day old nestlings with 0, 0.01, 0.1 or 1 mg lipopolysaccharide (LPS) per kg body mass, then collected a blood sample 24 h later to quantify multiple physiological metrics, including oxidative damage (i.e. d-ROMs), circulating amounts of triglyceride and glycerol, and levels of the acute phase protein haptoglobin. After birds had fledged, we identified and counted parasitic nest mites (*Dermanyssus* spp. and *Ornithonyssus* spp.). We found that only nestlings injected with 1 mg LPS kg^{-1} body mass, which is a common dosage in ecoimmunological studies, lost more body mass than individuals from other treatment groups. However, every dose of LPS resulted in a commensurate increase in oxidative damage. Parasitic mite abundance had no effect on oxidative damage across treatments. The amount of oxidative damage correlated with haptoglobin levels, suggesting compensatory mechanisms to limit self-damage during an immune response. We conclude that while only the highest-intensity immune challenges resulted in costs related to body mass, even low-intensity immune challenges result in detectable increases in oxidative damage.

KEY WORDS: Ecoimmunology, Infection intensity, LPS, Oxidative damage, Parasitism, Triglyceride

INTRODUCTION

Hosts benefit from immunologically resisting pathogens and parasites, yet hosts may avoid maximizing their response because of the high costs of an activated immune system (Sadd and Siva-Jothy, 2006). For free-living animals, the cost of the immune response can manifest within multiple contexts. Animals mounting an immune response demonstrate sickness behaviors (Lopes et al., 2021; Sköld-Chiriac et al., 2014) that can increase predation risk (Adelman et al., 2017). Animals may also experience physiological costs, including increases in metabolic requirements (Agugliaro et al., 2020), changes in circulating nutrient levels (Armour et al., 2020; Frisard et al., 2010), loss of body mass (Owen-Ashley et al., 2006; Palacios et al., 2011) or an increase in oxidative damage (Baylor and Butler, 2019).

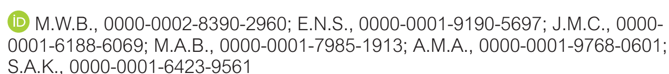
Some of these costs are integral to processes that result in the successful elimination of pathogens. For example, inducing oxidative damage in pathogens reduces their ability to survive and reproduce, resulting in their elimination (Cohen et al., 2018; West et al., 2011). Oxidative damage occurs when there is an excess production of reactive oxygen species (ROS) that chemically interact with, and thereby alter the function of, biomolecules such as protein, lipid and DNA (Huang and Li, 2020). However, not all ROS interact solely with the pathogenic target. Some ROS interact with the host's tissues, resulting in oxidative damage of those tissues. Thus, as part of the inflammatory response, organisms also deploy anti-inflammatory processes that limit pro-oxidant activity and consequently the extent of self-damage. For example, the acute phase protein haptoglobin is upregulated during inflammation (Raju et al., 2019) to promote anti-inflammatory processes and eliminate free hemoglobin, which has pro-oxidant properties (Nielsen and Moestrup, 2009). Thus, positive correlations between oxidative damage and haptoglobin (Armour et al., 2020; Costantini et al., 2015) are frequently interpreted within the framework that an increase in haptoglobin will mitigate oxidative damage (Fritze et al., 2019), although the nature of this relationship may depend upon disease severity (Sebastiano et al., 2018).

The underlying physiological processes of such immune response-related costs are not always clear. Loss of body mass during an immune challenge is often identified in ecoimmunological studies (Hegemann et al., 2012; Palacios et al., 2011; Sköld-Chiriac et al., 2014), and is likely the result of a reduction in food consumption (Ben-Hamo et al., 2017) or an increase in metabolic rate (Agugliaro et al., 2020). However, the changes in circulating nutrient levels that would be characteristic of such changes are not always consistent. During immune challenges, many animals exhibit a decrease in circulating triglyceride levels (Armour et al., 2020; Frisard et al., 2010; Griss et al., 2019; Shini et al., 2008), but others exhibit no change (Baylor and Butler, 2019) or even an increase (González et al., 2019) in circulating triglyceride levels. Circulating glycerol levels can increase in response to infection (Gimbo et al., 2015), or remain constant (Baylor and Butler, 2019).

Such variation in physiological metrics associated with an immune response could be a function of how robustly the individual is immunologically responding. The magnitude of immunological

[1]Department of Biology, Lafayette College, Easton, PA 18042, USA. [2]Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC 27695, USA. [3]Department of Ecology and Evolutionary Biology, University of Connecticut, Storrs, CT 06269, USA. [4]Institute for Systems Genomics, University of Connecticut, Storrs, CT 06269, USA.
*Present address: Tropical Conservation Biology and Environmental Science Program, University of Hawai'i, Hilo, HI 96720, USA. ‡Present address: College of Education, The Pennsylvania State University, University Park, PA 16802, USA.

§Author for correspondence (butlermw@lafayette.edu)


M.W.B., 0000-0002-8390-2960; E.N.S., 0000-0001-9190-5697; J.M.C., 0000-0001-6188-6069; M.A.B., 0000-0001-7985-1913; A.M.A., 0000-0001-9768-0601; S.A.K., 0000-0001-6423-9561

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costs is likely driven by infection intensity, as increases in infection intensity are associated with decreases in fitness (Adelman and Hawley, 2017). Dose–response curves for compounds that stimulate the immune system highlight the importance of dose. For example, investigations of fever-associated changes in body temperature have used dose–response experiments spanning multiple orders of magnitude of lipopolysaccharide (LPS) administration (Skold-Chiriac et al., 2015), a component of the cell walls of gram-negative bacteria that simulates bacterial infection. Recent work has demonstrated that there are detectable increases of oxidative damage at relatively low doses of LPS, while body mass is not affected unless relatively high doses are used (Armour et al., 2020).

Variation in infection intensity across a range of pathogens and parasites also highlights how immunological costs vary, with different metrics displaying different response patterns. While magnificent frigatebirds (*Fregata magnificens*) that demonstrate mild clinical signs of viral disease progression had elevated levels of oxidative damage, only individuals with severe clinical signs had elevated levels of the anti-inflammatory acute phase protein haptoglobin (Sebastiano et al., 2018). Inoculation of house finches (*Haemorhous mexicanus*) with different doses of bacteria (*Mycoplasma gallisepticum*) affected disease severity in complex patterns, with greater pathogen loads after primary infection, but reduced loads upon secondary infection (Leon and Hawley, 2017). Exposure to ectoparasites reduced levels of hemoglobin in Eastern bluebird (*Sialia sialis*) nestlings, but there was no effect on blood glucose levels (Grab et al., 2019). However, food-supplemented nestlings had increased immunological resistance to the parasites, which recovered hemoglobin levels (Knutie, 2020).

The goal of this study was to determine the physiological cost of simulated immune challenges in free-living nestling tree swallows, *Tachycineta bicolor* (Vieillot 1808). This species, in addition to being readily accessible because of its cavity-nesting behavior, has previously been used to address questions related to immune activation (Burness et al., 2018), oxidative damage (Stanton et al., 2017) and ectoparasitism (DeSimone et al., 2018). Specifically, we used a dose–response experimental design wherein we injected nestlings within a nest with concentrations of LPS that spanned two orders of magnitude to simulate variation in bacterial infection. We evaluated costs with respect to both the generation of oxidative damage and change in body mass. We also quantified metrics that would provide information on the underlying physiological processes of these costs, including how levels of haptoglobin were related to the generation of oxidative damage, and how circulating levels of triglyceride and glycerol relate to changes in body mass. We also characterized the nest parasite taxa, specifically parasitic mites (genera *Ornithonyssus* and *Dermanyssus*), to determine whether parasite load accounts for variation in metrics of nestling physiology in response to treatment.

MATERIALS AND METHODS

Nest monitoring, experimental procedure and sample collection

We monitored 83 Schwegler (Germany) wood-concrete nest boxes (3.8 cm hole diameter, item #110/8) that were mounted approximately 2.5 m above the ground on metal poles, checking each box at least twice per week. These boxes were located in an approximately 80 acre (∼32 ha) intramural athletics complex in eastern Pennsylvania, USA, that is composed predominantly of mown grass, and surrounded by farmed cropland and several trees. Once swallow eggs were laid, we monitored nests daily beginning 2 days prior to expected hatching date based on an 11 day incubation period from the day the last egg was laid (Winkler et al., 2011). Nests were then checked daily until all nestlings had hatched. When nestlings were 14 days old (Hogle and Burness, 2014; Pigeon et al., 2013), we affixed a Fisheries and Wildlife aluminium band to all individuals, measured body mass to the nearest 0.1 g, and measured right tarsus length to the nearest 0.1 mm. Each individual within a nest was randomly assigned a treatment, with no treatment represented twice in the nest until all other treatments were already represented. Specifically, individuals were injected in the apterium, anterior to the wing (Palacios et al., 2011), with 50 µl of a solution that resulted in doses of approximately 0, 0.01, 0.1 or 1.0 mg LPS kg^{-1} body mass (Armour et al., 2020).

Twenty-four hours later (mean: 24 h 02 min; range: 23 h 41 min to 24 h 16 min), which both aligns with LPS-induced increases in oxidative damage (Baylor and Butler, 2019; Marri and Richner, 2015) and controls for any diel effects in nutrient levels, we again measured body mass and collected approximately 250 µl of whole blood from each individual using heparinized capillary tubes. We placed blood samples on ice, and centrifuged whole blood at 12,000 ***g*** for 3 min within approximately 6 h of blood collection. Plasma was removed and stored at −80°C until further analysis (see below). Nestlings were returned to the nest, and we checked for nestling survival daily until all nestlings had fledged (n=90) or died (n=2). In total, we collected data from 92 nestlings in 22 nests. All applicable national and institutional guidelines for the care and use of animals were followed (IACUC protocols approved by Lafayette College, 19 April 2016, 27 January 2017).

Measurement of glycerol, triglyceride and haptoglobin

To quantify glycerol and triglyceride concentration in the plasma, we pipetted 240 µl glycerol reagent (F6428, Sigma-Aldrich) 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 ddH_2O water as the control blank and glycerol standard (G7793, Sigma-Aldrich) 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., 2016, 2020).

We measured haptoglobin using a colorimetric assay kit (TP-801, Tridelta DD, Kildare, Ireland). In accordance with kit directions, we added 60 µl of the provided reagent to each well of a clear, 96-well plate along with 5 µl of plasma sample or standard in duplicate. We then scanned the plate at 630 nm at 30°C to correct for initial differences in plasma color (Matson et al., 2012) using an Infinite M200Pro. Next, we added 84 µl of the chromogen reagent to each well and incubated the plate at 30°C for 5 min 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 damage

We quantified two different metrics of oxidative damage: TBARS and d-ROMs. TBARS quantifies thiobarbituric acid-reactive substances, with the predominant form being malondialdehyde (MDA), which is an end-product of lipid peroxidation. We quantified TBARS using a commercially available kit (TCA

method; 700870, Cayman Chemical, Ann Arbor, MI, USA). First, we mixed 50 µl of plasma with 50 µl of the provided trichloroacetic acid (TCA) solution, and then added 400 µl of the thiobarbituric (TBA)-based reagent. We vortexed this mixture and placed it in a heat block at 95°C for 1 h to allow MDA in the sample to bind with the TBA in the reagent. We then cooled the sample on ice for 10 min, and centrifuged all vials for 10 min at 1600 ***g*** at 4°C. We also prepared a standard curve based on the kit's directions and treated the standards identically to our samples. We then placed duplicate aliquots of 150 µl from each vial into a clear, 96-well plate and scanned each well at 533 nm.

We also measured organic hydroperoxides using the d-ROMs test (Diacron International). Hydroperoxides are oxidatively damaged biomolecules, including polyunsaturated fatty acids, proteins and nucleic acids (Costantini, 2016). We followed the kit's instructions, with the following modifications. First, we added 200 µl of the provided reagent (a 1:100 ratio of chromogen:buffer) to each well of a clear, 96-well plate. We then added 20 µl of plasma and the kit's calibrator in duplicate, and ddH_2O blank (as a control) in quadruplicate. We then incubated the plate at 37°C for 90 min and quantified absorbance at 505 nm.

Identifying and quantifying parasite abundance

We were able to collect 20 of the 22 nests within 48 h of when all nestlings had fledged. At this point, nests were carefully removed from the nest box and stored in a labeled, large (∼3.8 l) plastic bag. Nest material was then dissected over trays lined with a white piece of paper and all invertebrates were collected and preserved in 95% ethanol until identification. Nests contained parasitic, commensal and predatory mites and therefore we separated these groups before identifying the parasitic genera. Identifications were confirmed to major groups and specimens of parasitic Mesostigmata were slide-mounted using Hoyer's mounting medium, or by clearing first in 10% KOH at room temperature, followed by mounting in Hoyer's. All slides were cured on a slide warmer. Identifications of *Ornythonyssus* and *Dermanyssus* genera were performed under a dissecting microscope using published keys (Di Palma et al., 2012; Knee and Proctor, 2006; Murillo and Mullens, 2017).

Statistics

Data are available from Dryad (https://doi.org/10.5061/dryad.1vhhmgqt8). We analyzed all data using SAS (version 9.4, Cary, NC, USA), and *post hoc* tests compared LSMeans. Circulating glycerol and triglyceride levels were log-transformed, TBARS data were inverse-transformed, and haptoglobin levels were square root-transformed to meet the assumptions of normality. Using these transformations, residuals from all analyses were normally distributed using the Shapiro–Wilk test. To test for effects of treatment, we ran multiple linear mixed models (i.e. PROC MIXED) with nest as a random effect, treatment as a fixed effect, and pre-injection body mass, circulating glycerol circulating triglycerides, d-ROMs, TBARS and haptoglobin as dependent variables. We also ran the d-ROMs and TBARS models with circulating triglyceride levels as a covariate, as suggested (Pérez-Rodríguez et al., 2015). We also ran a mixed model with nest as a random effect, treatment as a fixed effect, pre-injection body mass as a covariate, and change in body mass during the 24 h experimental period as the dependent variable. To test for relationships between body mass and circulating lipid levels, we ran mixed models with nest as a random effect, pre-injection body mass and change in body mass as fixed effects, and either circulating glycerol or triglyceride levels as dependent variables. To investigate the relationship between haptoglobin and levels of oxidative damage (Sauerwein et al., 2005; Sebastiano et al., 2018), we ran mixed models with nest as a random effect, haptoglobin and circulating triglyceride level as fixed effects, and either d-ROMS or TBARS as dependent variables.

To examine the relationship between mite presence and nestling physiology, we categorized the 20 nests for which we had ectoparasite data as either having parasitic mites (n=12) or not (n=8). To test for the effect of parasitic mites on circulating haptoglobin, triglyceride or glycerol levels, we ran mixed models with nest as a random effect, and treatment, mite presence/absence and their interaction as fixed effects. We ran similar models for change in body mass as a dependent variable, but also included the covariate of pre-injection body mass. Lastly, we ran similar models for both d-ROMs and TBARS as dependent variables, but also included the covariate of circulating triglyceride levels.

RESULTS

Body mass prior to injection did not differ significantly between treatment groups, but individuals injected with 1.0 mg LPS kg^{-1} body mass lost more body mass than their nest mates during the 24 h experimental period (Table 1, Fig. 1). LPS had a dose-dependent effect on d-ROMs, with an increase in dose associated with an increase in levels of oxidative damage (Table 1, Fig. 2), although there was no effect on TBARS levels (Table 1). LPS administration did not affect circulating levels of glycerol, triglyceride or haptoglobin (Table 1).

Neither pre-injection body mass ($F_{1,67}$=0.10; P=0.75) nor change in body mass during the experimental period ($F_{1,67}$=1.74; P=0.19) was associated with circulating triglyceride levels. However, circulating glycerol levels were greater in individuals that lost more body mass during the experimental period ($F_{1,67}$=4.26; P=0.043), although there was no effect of pre-injection body

Table 1. Administration of lipopolysaccharide (LPS) affects maintenance of body mass and one metric of oxidative damage

Dependent variable	Covariate	*F*	d.f.	*P*
Body mass, pre-injection		0.64	3,67	0.59
Change in body mass		12.51	3,65	**<0.0001**
	Body mass, pre-injection	20.47	1,65	**<0.0001**
Circulating glycerol		1.99	3,66	0.12
Circulating triglyceride		0.19	3,66	0.90
d-ROMs		53.60	3,63	**<0.0001**
	Circulating triglyceride	6.25	1,63	**0.015**
TBARS		0.82	3,65	0.49
	Circulating triglyceride	16.05	1,65	**0.0002**
Haptoglobin		1.27	3,65	0.29

Circulating levels of glycerol and triglycerides were log transformed, TBARS was inverse-transformed, and haptoglobin was square-root transformed. Bold indicates significance.

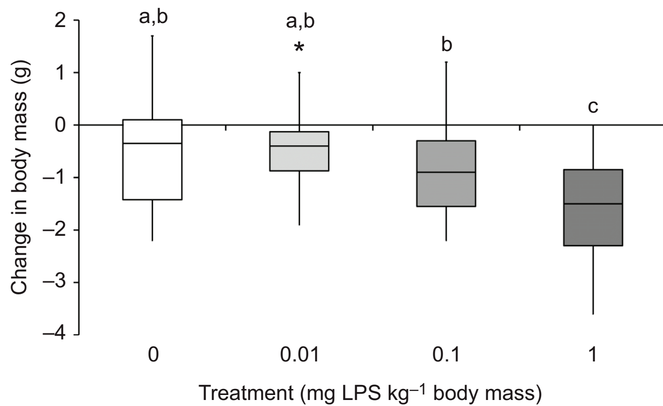

Fig. 1. A high dose of lipopolysaccharide (LPS) is associated with a loss of body mass. Box plots depict the median (central horizontal line), the interquartile range (box) and the range of observed values falling within 1.5× the interquartile range (whiskers), and any observed values falling outside this range (asterisks) based on 92 nestlings in 22 nests. Same for Fig. 2. Statistically significant differences (LSMeans, P<0.05) among treatment levels are denoted with different letters. Body mass pre-injection was a statistically significant covariate ($F_{1,65}$=20.47, P<0.0001). Raw data are plotted, and statistical models accounted for the non-independence of nest mates. Residuals were normally distributed.

mass ($F_{1,67}$=0.43; P=0.52). Both circulating triglyceride levels ($F_{1,65}$=5.11; P=0.027) and haptoglobin concentration ($F_{1,65}$=10.78; P=0.0017; Fig. 3) were positively related to levels of d-ROMs. However, while circulating triglyceride levels were positively related to TBARS levels ($F_{1,66}$=17.47; P<0.0001), haptoglobin concentration was not ($F_{1,66}$=1.07; P=0.31).

The presence of parasitic mites in a nest did not affect any morphological or physiological variable (Table 2), although there was a non-statistically significant trend for the presence of mites in a nest to be associated with greater haptoglobin levels within nestlings (Table 2; P=0.072). Inclusion of mite presence did not change the general statistical patterns associated with the effect of LPS on d-ROMs or change in body mass, and there were no statistically significant interactions between treatment and mite presence in any model (Table 2).

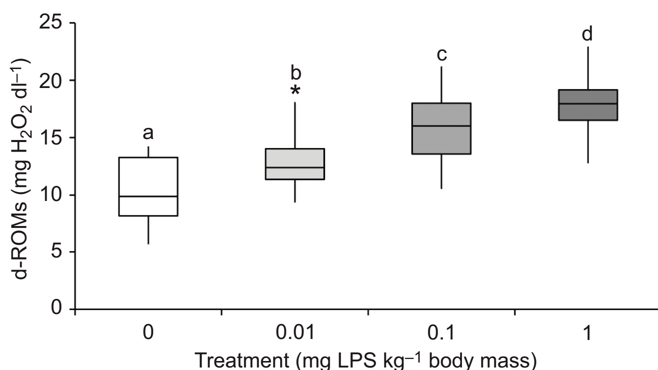

Fig. 2. An increased dose of LPS is associated with a greater amount of oxidative damage. Oxidative damage was assessed with d-ROMs. Box plots depict the median (central horizontal line), the interquartile range (box) and the range of observed values falling within 1.5× the interquartile range (whiskers) based on 92 nestlings in 22 nests. Statistically significant differences (LSMeans, P<0.05) among treatment levels are denoted with different letters. The log-transformed circulating triglyceride level was a statistically significant covariate ($F_{1,63}$=6.25, P=0.015). Raw data are plotted, and statistical models accounted for the non-independence of nest mates. Residuals were normally distributed.

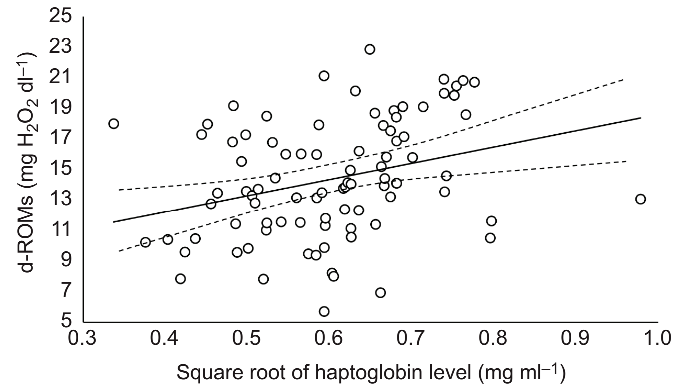

Fig. 3. Increased levels of circulating haptoglobin are associated with increased amounts of oxidative damage. Oxidative damage was assessed with d-ROMs. Raw data are plotted from 92 nestlings in 22 nests, and statistical models accounted for the non-independence of nest mates. Slope=10.53, intercept=8.05; 95% confidence interval is indicated by dashed curves.

DISCUSSION

We found that the highest levels of immune activation due to simulated infection intensity resulted in both the production of oxidative damage and loss of body mass, a pattern that is consistent with previously identified costs of immune activation (Armour et al., 2020; Palacios et al., 2011). However, we also found that intermediate and even low levels of simulated bacterial infection resulted in the significant production of oxidative damage, and that these costs occurred in the absence of effects on metabolism or body mass maintenance. Because increases in oxidative damage (Costantini and Dell'Omo, 2015; Stanton et al., 2017) and decreases in nestling body mass (Naef-Daenzer and Grüebler, 2016) can negatively affect survival, these data suggest that the degree of infection intensity can differentially affect traits associated with fitness.

To simulate bacterial infection, we administered LPS across a range of doses, including 1.0 mg LPS kg^{-1} body mass, which is similar to the dosage used in many avian ecoimmunological studies (Baylor and Butler, 2019; Merrill et al., 2017; Toomey et al., 2010), including work on tree swallows (Burness et al., 2018). LPS induces an inflammatory response (Batista et al., 2019) when it binds to toll-like receptors on macrophages (Park and Lee, 2013), and can thus be an important mediator of the intensity of an immune response (Dickson and Lehmann, 2019). Such pro-inflammatory conditions are associated with increases in oxidative damage (Hoffmann and Griffiths, 2018), and we detected a clear, dose-dependent effect of LPS on oxidative damage as assessed by organic hydroperoxide levels (i.e. d-ROMs). However, those patterns were not evident based on data from the TBARS assay, which targets MDA quantification. Such a disconnect between MDA and organic hydroperoxide values has been identified previously (Herborn et al., 2011), and there are two potential reasons for this discrepancy. First, d-ROMs quantifies organic hydroperoxides, which broadly encompasses oxidatively damaged biomolecules that also have the potential to result in further oxidative damage (Skrip and McWilliams, 2016), while TBARS putatively measures MDA, an end-product of lipid peroxidation. Thus, d-ROMs may have captured information related to an on-going oxidative process that had yet to result in sufficient accumulation of MDA. Second, the TBARS assay may not be specific to MDA, and can be affected by variation in circulating levels of aldehydes, sugar and urea

Table 2. Presence of parasitic mites does not affect any measured physiological markers

	LPS dose			Mite presence			Interaction			Covariate		
Physiological response	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
Change in body mass	10.60	3,51	**<0.0001**	0.16	1,51	0.69	0.68	3,51	0.57	9.62	1,51	**0.0031**
Circulating glycerol	1.53	3,52	0.22	0.21	1,52	0.65	0.32	3,52	0.81			
Circulating triglyceride	0.17	3,52	0.92	0.62	1,52	0.43	0.28	3,52	0.84			
d-ROMs	36.86	3,49	**<0.0001**	0.01	1,49	0.92	1.67	3,49	0.19	8.36	1,49	**0.0057**
TBARS	0.83	3,51	0.49	0.41	1,51	0.53	0.48	3,51	0.71	14.03	1,51	**0.0005**
Haptoglobin	0.96	3,51	0.42	3.37	1,51	0.072	0.91	3,51	0.44			

Effects on change in body mass included the covariate of pre-injection body mass, and effects on both d-ROMs and TBARS levels included the covariate of circulating triglyceride levels. Bold indicates significance.

(Langille et al., 2018). Based on this potential lack of specificity of the TBARS assay, the robustness of the d-ROMs assay to capture information related to oxidative damage (Costantini, 2016) and previously identified associations between administration of LPS and resulting organic hydroperoxide production (Armour et al., 2020; Baylor and Butler, 2019; Riedel et al., 2003), the d-ROMs assay appears to robustly capture variation in oxidative damage due to LPS administration.

This generation of oxidative damage in response to simulated bacterial infection can have important fitness-related consequences in free-living animals (Costantini, 2019). Higher levels of oxidative damage are associated with decreases in survival (Costantini and Dell'Omo, 2015), including in tree swallows (Stanton et al., 2017). However, the amount of oxidative damage does not always predict survival probability (Boonekamp et al., 2018), particularly if oxidative damage is quantified as MDA (Bodey et al., 2020; Losdat et al., 2018; Meniri et al., 2020). This lack of a consistent correlation between oxidative damage and survival is unsurprising, considering different studies compare different biochemical markers in varying tissues at different time points in an animal's life (Costantini, 2019). Thus, it is unclear the extent to which the variation in oxidative damage we detected predicts future survival. More concretely, it is unclear whether it is the pathogen, the energetically expensive activated immune response, or the resulting generation of oxidative damage that is the causal factor in affecting subsequent survival probability in immune-challenged individuals. Disentangling the extent to which various components of infection or parasitism affect fitness, including the generation of oxidative damage during an immune response, will allow for greater precision of predictions that relate the degree of infection intensity to fitness.

Although we found a robust pattern linking the generation of oxidative damage and simulated bacterial infection, we did not detect any effect of parasitic mite presence on the generation of oxidative damage. Several potential explanations can account for this lack of relationship. First, the correlational nature of this study, wherein ectoparasite levels were not manipulated, allows for the possibility that inter-nest variation in ectoparasite load may be driven by some additional factor (e.g. nest location; Veiga and Valera, 2020) that obscured any potential relationships between the presence of parasitic mites and oxidative damage. Second, the overall levels of parasitism were relatively low, as the number of individual parasitic mites was in the single digits for all but two nests. Thus, nest mite abundance may have simply been too low for immune system detection and thus had no effect alone, or with the LPS treatment, on oxidative damage. Lastly, it is also possible that nestlings are more tolerant of mites (e.g. by reducing parasite damage without reducing parasite fitness), rather than immunologically resistant, as observed with other avian host–nest parasite studies (Grab et al., 2019; Knutie et al., 2016). To address these explanations, future studies could experimentally manipulate parasite abundance (increase and decrease load) and characterize mechanisms of resistance and/or tolerance.

In addition to oxidative damage, large immune responses are energetically demanding (Burness et al., 2010; Demas et al., 2003; Hegemann et al., 2012; Lochmiller and Deerenberg, 2000) and associated with loss of body mass (Cornelius et al., 2017; Smith et al., 2017). In swallow nestlings, we found that a reduction of body mass occurred only at the highest dose of LPS. Because low nestling body mass can be associated with reduced subsequent survival (Brown and Brown, 2018; Naef-Daenzer and Grüebler, 2016), a large infection or just a large immune response could negatively affect fitness. Additionally, plasma glycerol levels are predicted to be highest when animals are relying on energetic reserves, and thus engaging in lipolysis (Fokidis et al., 2012; Guglielmo et al., 2005; Neuman-Lee et al., 2015). Given the LPS-dependent loss of body mass, and an inverse relationship between change in body mass and circulating glycerol levels, our data collectively support the energetically expensive nature of a robust immune response, requiring the mobilization of stored nutrients. Such mobilization of stored nutrients has been associated with increases in oxidative damage (Webb et al., 2019), highlighting the overlapping of physiological pathways associated with oxidative physiology, metabolism and the immune response. However, the ability to maintain body mass except with the highest dose of LPS stands in contrast to oxidative costs that occurred across all ranges of simulated bacterial infection. Thus, while a range of immune responses entails costs, these costs are not necessarily proportional, and selection may favor individuals that can eliminate infections when infection intensities are low, prior to the development of high levels of infection that require disproportionately high costs.

One approach to evaluating the mechanisms underlying variation in costs of infection is to investigate the relationships among different physiological pathways. For example, we found a positive correlation between organic hydroperoxide production and circulating haptoglobin concentration. However, it is unlikely that haptoglobin is causing an increase in oxidative damage; rather, haptoglobin is upregulated during inflammation (Raju et al., 2019) and likely functions to reduce the scope of oxidative damage (Fritze et al., 2019) and limit pro-inflammatory processes (Arredouani et al., 2005; Nielsen and Moestrup, 2009). Thus, selection may favor a proportional increase in haptoglobin in response to a pro-inflammatory stimulus such that the inflammatory response is robust enough to decrease pathogen fitness, while reducing the likelihood of an overly damaging and energy-intensive inflammatory response. However, because haptoglobin is also released in response to injury (Lee et al., 2015) and generally mitigates the pro-oxidant effects of heme (Loomis et al., 2017), its antioxidant function may be differentially expressed according to

the specific immunological context. Future investigations into the relative investment in physiological processes for different stimuli or infection intensities could elucidate the immunological strategy individuals evince at different levels of infection.

In conclusion, we found that the highest levels of simulated bacterial infection resulted in changes to body mass, as well as oxidative damage. However, physiological costs were not restricted to just the highest levels of infection, as substantially lower levels of simulated bacterial infection also entailed increased levels of oxidative damage. This association between LPS and oxidative damage in free-living, nestling tree swallows is similar to the pattern identified in adult captive quail (Armour et al., 2020). Together, these studies suggest that a robust relationship between simulated bacterial infection and the degree of oxidative damage may be pervasive within Aves. To evaluate the generality of this relationship, studies at various life stages (e.g. during breeding or migration), and using species that occupy different ecological niches across multiple avian orders will be critical. Additionally, the fitness-related consequences of these moderate increases in oxidative damage remain an underexplored avenue for future research.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.W.B., S.A.K.; Methodology: M.W.B., S.A.K.; Formal analysis: M.W.B.; Investigation: M.W.B., E.N.S., J.M.C., M.A.B., A.M.A., S.A.K.; Writing - original draft: M.W.B.; Writing - review & editing: E.N.S., J.M.C., M.A.B., A.M.A., S.A.K.; Visualization: M.W.B.; Supervision: M.W.B.

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Data availability
Data are available from the Dryad digital repository (Butler, 2021): dryad.1vhhmgqt8

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