Effects of a Bacterial Infection on Mitochondrial Function and Oxidative Stress in a Songbird

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ABSTRACT

As a major physiological mechanism involved in cellular renewal and repair, immune function is vital to the body's capacity to support tissue maintenance and organismal survival. Because immune defenses can be energetically expensive, the activities of metabolically active organs, such as the liver, are predicted to increase during infection by most pathogens. However, some pathogens are immunosuppressive, which might reduce the metabolic capacities of select organs to suppress immune response. *Mycoplasma gallisepticum* (MG) is a well-known immunosuppressive bacterium that infects domestic chickens and turkeys as well as songbirds. In the house finch (*Haemorhous mexicanus*), which is the primary host for MG among songbird species, MG infects both the respiratory system and the conjunctiva of the eye, causing conspicuous swelling. To study the effect of a systemic bacterial infection on cellular respiration and oxidative damage in the house finch, we measured mitochondrial respiration, mitochondrial membrane potential, reactive oxygen species production, and oxidative damage in the livers of house finches that were wild caught and either infected with MG, as indicated by genetic screening for the pathogen, or free of MG infection. We observed that MG-infected house finches showed significantly lower oxidative lipid and protein damage in liver tissue compared with their uninfected counterparts. Moreover, using complex II substrates, we documented a nonsignificant trend for lower state 3 respiration of liver mitochondria in MG-infected house finches compared with uninfected house finches ($P = 0.07$). These results are consistent with the hypothesis that MG suppresses organ function in susceptible hosts.

Keywords: Mycoplasma gallisepticum, mitochondrial membrane potential, oxidative phosphorylation (OXPHOS), immunosuppression.

Introduction

When an animal host is faced with an immune challenge, the body typically responds by upregulating both innate and adaptive immune defenses that reduce or eliminate the pathogen (Hoebe et al. 2004). Most hosts also upregulate renewal and repair processes to mitigate damage (McDade 2005; Rauw 2012). The energetic demands of such responses to a systemic pathogen infection have been shown to necessitate an increase in the basal metabolic rate, typically by 5%–15% (Martin et al. 1990; Demas et al. 1997; Ots et al. 2001; Nilsson 2003; Hasselquist and Nilsson 2012; King and Swanson 2013). Because the energy investment required to eliminate a pathogen from an animal's system can be high, most hosts rely on some degree of tolerance to mitigate pathogen invasion (Bonneaud et al. 2017). Yet this paradigm of defense and tolerance by the host is not consistent across all host-pathogen interactions because some pathogens have evolved mechanisms to manipulate the response by the host, including downregulation of the host's protective immune system (Bonneaud et al. 2012, 2018).

The production of proteins and the fueling of processes required to mount an immune response require substantial energy production by mitochondria (Demas 2004; Lane and Martin 2010; Koch et al. 2017), so assessment of mitochondrial function can potentially provide key insights into the energy investment by and energy manipulation of the host. The behavior of mitochondria can be quantified using absolute respiration rates, which thereby serve as straightforward indicators of the mitochondrial function (Brand and Nicholls 2011). Mitochondrial state 2 respiration is often quantified as an indicator of leak respiration. It is measured with either complex I or complex II substrates but without ADP (Nicholls and Ferguson 2002). The addition of ADP following state 2 respiration

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allows the ATP synthase to function, the mitochondrial membrane potential (MMP) to drop, and the electron transport to accelerate to its maximal level. State 3 respiration is defined as the measure of maximum oxygen utilization by the mitochondria. When the ATP-to-ADP ratio approaches equilibrium, MMP rises, proton reentry through the ATP synthase stops, and respiration slows to eventually reach mitochondrial state 4 respiration. State 4 respiration is a measure of the basal respiration rate of the mitochondria (Wong et al. 2017). The mitochondrial respiratory control ratio (RCR) is defined as the ratio of state 3 to state 4 respiration. RCR varies with the coupling efficiency of the mitochondria and is a strong indicator of respiratory capacity. Combining these parameters of mitochondrial respiration, ideally in parallel with MMP, becomes an important assessment of organismal bioenergetics performance (Brand and Nicholls 2011).

Along with ATP, free radicals are inevitably produced during oxidative phosphorylation (Barja 2007; Bratic and Trifunovic 2010). When the production of free radicals (reactive species) exceeds the capacity of cells to reduce their reactivity, cells are said to experience oxidative stress (Zhang et al. 2017). Damage to lipids, proteins, and DNA that is caused by reactive species may ultimately reduce mitochondrial and cellular performance, which could have fitness consequences (Speakman and Garratt 2014). The liver could be one of the important organs in these processes because it not only ensures sufficient supplies of energy and substrates during immune responses (Knolle and Gerken 2000) but also contains many immunologically active cells, which interact directly with antigens (Racanelli and Rehermann 2006).

Mycoplasma gallisepticum (MG) is a bacterium that is a well-known pathogen of domestic poultry. In 1994, a poultry lineage of MG underwent a significant host shift in Maryland, infecting a population of house finches (*Haemorhous mexicanus*; Ley et al. 1996; Hochachka and Dhondt 2000; Farmer et al. 2002). MG spread rapidly through the house finch population in eastern North America (Dhondt et al. 1998) and subsequently spread across the northern states to California and then to Arizona (Duckworth et al. 2003; Staley et al. 2017). In poultry, MG is primarily a respiratory disease (Yoder 1991), but in the house finch, in addition to causing a respiratory infection, MG infects the conjunctiva of the eye, causing conspicuous swelling (Roberts et al. 2001).

Interestingly, MG has been shown to have immune- and metabolic-suppressive properties (Rharbaoui et al. 2002; Ganapathy and Bradbury 2003; Mohammed et al. 2007). Mitochondria could be a prime target for such suppression because they both provide the source of energy required to mount an immune response and are directly involved in immune activation and signaling (West et al. 2011; Weinberg et al. 2015; Koch et al. 2017). For example, mitochondria communicate with the immune system through its redox status and tricarboxylic acid cycle metabolites. Recent studies on mitochondrial complex II have identified that complex II substrate (succinate) serves as a direct link between the mitochondrial respiratory chain and immune activation (Murphy and O'Neill 2018). Consequently, targeting mitochondria could be a potential mechanism for the immune- and metabolic-suppressive properties (such as host cells avoiding pathogen-killing reactive oxygen species [ROS]) of MG, resulting in less energy demand and reduced levels of oxidative damage in tissues.

MG has been found in the liver of infected chickens, and histological changes to the liver have also been reported (Kerr and Olson 1967). Moreover, livers of MG-infected birds had decreased levels of antioxidants such as catalase, glutathione, and glutathione-S-transferase, but glutathione reductase levels remained unchanged (Vitula et al. 2011). Given the vital importance of the liver in supporting energetically demanding processes, an MG-induced immune response will likely alter energy allocation to other demanding physiological processes. This is exemplified in the work of Nolan et al. (2004), who reported a trade-off between reproduction and the immune response in MG-infected house finches.

In this study, we assessed the effect of MG infection in house finches on the performance of liver mitochondria. We hypothesized that redox homeostasis (steady state of redox status; see Ursini et al. 2016) of the liver and the function of mitochondria should be affected by pathogen exposure in a manner that enhances an individual's ability to cope with the demands of an immune challenge. We predicted that if the immune system activation and response of MG infection are energy demanding, then mitochondrial respiratory capacities should be elevated. In addition, higher respiratory capacity should result in high MMP that would enable birds to cope with the high energy and nutrient demands induced by the immune defense system. We further predicted that MG-infected birds would exhibit elevated ROS production and higher oxidative damage compared with healthy birds. Alternatively, if MG suppresses the host's immune and metabolic system by targeting mitochondria, lower mitochondrial respiratory function and oxidative damage would be observed.

Material and Methods

Bird Capture and Tissue Collection

All animal handling and tissue collection procedures were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2016–2922). Juvenile house finches were collected with traps at feeding stations in August 2017 in Auburn, Alabama (Hill 2002). This population of house finches has been coevolving with MG since 1995, 24 yr at the time of this study (Bonneaud et al. 2011). After capture, birds were transferred to the lab, where a choanal swab (FLOQSwabs, Copan, Murrieta, CA) was collected from each bird. At the same time, the severity of disease in both eyes of each bird was scored on a five-point scale by three different observers according to Farmer et al. (2002). House finches were then euthanized with an overdose of isoflurane (~30 s of exposure). This brief exposure to isoflurane is predicted to have little impact on mitochondrial performance (Lee et al. 2015). After the birds' death, livers were quickly removed and weighed. The left lobe and most of the right lobe of the liver were used for mitochondrial isolation. A small

piece of the right lobe was removed, flash frozen in liquid nitrogen, then stored at −80°C for later analyses. Choanal swabs were shipped on dry ice to the University of Exeter for quantification of MG infection.

Mitochondrial Measurements

Mitochondrial Isolation. Mitochondria were isolated following procedures outlined previously (Hill et al. 2019). The mitochondrial isolation and function measurements were optimized specifically for house finches. Briefly, the fresh liver was minced and then homogenized in a Potter-Elvehjem PTFE pestle and glass tube in isolation buffer (250 mM sucrose, 5 mM HEPES, 1 mM EGTA, pH = 7.4 at 40°C; Hill et al. 2019). The resulting homogenate was centrifuged, and the supernatant was then decanted through cheesecloth and centrifuged again. The resulting supernatant was discarded, and the mitochondria pellet was washed in liver isolation solution. This solution was centrifuged again, and the final mitochondria pellet was suspended in a mannitol-sucrose solution.

Mitochondrial Respiration. Mitochondrial respiration was determined polarigraphically (Oxytherm, Hansatech Instruments, Norfolk, UK) following procedures outlined previously (Hill et al. 2019). The temperature for the chambers was set at 40°C, matching avian body temperature. Mitochondrial respiration buffer included 100 mM KCl, 50 mM MOPS, 10 mM KH_2PO_4, 10 mM $MgCl_2$, 1 mM EGTA, 70 mM sucrose, 220 mM mannitol, and 0.2% w/v free fatty acid BSA, pH = 7.4 at 40°C. In the respiration chamber, isolated mitochondria were added to respiration buffer at a concentration of 0.35 mg/mL of mitochondrial protein. Mitochondrial protein levels were measured by a Bradford assay (VWR Scientific, Radnor, PA). In one chamber, respiration was measured using 2 mM pyruvate, 2 mM malate, and 10 mM glutamate as a substrate. In the second chamber, respiration was measured using 5 mM succinate as a substrate with 5 μM rotenone to inhibit complex I. State 2 respiration was defined as the rate of respiration in the presence of mitochondria and substrates. State 3 respiration (maximum mitochondrial respiration) was defined as the rate of respiration in the presence of ADP and was initiated by adding 0.25 mM ADP to the chamber containing buffered mitochondria and respiratory substrates. State 4 respiration (basal mitochondrial respiration) was measured after the phosphorylation of ADP was complete. States 2, 3, and 4 respiration were normalized to mitochondrial protein level. RCR was calculated by dividing state 3 respiration by state 4 respiration.

Mitochondrial Membrane Potential. We measured MMP by following Lambert et al. (2008) using the potential-sensitive dye safranin O. Isolated mitochondria were incubated in standard buffer containing 3 mM HEPES, 1 mM EGTA, 0.3% (w/v) BSA, 1 μg/mL of oligomycin, and 120 mM potassium chloride (pH = 7.2 at 40°C) at a concentration of 0.35 mg/mL of mitochondrial protein in standard buffer with 5 μM safranin O. Change in fluorescence was measured in a cuvette using Spectramax M (Molecular Devices, Sunnyvale, CA) at an excitation of 533 nm and an emission of 576 nm at 40°C. At the end of each run, membrane potential was dissipated by adding 2 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). The relative decrease in fluorescent signal on energization of the mitochondria is used to represent the membrane potential. Results are reported as the absolute magnitude of this change in fluorescence, with larger changes in relative fluorescence units indicating higher membrane potentials. Relative fluorescence units were normalized to mitochondrial protein level.

H_2O_2 Emission. The H_2O_2 emission in isolated mitochondria was quantified using Amplex Red (Thermofisher, Waltham, MA; Zhang et al. 2017; Hill et al. 2019). Formation of resorufin (Amplex Red oxidation) by H_2O_2 was measured at an excitation wavelength of 545 nm and an emission wavelength of 590 nm using a Synergy H1 hybrid plate reader (BioTek, Winooski, VT) at 37°C in a 96-well plate using succinate. To eliminate carboxylesterase interference, 100 μM phenylmethyl sulfonyl fluoride was added to the experimental medium immediately before measurement according to Miwa et al. (2016). Readings of resorufin formation were recorded every 5 min for 15 min, and a slope (rate of formation) was produced from these. The obtained slope was then converted to the rate of H_2O_2 production using a standard curve and normalized to mitochondrial protein levels.

Mitochondrial Density. The citrate synthase (CS) activity was measured in liver homogenate and used as a proxy for mitochondrial density (Spinazzi et al. 2012). Buffer for the CS activity assay included 100 mM Tris at pH = 8.0, 0.1% (v/v) Triton X-100, 100 μM DTNB, and 300 μM acetyl-CoA with 1 μg of isolated liver mitochondria. Baseline activity was measured for 2 min, and reactions were started by adding the final concentration of 0.5 mM oxaloacetic acid and measured for 3 min at 412 nm using a Synergy H1 hybrid plate reader. CS activity is widely used as a biomarker for mitochondrial content in tissues (Larsen et al. 2012).

Western Blots

Western blots were conducted on liver samples to analyze peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α, a key regulator of mitochondrial biogenesis; GTX37356, GeneTex), a marker of lipid peroxidation (4-hydroxynonenal [4-HNE]; ab46545, Abcam, Cambridge, MA), and a marker of protein oxidation (protein carbonyls; OxyBlot S7150, EMD Millipore, Billerica, MA). Each membrane was stained with Ponceau and was used as the loading and transfer control. A chemiluminescent system was used to visualize marked proteins (GE Healthcare Life Sciences, Pittsburgh, PA). Images were taken and analyzed with the ChemiDoc-It imaging system (UVP, Upland, CA).

MG Load

Bacterial load was measured by quantitative amplification of MG DNA from conjunctival swabs obtained at capture. DNA

was extracted using a QIAGEN DNeasy blood and tissue kit according to the manufacturer's standard protocol. Multiplex quantitative polymerase chain reactions (PCRs) for *mgc2* and *rag1* in each sample were then conducted using an Applied Biosystems StepOnePlus real-time PCR system (Tardy et al. 2019). Reactions were run in a 20-μL volume containing 2μL of sample genomic DNA template, 1 μL each of 10 μM mgc110-F/R and rag1-102-F/R primers (total of 4 uL), 0.5 μL each of 10 μM MGc110-JOE and Rag1-102-6FAM fluorescent hydrolysis probes (total of 1 μL), 10 μL of 2 × qPCRBIO Probe Mix Hi-ROX (PCR Biosystems), and 3 μL of nuclease-free water (Ambion). Reactions were run at 95°C for 3 min, followed by 45 cycles at 95°C for 1 s and 60°C for 20 s. Samples were run in duplicate alongside serial dilutions of plasmid-based standards. Amplification data were exported to LinRegPCR version 2017.1 for calculation of individual reaction efficiencies and quantification of low-amplification samples; between-run variation was normalized using Factor-qPCR version 2016.0, with plasmid standard serial dilutions used for factor correction.

Statistics

We first used linear models including the factor sex to compare markers between MG-infected and uninfected birds. However, adding or removing sex as a covariant yields similar results for mitochondrial respiration and oxidative status markers, so it was removed from statistical analyses (table A1). We employed analysis comparing MG-infected birds with uninfected birds for mitochondrial respiration, MMP, H_2O_2 production rate, CS activity, and oxidative damage markers using two-sample *t*-tests. We then tested the severity of the MG infection using Pearson's correlation coefficient between MG load and all markers in only MG-infected birds (table A2). Linear models were performed with R (R Development Core Team 2013). Other statistical analyses were performed with SigmaStat 3.5 (Systat Software, Point Richmond, CA). Significance was established at $\alpha = 0.05$.

Results

In this study, 55 house finches (36 males, 19 females) were captured. After quantification of MG load in these house finches, 28 birds were MG positive, and 27 birds were MG negative (no quantitative PCR amplification). MG loads were positively correlated with the eye score using Spearman's rank correlation coefficient ($r_s = 0.86$, $P < 0.01$; fig. 1).

Mitochondrial isolations failed in four (two MG-infected and two uninfected) birds. When complex I substrates (glutamate, malate, and pyruvate) were used, mitochondrial states 2, 3, and 4 respiration (fig. 2*A*), RCR (fig. 2*C*), and MMP (fig. 2*D*) did not differ between MG-infected and uninfected birds ($P > 0.10$).

When complex II substrate (succinate) was used, mitochondrial states 2 and 4 respiration (fig. 2*B*), RCR (fig. 2*C*), and MMP (fig. 2*D*) did not differ between groups ($P > 0.36$). However, there was a nonsignificant trend where MG-infected birds had lower state 3 respiration compared with uninfected birds ($t_{49} = 1.83$, $P = 0.07$; fig. 2*B*).

Both the H_2O_2 production rate from isolated liver mitochondria and CS activities in liver tissue did not vary between MG-infected and uninfected birds ($P > 0.32$; fig. 3*A*, 3*B*). Interestingly, both oxidative damage markers for lipid (4-HNE: $t_{53} = 2.19$, $P = 0.03$) and protein (protein carbonyl: $t_{53} = 2.34$, $P = 0.02$) oxidation were lower in MG-infected birds compared with uninfected birds (fig. 3*C*). PGC-1α (key regulator of mitochondrial biogenesis) protein levels were not different between MG-infected and uninfected birds ($P = 0.24$; fig. 3*C*).

Next, we asked whether the severity of the MG infection impacted mitochondrial performance. Relative MG load only showed negative but nonsignificant correlation ($r = -0.36$,

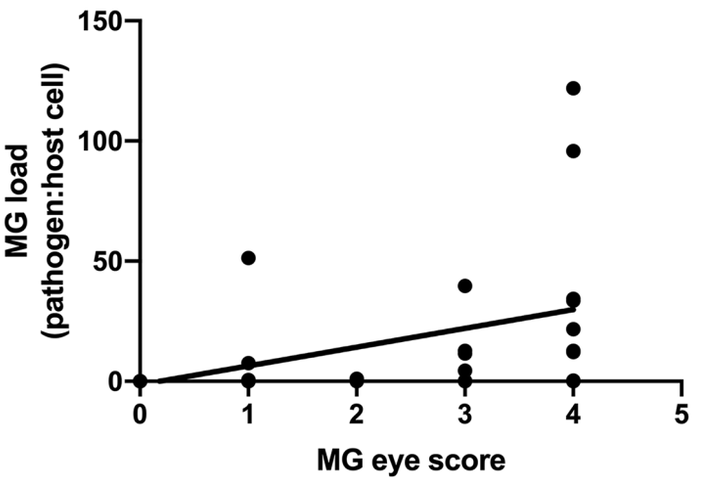

Figure 1. Correlation between *Mycoplasma gallisepticum* (MG) eye score and MG load in house finches.

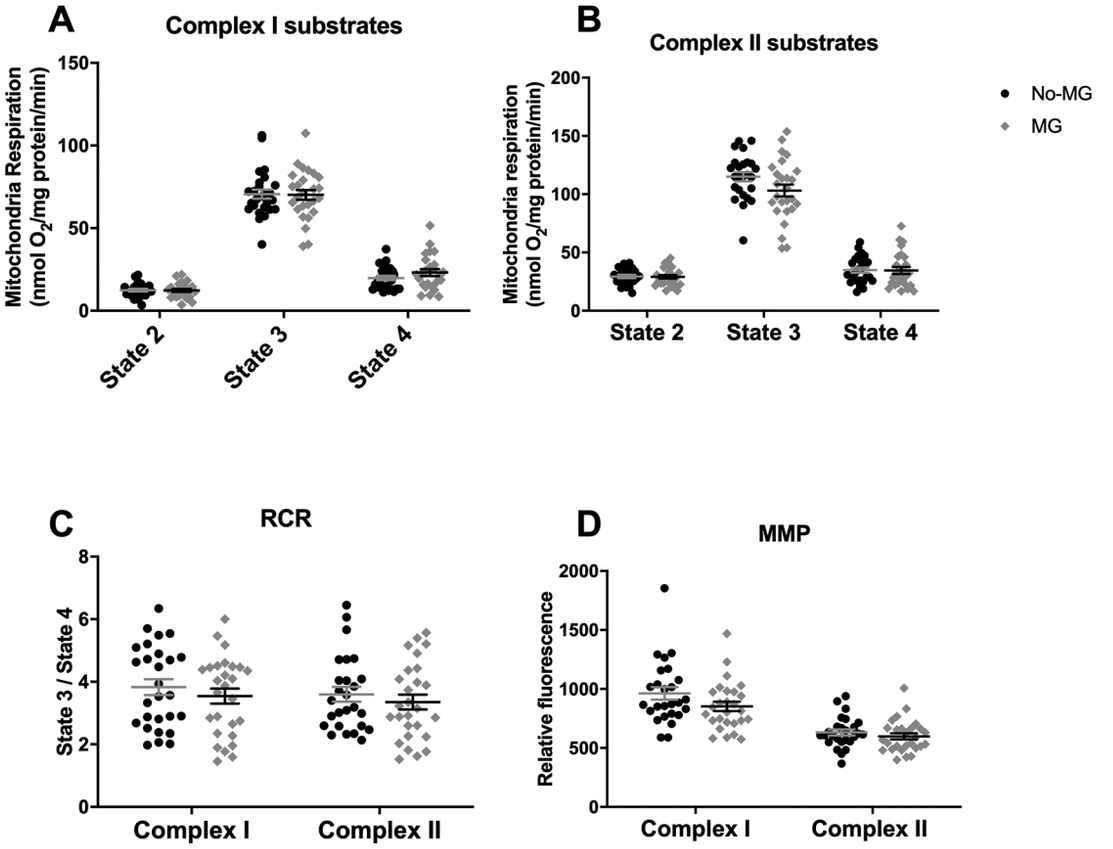

Figure 2. Mitochondrial respiration and mitochondrial membrane potential (MMP) from isolated liver mitochondria from birds infected with *Mycoplasma gallisepticum* (MG) and uninfected birds. Data include states 2, 3, and 4 respiration rates with complex I (2 mM pyruvate, 2 mM malate, and 10 mM glutamate; *A*) and complex II (5 mM succinate with 5 μM rotenone; *B*) substrates, respiration control ratio (RCR; *C*), and MMP (*D*). Dots represent uninfected birds, and diamonds represent MG-infected birds. Data are presented as mean ± SEM.

$P = 0.06$) with RCR using complex I substrates in MG-infected birds. Relative MG load was not significantly correlated with other mitochondrial respiration, MMP, H_2O_2 production rate, CS activity, or oxidative damage markers ($P > 0.66$; table A2).

Discussion

We investigated the effects of MG infection on mitochondrial respiratory function, MMP, H_2O_2 production rate, and oxidative damage in the livers of wild house finches. As the metabolic hub of organisms, the liver plays a central role in carbohydrate, lipid, and protein metabolism and provides both nutrients and substrates to the immune system (Rui 2011). Lymphocyte T cells and B cells are found scattered throughout the liver, where they play critical roles in first-line immune defense against invading pathogens (Racanelli and Rehermann 2006). Consequently, redox homeostasis of the liver and the function of mitochondria should be affected by pathogen exposure in a manner that enhances an individual's ability to cope with the demands of an immune challenge (Costantini 2019). The obvious prediction at the initiation of this study was that MG-infected house finches would have higher respiration and greater oxidative damage in liver cells than uninfected individuals. Contrary to this prediction, we found that MG-infected house finches showed decreased oxidative lipid and protein damage compared with uninfected house finches. Moreover, a nonsignificant trend for lower mitochondrial respiration using complex II substrates was observed for MG-infected birds compared with their uninfected counterparts. These observations suggest that MG-infected individuals may have lower liver respiratory rates with less oxidative damage than uninfected birds.

Mycoplasmas, including MG, can suppress the immune system to evade detection (Rharbaoui et al. 2002; Ganapathy and Bradbury 2003; Mohammed et al. 2007). One explanation for the observations in the current study is that MG suppressed the immune systems of infected house finches, leading to decreased energy demand and reduced levels of oxidative damage in livers. To upregulate aerobic capacity, cells can modify either mitochondrial density or mitochondrial respiratory functions. In this study, mitochondrial density, which is represented by CS activity, did not vary with the presence or absence of MG, and it did not correlate with MG load in the house finch liver. However, state 3

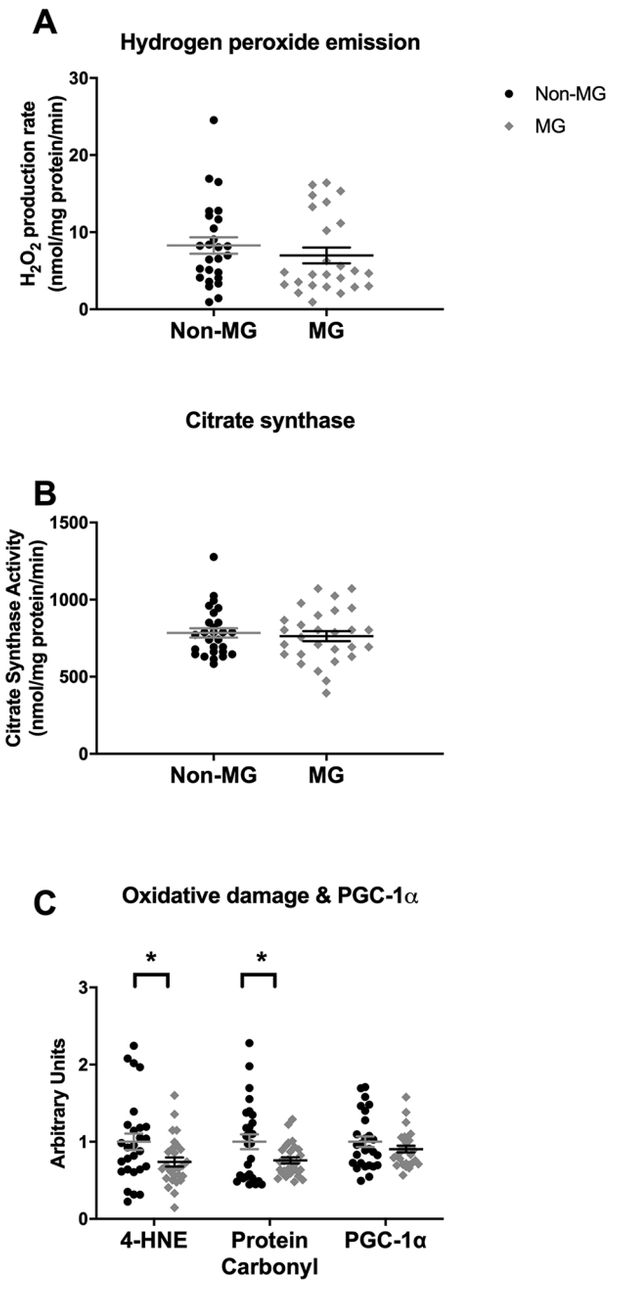

Figure 3. H_2O_2 production rate from isolated liver mitochondria (*A*), citrate synthase activities in liver tissues (*B*), and relative protein levels of lipid peroxidation (4-hydroxynonenal [4-HNE]), protein carbonyl, and peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α; *C*) in birds infected with *Mycoplasma gallisepticum* (MG) and uninfected birds. Dots represent uninfected birds, and diamonds represent MG-infected birds. Data are presented as mean ± SEM. Asterisks indicate statistical significance at $P < 0.05$.

respiration with complex II substrates might be suppressed by MG infection. Thus, our data suggest that such suppression was achieved by manipulating mitochondrial functions other than mitochondrial density. Results of PGC-1α, a mitochondrial biogenesis marker (LeBleu et al. 2014), further support the assertion that mitochondrial biogenesis, turnover rate, and density were not affected by MG infection.

Research by Gaunson et al. (2000) and Bonneaud et al. (2011) indicates that MG is immunosuppressive in both chickens and house finches, especially during the later stages of the immune response. Furthermore, microarray data showed that, following experimental infection, susceptible individuals (i.e., nongenetically resistant) displayed lower levels of expression of genes regulating metabolism, such as prosaposin and spermidine/spermine N1-acetyltransferase, than genetically resistant individuals (Bonneaud et al. 2011). The cDNA macroarray also indicated that a number of genes associated with redox status and the electron transport system, such as cytochrome oxidase subunit I and III and NADH dehydrogenase subunit 4, are downregulated in the MG-infected house finches (Wang et al. 2006). Together, these findings suggest that the immunosuppression by MG modulated not only the capacity of the host's immune system in order to evade detection but also the respiratory functions of mitochondria in the liver.

In previous studies with this same population of house finches, which has coevolved with MG for about 24 yr at the time of this study, observations suggested that birds in Alabama (same location as house finches in this study) were less susceptible to immunosuppression than house finches from naive western populations (Bonneaud et al. 2011). However, those conclusions were drawn from observations of infection experiments in which MG infection was imposed on randomly sampled birds (Bonneaud et al. 2011). In a follow-up study on this coadapted population, evidence for immunosuppression was observed in birds with drab feather coloration (low-condition birds) and not in birds with bright feather coloration (high-condition birds; Balenger et al. 2015). In the current study, we compared naturally infected house finches with house finches that had avoided or resisted infection in the same environment. In such a comparison, the sample of infected birds is not randomly drawn from the population but is likely to be biased toward birds that are susceptible to infection and immunosuppression.

The most surprising finding of this study is the lower levels of oxidative lipid and protein damage in MG-infected birds compared with uninfected birds. Moreover, we did not observe any difference in the ROS production rate from liver mitochondria between MG-infected and uninfected house finches. Consequently, the observed decrease in oxidative damage could result from (1) a decrease in other cellular ROS-producing sites (such as NADPH oxidases), (2) an increase of antioxidant levels, or (3) an increase in systems that repair and replace oxidative damage. Unfortunately, we did not measure these parameters in this study, so we cannot distinguish between these explanations. Previous studies have proposed that oxidative damage in host cells and tissues increases in response to MG infection as a consequence of an increase in ROS generated by the host's immune system (Nunoya et al. 1987; Vitula et al. 2011). However, there have been few direct measures of oxidative damage in MG-infected birds. Using total thiobarbituric acid reactive species (TBARS) as a marker, Vitula et al. (2011) found that lipid peroxidation was lower in plasma but higher in the lungs and unchanged in other organs, including the liver, in MG-infected birds. Using the same marker, McGraw

et al. (2013) did not observe a difference in TBARS levels in plasma between MG-infected versus uninfected house finches. However, owing to the lack of full validation of TBARS measurements in biological fluids, the reliability of TBARS as a biomarker of lipid peroxidation has been questioned (Khoubnasabjafari et al. 2015).

A number of studies documented that antioxidant defense systems were suppressed by MG in most host tissues, especially liver tissue (Nunoya et al. 1987; Jenkins et al. 2008; Vitula et al. 2011). As a result, it is unlikely that the observed low level of oxidative damage in MG-infected birds was due to the upregulation of antioxidant defense systems. At the same time, we did not observe any differences in the H_2O_2 production rate in isolated liver mitochondria from the MG-infected or uninfected group. There are a few potential explanations for these observations. First, H_2O_2 production was measured with succinate as the substrate without the presence of ADP (state 2 respiration) in this study. Using succinate at this respiratory state, most H_2O_2 was produced from the quinone site of complex I and the Q_o site of complex III (Quinlan et al. 2013). Although I_Q and III_{Q_o} sites are the primary sites of H_2O_2 production during the resting stage in normal mitochondria, it is possible that other sites of H_2O_2 production were decreased because of MG infection (Brand 2016). Second, in this study, the H_2O_2 production rate was measured with unlimited substrates. During MG infection, it is likely that ATP demand increased for MG-infected birds. As a result, the redox state may have been more oxidized for MG-infected birds, resulting in low H_2O_2 production and eventually leading to low oxidative damage (Murphy 2009). Third, other H_2O_2 production sites, such as the NOX family of NADPH oxidases, may have been influenced by MG infection (Bedard and Krause 2007). The oxidases are often employed for pathogen killing in host cells (Segal et al. 2012), where it is possible for MG to suppress ROS production to avoid the immune defense system of the host cells. In sum, MG-infected house finches exhibited reduced levels of lipid peroxidation and protein carbonylation, which is likely due to the immune- and metabolic-suppressive properties of MG.

It is important to note that in the current study, MG-infected birds contracted MG in the wild. Consequently, birds were captured and sampled across a range of infection stages. Moreover, we do not know whether MG-negative birds had never been infected with MG or had been previously infected and recovered. However, because all birds that we sampled had hatched less than 5 mo before capture, it is likely that this was the first bout of MG experienced by infected birds and that MG-negative birds had never been infected. Moreover, MG can be found in the livers of infected house finches (Kerr and Olson 1967), but MG load in the livers of these finches was not measured. Further studies on the immunosuppressive properties of MG and bioenergetics should focus on MG load in tissues, mitochondrial respiration, and oxidative stress levels for birds at different stages of MG infection. Another factor that may affect the results is the sex of the animal. In the current study, sex did not influence much in the parameters measured (table A1), but future experiments that can further discern, among other parameters, the complex relationships between sex, age, and infection load should be planned.

Mock immune challenges without pathogen infection have been shown to result in upregulations of basal and maximal metabolic rates (Martin et al. 2003; Eraud et al. 2005; King and Swanson 2013; Bonneaud et al. 2016), mitochondrial respiration (Frisard et al. 2015), and oxidative stress (Bertrand et al. 2006). Such immune challenges, however, cannot mimic potential immunosuppressive effects of a pathogen infection. Moreover, it is unclear whether the MG-infected house finches were in the acute or chronic stages of the infection. Hence, novel insights, such as our observation of the limited effects of pathogen infection on oxidative stress and respiratory function following MG infection, should not be unexpected when the physiological strategies of both the host and the pathogen are allowed to play out.

Oxidative stress has been used as a marker to study immune response trade-offs in avian species (Costantini 2008; Costantini and Møller 2009). Moreover, much has been written in recent years about the need to include direct measures of oxidative damage and/or ROS rather than only measures of antioxidants as a proxy measure (Monaghan et al. 2009; Isaksson et al. 2011*a*, 2011*b*; Selman et al. 2012; Metcalfe and Monaghan 2013; Speakman et al. 2015). We encourage researchers to not only measure ROS levels and oxidative defense and damage levels but also incorporate bioenergetics parameters to achieve a multitarget evaluation (Zhang et al. 2018). For the current study, if we had measured only markers of oxidative damage, we would have incorrectly concluded that MG has beneficial effects on an avian host. Such a conclusion would have missed key immune- and metabolic-suppressive consequences of MG infection. These outcomes further emphasize the importance of using mitochondrial bioenergetic markers when studying oxidative stress as physiological mechanisms underlying life-history strategies (Zhang and Hood 2016; Hood et al. 2018*a*, 2018*b*).

In conclusion, we have shown that both lipid peroxidation and protein carbonylation were lower in MG-infected house finches compared with uninfected house finches. Moreover, nonsignificant trends were observed for low state 3 respiration with complex II substrates in MG-infected house finches compared with their control. Such reduction in oxidative damage and mitochondrial respiration markers demonstrated the immune- and metabolic-suppressive properties of MG. Future studies of mechanisms underlying such immune and metabolic suppression for MG are needed.

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APPENDIX

Effect of Sex on the Measure of Mitochondrial Function

For this investigation, we collected samples for both male and female house finches. Using a linear model, we tested for an effect of sex on all of the variables described herein. Because sex had a significant effect on only one variable (mitochondrial membrane potential), we reran the comparison as a *t*-test and reported those values. We report the results of both models in table A1.

Correlations among Variables

While the relationships between mitochondrial physiology variables are complex, evaluating the correlations among variables can be informative. We present a correlation matrix in table A2.

Table A1: Full linear model results between birds infected with *Mycoplasma gallisepticum* (MG) and uninfected birds including sex as a covariant for all measurements and two-sample *t*-test results between MG-infected and uninfected birds not including sex

	Results of linear model					Results of *t*-test		
		Effect of sex		Effect of MG				
	df	*t*	*P*	*t*	*P*	df	*t*	*P*
Complex I substrates:								
State 2 (nmol O_2/mg protein/min)	2, 48	.55	.59	.08	.94	49	.12	.91
State 3 (nmol O_2/mg protein/min)	2, 49	.59	.56	.31	.76	50	.10	.92
State 4 (nmol O_2/mg protein/min)	2, 49	.64	.52	1.03	.31	50	1.35	.18
RCR (state 3/state 4 respiration)	2, 51	1.29	.20	.32	.75	52	.82	.42
MMP (relative fluorescence)	2, 50	3.52	<.01	2.93	<.01	51	1.67	.10
Complex II substrates:								
State 2 (nmol O_2/mg protein/min)	2, 48	.68	.50	.06	.95	49	.19	.85
State 3 (nmol O_2/mg protein/min)	2, 48	.19	.85	1.63	.11	49	1.83	.07
State 4 (nmol O_2/mg protein/min)	2, 48	.33	.74	.05	.96	49	.07	.94
RCR (state 3/state 4 respiration)	2, 50	.34	.73	.81	.42	51	.74	.46
MMP (relative fluorescence)	2, 50	2.19	.03	1.65	.10	51	.93	.36
ROS, oxidative damage, and biogenesis:								
H_2O_2 (nmol/min/mg protein)	2, 48	.78	.44	1.11	.27	49	.88	.38
4-HNE adducts (arbitrary units)	2, 52	.81	.42	1.79	.08	53	2.19	.03
Protein carbonyl (arbitrary units)	2, 52	1.82	.07	1.66	.10	53	2.34	.02
PGC-1α (arbitrary units)	2, 52	.29	.78	1.20	.24	53	1.18	.24
Citrate synthase (nmol/min/mg protein)	2, 52	1.29	.20	.02	.98	53	.48	.64

Note. Complex I substrates include 2 mM pyruvate, 2 mM malate, and 10 mM glutamate. Complex II substrates include 5 mM succinate with 5 μM rotenone. RCR = respiratory control ratio; MMP = mitochondrial membrane potential; ROS = reactive oxygen species; 4-HNE = 4-hydroxynonenal; PGC-1α = peroxisome proliferator-activated receptor gamma coactivator 1α.

Table A2: Pearson's correlation coefficient of *Mycoplasma gallisepticum* (MG) load, mitochondrial respiration, and oxidative damage markers in MG-infected birds

	Complex I substrates					Complex II substrates					ROS, oxidative damage, and biogenesis				
	State 2	State 3	State 4	RCR	MMP	State 2	State 3	State 4	RCR	MMP	4-HNE	Protein-CO	PGC-1α	CS	H_2O_2
MG load	.09	−.03	.24	−.36	.16	.13	.03	.11	−.01	−.05	.04	−.09	.02	.02	.08
Complex I substrates:															
State 2		.04	.19	−.18	.05	.44*	.21	.34	−.19	.36	−.14	−.08	−.16	−.06	.20
State 3			.37	−.04	.37	.34	.66**	.29	.12	.41*	.21	−.26	.21	.20	.40
State 4				−.87**	.49*	.33	.40*	.67**	−.39*	.38	−.14	−.16	.11	−.29	−.09
RCR					−.39*	−.15	−.06	−.51**	.43*	−.24	.21	.06	−.13	.38	.22
MMP						.28	.29	.43*	−.24	.34	.00	−.01	.40*	.06	.05
Complex II substrates:															
State 2							.72**	.48*	−.04	.50**	.01	−.23	−.24	−.12	.23
State 3								.504**	.13	.54**	.19	−.10	−.13	.07	.30
State 4									−.74**	.46*	−.29	.00	−.23	−.28	−.05
RCR										−.09	.54**	.00	.34	.25	.16
MMP											.06	−.34	.08	.04	.09
ROS, oxidative damage, and biogenesis:															
4-HNE												−.17	.34	.03	−.13
Protein-CO													.07	−.21	−.25
PGC-1α														−.01	−.38
CS															.48*

Note. Complex I substrates include 2 mM pyruvate, 2 mM malate, and 10 mM glutamate. Complex II substrates include 5 mM succinate with 5 μM rotenone. RCR = respiratory control ratio; MMP = mitochondrial membrane potential; ROS = reactive oxygen species; 4-HNE = 4-hydroxynonenal; PGC-1α = peroxisome proliferator-activated receptor gamma coactivator 1α; protein-CO = protein carbonyl; CS = citrate synthase.

*$P < 0.05$.

**$P < 0.01$.

Literature Cited

Balenger S.L., C. Bonneaud, S.A. Sefick, S.V. Edwards, and G.E. Hill. 2015. Plumage color and pathogen-induced gene expression in a wild bird. Behav Ecol 26:1100–1110.

Barja G. 2007. Mitochondrial oxygen consumption and reactive oxygen species production are independently modulated: implications for aging studies. Rejuvenation Res 10: 215–224.

Bedard K. and K.H. Krause. 2007. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87:245–313.

Bertrand S., F. Criscuolo, B. Faivre, and G. Sorci. 2006. Immune activation increases susceptibility to oxidative tissue damage in zebra finches. Funct Ecol 20:1022–1027.

Bonneaud C., S.L. Balenger, G.E. Hill, and A.F. Russell. 2012. Experimental evidence for distinct costs of pathogenesis and immunity against a natural pathogen in a wild bird. Mol Ecol 21:4787–4796.

Bonneaud C., S.L. Balenger, A.F. Russell, J. Zhang, G.E. Hill, and S.V. Edwards. 2011. Rapid evolution of disease resistance is accompanied by functional changes in gene expression in a wild bird. Proc Natl Acad Sci USA 108:7866–7871.

Bonneaud C., M. Giraudeau, L. Tardy, M. Staley, G.E. Hill, and K.J. McGraw. 2018. Rapid antagonistic coevolution in an emerging pathogen and its vertebrate host. Curr Biol 28: 2978–2983.

Bonneaud C., I. Sepil, L. Wilfert, and R. Calsbeek. 2017. Plasmodium infections in natural populations of *Anolis sagrei* reflect tolerance rather than susceptibility. Integr Comp Biol 57:352–361.

Bonneaud C., R.S. Wilson, and F. Seebacher. 2016. Immune-challenged fish up-regulate their metabolic scope to support locomotion. 11:e0166028.

Brand M.D. 2016. Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. Free Radic Biol Med 100:14–31.

Brand M.D. and D.G. Nicholls. 2011. Assessing mitochondrial dysfunction in cells. Biochem J 435:297–312.

Bratic I. and A. Trifunovic. 2010. Mitochondrial energy metabolism and ageing. Biochim Biophys Acta 1797:961–967.

Costantini D. 2008. Oxidative stress in ecology and evolution: lessons from avian studies. Ecol Lett 11:1238–1251.

———. 2019. Understanding diversity in oxidative status and oxidative stress: the opportunities and challenges ahead. J Exp Biol 222:jeb194688.

Costantini D. and A.P. Møller. 2009. Does immune response cause oxidative stress in birds? a meta-analysis. Comp Biochem Physiol A 153:339–344.

Demas G.E. 2004. The energetics of immunity: a neuroendocrine link between energy balance and immune function. Horm Behav 45:173–180.

Demas G.E., V. Chefer, M.I. Talan, and R.J. Nelson. 1997. Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. Am J Physiol 273: R1631–R1637.

Dhondt A.A., D.L. Tessaglia, and R.L. Slothower. 1998. Epidemic mycoplasmal conjunctivitis in house finches from eastern North America. J Wildl Dis 34:265–280.

Duckworth R.A., A.V. Badyaev, K.L. Farmer, G.E. Hill, and S.R. Roberts. 2003. First case of *Mycoplasma gallisepticum* infection in the western range of the house finch (*Carpodacus mexicanus*). Auk 120:528–530.

Eraud C., O. Duriez, O. Chastel, and B. Faivre. 2005. The energetic cost of humoral immunity in the collared dove, *Streptopelia decaocto*: is the magnitude sufficient to force energy-based trade-offs? Funct Ecol 19:110–118.

Farmer K.L., G.E. Hill, and S.R. Roberts. 2002. Susceptibility of a naive population of house finches to *Mycoplasma gallisepticum*. J Wildl Dis 38:282–286.

Frisard M.I., Y. Wu, R.P. McMillan, K.A. Voelker, K.A. Wahlberg, A.S. Anderson, N. Boutagy, K. Resendes, E. Ravussin, and M.W. Hulver. 2015. Low levels of lipopolysaccharide modulate mitochondrial oxygen consumption in skeletal muscle. Metabolism 64:416–427.

Ganapathy K. and J. Bradbury. 2003. Effects of cyclosporin A on the immune responses and pathogenesis of a virulent strain of *Mycoplasma gallisepticum* in chickens. Avian Pathol 32:495–502.

Gaunson J.E., C.J. Philip, K.G. Whithear, and G.F. Browning. 2000. Lymphocytic infiltration in the chicken trachea in response to *Mycoplasma gallisepticum* infection. Microbiology 146:1223–1229.

Hasselquist D. and J.-Å. Nilsson. 2012. Physiological mechanisms mediating costs of immune responses: what can we learn from studies of birds? Anim Behav 83:1303–1312.

Hill G.E. 2002. A red bird in a brown bag: the function and evolution of colorful plumage in the house finch. Oxford University Press, Oxford.

Hill G.E., W.R. Hood, Z. Ge, R. Grinter, C. Greening, J.D. Johnson, N.R. Park, H.A. Taylor, V.A. Andreasen, and M.J. Powers. 2019. Plumage redness signals mitochondrial function in the house finch. Proc R Soc B 286:20191354.

Hochachka W.M. and A.A. Dhondt. 2000. Density-dependent decline of host abundance resulting from a new infectious disease. Proc Natl Acad Sci USA 97:5303–5306.

Hoebe K., E. Janssen, and B. Beutler. 2004. The interface between innate and adaptive immunity. Nat Immunol 5: 971.

Hood W.R., S.N. Austad, P. Bize, A.G. Jimenez, K.L. Montooth, P.M. Schulte, G.R. Scott, I. Sokolova, J.R. Treberg, and K. Salin. 2018*a*. The mitochondrial contribution to animal performance, adaptation, and life-history variation. Integr Comp Biol 58:480–485.

Hood W.R., Y. Zhang, A. Mowry, H. Hyatt, and A. Kavazis. 2018*b*. Life history trade-offs within the context of mitochondrial hormesis. Integr Comp Biol 58:567–577.

Isaksson C., B.C. Sheldon, and T. Uller. 2011*a*. The challenges of integrating oxidative stress into life-history biology. Bioscience 61:194–202.

Isaksson C., G.M. While, M. Olsson, J. Komdeur, and E. Wapstra. 2011*b*. Oxidative stress physiology in relation to

life history traits of a free-living vertebrate: the spotted snow skink, *Niveoscincus ocellatus*. Integr Zool 6:140–149.

Jenkins C., R. Samudrala, S.J. Geary, and S.P. Djordjevic. 2008. Structural and functional characterization of an organic hydroperoxide resistance protein from *Mycoplasma gallisepticum*. J Bacteriol 190:2206–2216.

Kerr K.M. and N.O. Olson. 1967. Pathology in chickens experimentally inoculated or contact-infected with *Mycoplasma gallisepticum*. Avian Dis 11:559–578.

Khoubnasabjafari M., K. Ansarin, and A. Jouyban. 2015. Reliability of malondialdehyde as a biomarker of oxidative stress in psychological disorders. BioImpacts 5:123–127.

King M.O. and D.L. Swanson. 2013. Activation of the immune system incurs energetic costs but has no effect on the thermogenic performance of house sparrows during acute cold challenge. J Exp Biol 216:2097–2102.

Knolle P.A. and G. Gerken. 2000. Local control of the immune response in the liver. Immunol Rev 174:21–34.

Koch R.E., C.C. Josefson, and G.E. Hill. 2017. Mitochondrial function, ornamentation, and immunocompetence. Biol Rev 92:1459–1474.

Lambert A.J., J.A. Buckingham, H.M. Boysen, and M.D. Brand. 2008. Diphenyleneiodonium acutely inhibits reactive oxygen species production by mitochondrial complex I during reverse, but not forward electron transport. Biochim Biophys Acta 1777:397–403.

Lane N. and W. Martin. 2010. The energetics of genome complexity. Nature 467:929.

Larsen S., J. Nielsen, C.N. Hansen, L.B. Nielsen, F. Wibrand, N. Stride, H.D. Schroder, R. Boushel, J.W. Helge, and F. Dela. 2012. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. J Physiol 590: 3349–3360.

LeBleu V.S., J.T. O'Connell, K.N. Gonzalez Herrera, H. Wikman, K. Pantel, M.C. Haigis, F.M. de Carvalho, et al. 2014. PGC-1α mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. Nat Cell Biol 16:992.

Lee Y.-M., B.C. Song, and K.-J. Yeum. 2015. Impact of volatile anesthetics on oxidative stress and inflammation. BioMed Res Int 2015:242709.

Ley D.H., J.E. Berkhoff, and J.M. McLaren. 1996. *Mycoplasma gallisepticum* isolated from house finches (*Carpodacus mexicanus*) with conjunctivitis. Avian Dis 40:480–483.

Martin A., E.A. Dunnington, W.B. Gross, W.E. Briles, R.W. Briles, and P.B. Siegel. 1990. Production traits and alloantigen systems in lines of chickens selected for high or low antibody responses to sheep erythrocytes. Poult Sci 69:871–878.

Martin L.B., A. Scheuerlein, and M. Wikelski. 2003. Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? Proc R Soc B 270: 153–158.

McDade T.W. 2005. Life history, maintenance, and the early origins of immune function. Am J Hum Biol 17:81–94.

McGraw K.J., M. Giraudeau, G.E. Hill, M.B. Toomey, and M. Staley. 2013. Ketocarotenoid circulation, but not retinal carotenoid accumulation, is linked to eye disease status in a wild songbird. Arch Biochem Biophys 539:156–162.

Metcalfe N.B. and P. Monaghan. 2013. Does reproduction cause oxidative stress? an open question. Trends Ecol Evol 28:347–350.

Miwa S., A. Treumann, A. Bell, G. Vistoli, G. Nelson, S. Hay, and T. von Zglinicki. 2016. Carboxylesterase converts Amplex Red to resorufin: implications for mitochondrial H_2O_2 release assays. Free Radic Biol Med 90:173–183.

Mohammed J., S. Frasca Jr., K. Cecchini, D. Rood, A.C. Nyaoke, S.J. Geary, and L.K. Silbart. 2007. Chemokine and cytokine gene expression profiles in chickens inoculated with *Mycoplasma gallisepticum* strains R_{low} or GT5. Vaccine 25:8611–8621.

Monaghan P., N.B. Metcalfe, and R. Torres. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecol Lett 12:75–92.

Murphy M.P. 2009. How mitochondria produce reactive oxygen species. Biochem J 417:1–13.

Murphy M.P. and L.A.J. O'Neill. 2018. Krebs cycle reimagined: the emerging roles of succinate and itaconate as signal transducers. Cell 174:780–784.

Nicholls D.G. and S.J. Ferguson. 2002. Bioenergetics. Elsevier, Amsterdam.

Nilsson J.-Å. 2003. Ectoparasitism in marsh tits: costs and functional explanations. Behav Ecol 14:175–181.

Nolan P.M., S.R. Roberts, and G.E. Hill. 2004. Effects of *Mycoplasma gallisepticum* on reproductive success in house finches. Avian Dis 48:879–885.

Nunoya T., M. Tajima, and T. Yagihashi. 1987. Decrease in catalase activity of cultured cells by *Mycoplasma gallisepticum* infection. Vet Microbiol 13:343–351.

Ots I., A.B. Kerimov, E.V. Ivankina, T.A. Ilyina, and P. Horak. 2001. Immune challenge affects basal metabolic activity in wintering great tits. Proc R Soc B 268:1175–1181.

Quinlan C.L., I.V. Perevoshchikova, M. Hey-Mogensen, A.L. Orr, and M.D. Brand. 2013. Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. Redox Biol 1:304–312.

Racanelli V. and B. Rehermann. 2006. The liver as an immunological organ. J Hepatology 43:S54–S62.

Rauw W.M. 2012. Immune response from a resource allocation perspective. Front Genet 3:267.

R Development Core Team. 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.

Rharbaoui F., B. Drabner, S. Borsutzky, U. Winckler, M. Morr, B. Ensoli, P.F. Mühlradt, and C.A. Guzmán. 2002. The *Mycoplasma*-derived lipopeptide MALP-2 is a potent mucosal adjuvant. Eur J Immunol 32:2857–2865.

Roberts S.R., P.M. Nolan, and G.E. Hill. 2001. Characterization of *Mycoplasma gallisepticum* infection in captive house finches (*Carpodacus mexicanus*) in 1998. Avian Dis 45:70–75.

Rui L. 2011. Energy metabolism in the liver. Comp Physiol 4: 177–197.

Segal B.H., M.J. Grimm, A.N.H. Khan, W. Han, and T.S. Blackwell. 2012. Regulation of innate immunity by NADPH oxidase. Free Radic Biol Med 53:72–80.

Selman C., J.D. Blount, D.H. Nussey, and J.R. Speakman. 2012. Oxidative damage, ageing, and life-history evolution: where now? Trends Ecol Evol 27:570–577.

Speakman J.R., J.D. Blount, A.M. Bronikowski, R. Buffenstein, C. Isaksson, T.B.L. Kirkwood, P. Monaghan, et al. 2015. Oxidative stress and life histories: unresolved issues and current needs. Ecol Evol 5:5745–5757.

Speakman J.R. and M. Garratt. 2014. Oxidative stress as a cost of reproduction: beyond the simplistic trade-off model. Bioessays 36:93–106.

Spinazzi M., A. Casarin, V. Pertegato, L. Salviati, and C. Angelini. 2012. Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. Nat Protoc 7:1235–1246.

Staley M., C. Bonneaud, K.J. McGraw, C.M. Vleck, and G.E. Hill. 2017. Detection of *Mycoplasma gallisepticum* in house finches (*Haemorhous mexicanus*) from Arizona. Avian Dis 62:14–17.

Tardy L., M. Giraudeau, G.E. Hill, K.J. McGraw, and C. Bonneaud. 2019. Contrasting evolution of virulence and replication rate in an emerging bacterial pathogen. Proc Natl Acad Sci USA 116:16927–16932.

Ursini F., M. Maiorino, and H.J. Forman. 2016. Redox homeostasis: the golden mean of healthy living. Redox Biol 8: 205–215.

Vitula F., L. Peckova, H. Bandouchova, M. Pohanka, L. Novotny, D. Jira, J. Kral, K. Ondracek, J. Osickova, and D. Zendulkova. 2011. *Mycoplasma gallisepticum* infection in the grey partridge *Perdix perdix*: outbreak description, histopathology, biochemistry and antioxidant parameters. BMC Vet Res 7:34.

Wang Z., K. Farmer, G.E. Hill, and S.V. Edwards. 2006. A cDNA macroarray approach to parasite-induced gene expression changes in a songbird host: genetic response of house finches to experimental infection by *Mycoplasma gallisepticum*. Mol Ecol 15:1263–1273.

Weinberg S.E., L.A. Sena, and N.S. Chandel. 2015. Mitochondria in the regulation of innate and adaptive immunity. Immunity 42:406–417.

West A.P., G.S. Shadel, and S. Ghosh. 2011. Mitochondria in innate immune responses. Nat Rev Immunol 11:389–402.

Wong H.-S., P.A. Dighe, V. Mezera, P.-A. Monternier, and M.D. Brand. 2017. Production of superoxide and hydrogen peroxide from specific mitochondrial sites under different bioenergetic conditions. J Biol Chem 292:16804–16809.

Yoder H., Jr. 1991. *Mycoplasma gallisepticum* infection. Dis Poult 9:198–212.

Zhang Y., A.L. Brasher, N.R. Park, H.A. Taylor, A.N. Kavazis, and W.R. Hood. 2018. High activity before breeding improves reproductive performance by enhancing mitochondrial function and biogenesis. J Exp Biol 221:jeb177469.

Zhang Y. and W.R. Hood. 2016. Current versus future reproduction and longevity: a re-evaluation of predictions and mechanisms. J Exp Biol 219:3177–3189.

Zhang Y., F. Humes, G. Almond, A.N. Kavazis, and W.R. Hood. 2017. A mitohormetic response to pro-oxidant exposure in the house mouse. Am J Physiol 314:R122–R134.