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Corticosterone and Mitochondrial Efficiency Are Associated With Changes in DNA Oxidative Damage During an Acute Stress Response in Leach's Storm-Petrels (*Hydrobates leucorhous*)

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ABSTRACT

The ability of organisms to effectively respond to challenges is critical for survival. We investigated how an acute stressor affected corticosterone, mitochondrial function, and DNA oxidative damage in a wild population of Leach's storm-petrels (*Hydrobates leucorhous*). We conducted a standardized 20-min handling procedure on storm-petrel chicks and collected baseline and post-handling blood samples. We measured plasma corticosterone and red blood cell DNA oxidative damage levels through the detection of a mutated DNA base 8-Hydroxy-2'-deoxyguanosine (8-OHdG). In addition, we quantified six measures of mitochondrial aerobic metabolism from red blood cells. Overall, the handling stressor increased plasma corticosterone levels and decreased mitochondrial efficiency to produce ATP. Although the increase in corticosterone was inversely related to the change in DNA oxidative damage, the decrease in mitochondrial efficiency was positively correlated with the change in DNA oxidative damage. Thus, over an acute stress response, individuals who had the largest increase in corticosterone also had the least amount of oxidative damage. In addition, individuals who prioritized ATP production during the acute stress also showed higher levels of oxidative damage. This work highlights the complex pathways by which corticosterone and mitochondrial efficiency affect oxidative damage during acute stress, providing new insights into the trade-offs underlying physiological responses in wild animals.

1 | Introduction

Overcoming challenges—whether internal or environmental—is a central component of fitness. Effectively responding to a challenge (or stressor) requires reallocation of energy and both behavioral and physiological modifications. The hormones and feedback loops of the endocrine system have received extensive attention as important mediators of responses to challenges

(Charmandari et al. 2005). Glucocorticoid hormones primarily regulate energy mobilization, a critical function that occurs even in the absence of a stressor (reviewed in MacDougall-Shackleton et al. 2019) by exerting complex effects on several physiological systems (Sapolsky 2000). As a result, glucocorticoids affect almost all organs and tissues in the body and have been estimated to influence approximately 20% of the expressed human genome (Chrousos and Kino 2005). Despite this

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Summary

- In response to an acute handling stressor, Leach's storm-petrel chicks who had the highest corticosterone responses exhibited reduced DNA oxidative damage, and individuals who prioritized ATP production experienced higher DNA oxidative damage.
- Additionally, corticosterone and mitochondrial efficiency independently predict DNA oxidative damage.

functional versatility, glucocorticoids have traditionally been studied in the context of the stress response, a focus established by Hans Selye's pioneering work (Selye 1936). However, while glucocorticoids are traditionally equated with stress (but see Jimeno and Verhulst 2023; MacDougall-Shackleton et al. 2019), measuring their levels alone does not provide insights into the molecular mechanisms that underlie the inevitable trade-offs associated with responding to a stressor (Wada 2019).

Over the past decade, growing interest has emerged in expanding stress biomarkers to provide a more integrative perspective on stress (Wada 2019; Romero et al. 2015; Bonier et al. 2009). Ultimately, responding to challenges to maintain homeostasis is an energy-driven process, a core principle of allostasis (Picard et al. 2018). Allostasis is the process by which the body actively adjusts and adapts to changing demands by dynamically regulating physiological systems to maintain stability through change (McEwen and Wingfield 2010). During a stress response, allostasis requires energy to drive gene expression, cellular responses, and broad integrative changes across behavioral and physiological systems, with this energetic demand primarily met by mitochondrial aerobic metabolism (Picard et al. 2018).

Mitochondria are organelles that function at the center of energy flow in animal cells (Koch et al. 2021; Picard et al. 2018), and thus play a major role in allostasis. Their role as a communication hub is highlighted by the fact that mitochondria are also the sites of steroid production (Picard et al. 2018). However, not only do mitochondria produce hormones, like glucocorticoids, but they are also a major target of these steroids (Picard et al. 2018; Lee et al. 2013; Du et al. 2009). By binding to mitochondrial glucocorticoid receptors, glucocorticoids enable mitochondria to actively respond to metabolic signals, underscoring their role in integrated stress physiology (Picard et al. 2018).

Energy in the mitochondria is harnessed by the electron transfer system, which is embedded in the inner mitochondrial membrane and drives ATP production by efficiently tying oxygen consumption to electron energy transfer (Berry et al. 2018; Brand 2000; Figure 1A). In this system, electrons derived from the breakdown of food molecules are passed down a series of protein complexes, providing energy to pump protons from the mitochondrial matrix to the intermembrane space, ultimately building an electrochemical gradient called the proton motive force (PMF; Brand 2000; Figure 1A). The stored energy in the PMF is harnessed when protons flow down their gradient into the matrix through ATP synthase, driving the phosphorylation of ADP to produce ATP (Brand 2000;

Figure 1B). Additionally, some protons may flow down the PMF and bypass ATP synthase, either through leaking across the membrane or through specialized proteins (together termed proton leak; Zhao et al. 2019; Figure 1C).

While a high PMF benefits ATP production, it also comes with a cost as it causes the flow of electrons through the electron transfer system to slow (Brand 2000). This delay increases the likelihood that electrons will leak away from the proteins (termed electron leak) and react directly with oxygen, producing reactive oxygen species (ROS). ROS are a double-edged sword. At normal levels, ROS are important cell-signaling molecules (Zhao et al. 2019; Li et al. 2017; Dröge 2002), but when overproduced, ROS can cause oxidative damage to biomolecules such as DNA, proteins, and lipids (Monaghan et al. 2009). Therefore, the benefit of increased ATP production must be balanced against the risk of increased ROS production and subsequent oxidative damage. ROS attack on DNA most commonly leads to the oxidation of guanosine and the generation of 8-Hydroxy-2'-deoxyguanosine (8-OHdG; Valavanidis et al. 2009; Kasai 1997). The accumulation of 8-OHdG can lead to detrimental consequences such as cellular dysfunction if unrepaired (Valko et al. 2007; Bartkova et al. 2006).

The costs of accumulating oxidative damage have driven the evolution of elegant ROS defense and repair mechanisms (Sies et al. 2017; Valko et al. 2007). Once ROS are produced, scavenging by both enzymatic and nonenzymatic antioxidants can neutralize ROS to shield macromolecules from damage (Monaghan et al. 2009). If ROS escape antioxidant defense and cause damage, there are a host of mechanisms that can remove or repair the damaged molecules (Halliwell and Gutteridge 2015). While downstream defense mechanisms are important, a more efficient way could be to limit ROS production at the source. Because both proton and electron leak decrease the PMF (Figure 1D), increasing proton leak can decrease electron leak and subsequent ROS production, although this strategy does come at the cost of decreased ATP synthesis (Jastroch et al. 2010; Brand 2000; Figure 1E). Proton leak is mostly influenced by membrane composition but also through inducible pathways such as the adenine nucleotide translocase and uncoupling proteins (Zhao et al. 2019). While increasing proton leakage is less efficient in terms of ATP production, it can still be favorable if it contributes to maintaining low ROS production and thus lower oxidative damage (Zhao et al. 2019).

Oxidative damage is generally increased by both acute and chronic stressors, as well as elevated glucocorticoids (Jimeno et al. 2023; Gormally et al. 2019; Majer et al. 2019; Treidel et al. 2013; Costantini et al. 2011; Flint et al. 2007). While the underlying mechanisms remain unclear, one possibility is that stress generally, and perhaps glucocorticoids more specifically, modify the electron transfer system and ROS production (Berry et al. 2018; Valko et al. 2007). The measurement of mitochondrial aerobic respiration through high-resolution respirometry (HRR) is a valuable tool that provides an in-depth analysis of an individual's mitochondrial profile (Koch et al. 2021; Stier et al. 2017; Djafarzadeh and Jakob 2017). Such tools may allow us to better understand the molecular consequences of stressors and the associated fitness-related trade-offs (Wada 2019).

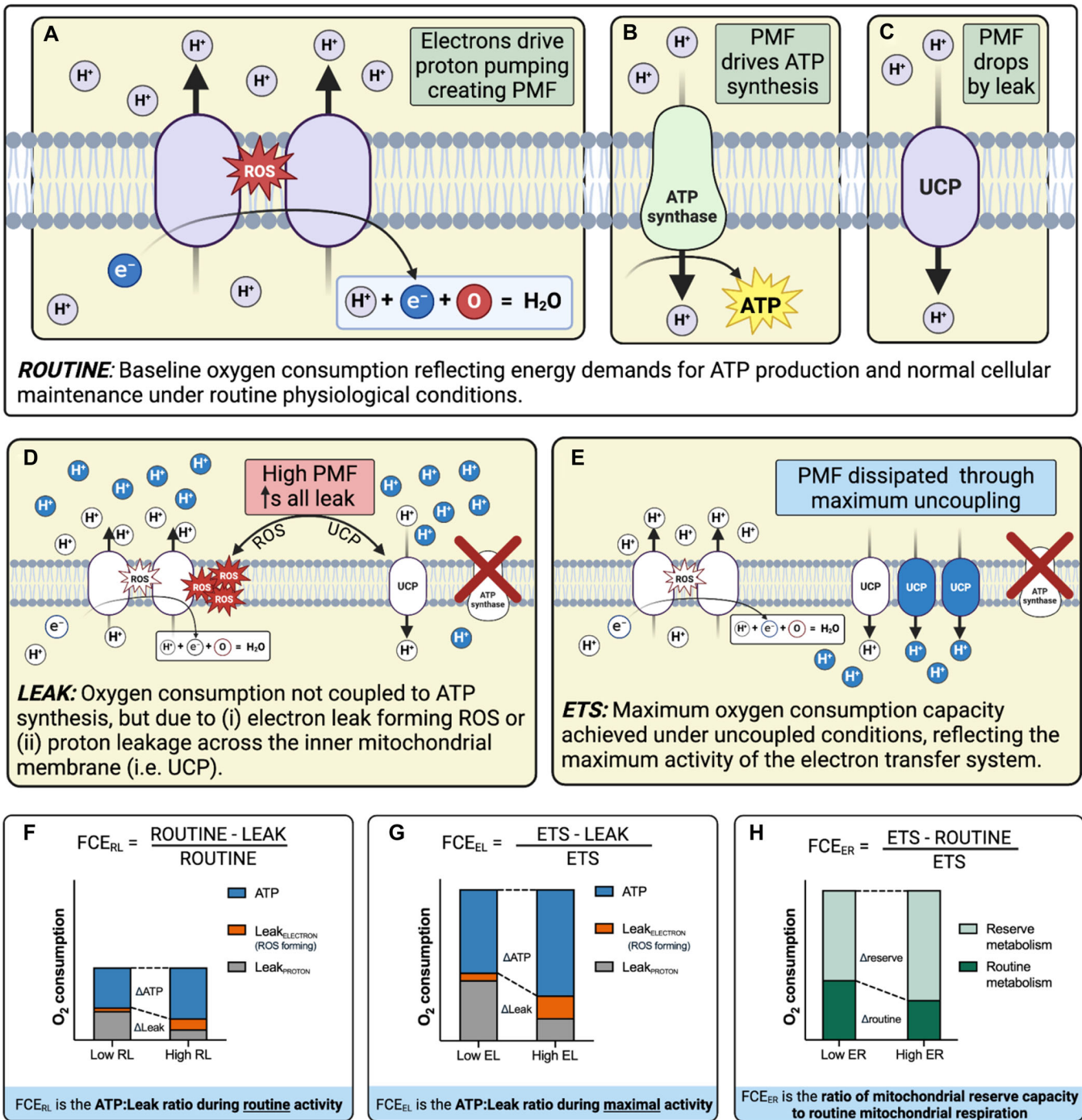


FIGURE 1 | Legend on next page.

The relationships between challenges and the associated mitochondrial responses are likely to vary with current environmental conditions, an individual's developmental stage, and a species' life history. Currently, these types of studies are few, but Koch et al. (2021) in a recent review, emphasizes the power of integrating mitochondrial metabolism into studies of ecology and evolution. In this study, we explored how an acute handling stressor affected corticosterone, mitochondrial function, and associated DNA oxidative damage in a long-lived species. Leach's storm-petrels (*Hydrobates leucorhous*) are pelagic seabirds with a life history characterized by exceptional longevity and low reproductive rates (Pollet et al. 2021). This life history strategy may predict that individuals should be able to effectively mediate the fitness-related consequences of an acute stressor, and we provide evidence that Leach's storm-petrel chicks demonstrate exceptional plasticity during a stress response.

2 | Methods

2.1 | Birds

Chicks were selected from a breeding colony of 20,000 pairs of Leach's storm-petrels at the Bowdoin College Scientific Station on Kent Island, New Brunswick, Canada (66°45' W, 44°35' N). All procedures were approved by the Bowdoin College Institutional Animal Care and Use Committee. Starting in May 2024, burrows were checked daily for the presence of an egg. Upon egg detection, the burrow was left undisturbed for 38 days during the egg incubation period to minimize human disturbance and parental abandonment. On day 38, burrows were checked daily for chick hatching to determine hatching success and chick age. Once a chick hatched, the burrow was left undisturbed again for 7 days (Vermeer et al. 1988). Beginning on day 8 post-hatch, chicks were weighed every 24 h between 0800 and 0900 h. Previous work on this population has shown that repeated handling of chicks does not affect the acute stress response later in development (Fiske et al. 2013). For daily mass measurements, chicks were briefly removed from the burrow,

placed on a digital field scale, and immediately returned back into the burrow. Of the 60 occupied burrows with chicks, a subset of 11 individuals was selected based on hatch date to allow for adequate spacing between sampling and mitochondrial measures. The limited sample size of this study reflects the time-intensive nature of mitochondrial respirometry work which requires daily instrument maintenance, calibrations, limited sample throughput, and extensive cleaning protocols (Walsh et al. 2023).

2.2 | Chick Processing Protocol

While chick masses were recorded every day, on day 30.1 ± 1.3 (SD) post hatch, we measured an acute stress response in the chicks ($n = 11$) using a chick processing protocol. Chicks were removed from the burrow, and a baseline blood sample was collected at 1.9 ± 0.7 (SD) minutes of initial contact with the burrow entrance. Chicks then underwent a standardized handling procedure in which the individual was walked to a central processing station for morphometric measurements of wing length, mass, and banding. From initial contact with the burrow, chicks were handled for 20 min according to a standardized handling protocol, and then a second "stress-induced" blood sample was collected at 21.7 ± 0.8 (SD) minutes. Due to the time and instrumental limitations of processing two samples (baseline and stress-induced) per chick, we handled one chick in the morning ($1116 \text{ h} \pm 24$ (SD) minutes) and one chick in the afternoon ($1600 \text{ h} \pm 21.2$ (SD) minutes).

2.3 | Blood Sampling

Blood samples were drawn from the brachial vein of the wing in 100 μL heparinized capillary tubes, and the amount of collected blood did not exceed 1% of the chick's body mass (McGuill and Rowan 1989). After sample collection, we applied cotton with pressure to promote clotting. The blood sample was immediately placed in a chilled styrofoam cooler and returned to the

FIGURE 1 | A simplified view of mitochondrial aerobic respiration. (A) Electrons (e^-), released from breaking down food, are passed down the proteins of the electron transfer system in the inner mitochondrial membrane. Some of these proteins pump protons (H^+) from the mitochondrial matrix to the intermembrane space, creating a H^+ gradient known as the proton motive force (PMF). When electrons leave the system they combine with protons and oxygen to form water. However, some e^- can escape the system to prematurely react with oxygen, forming reactive oxygen species (ROS). (B) The generated PMF drives H^+ back into the mitochondrial matrix through ATP synthase converting ADP and inorganic phosphate (not shown) into ATP. (C) As the PMF builds, the speed of e^- traveling through the system slows, making the premature escape of e^- more likely, increasing ROS production. The PMF can be dissipated by proton leak either directly through the membrane (not shown) or through inducible proteins like uncoupling proteins (UCP). Together, panels (A, B, and C) represent ROUTINE respiration, which represents oxygen consumption to fuel basal ATP production and leak. ROUTINE metabolism is manipulated through the addition of different chemicals to gain a deeper understanding of mitochondrial function shown in (D and E). In these panels, components of the electron transfer system that do not change have a white fill, while changed or additional components have colored fills for ease of interpretation. (D) Leak respiration. Oligomycin inhibits (red X) ATP synthase activity, causing an increase in the PMF. This increased PMF can promote proton leak through the membrane (not shown) or through inducible agents, like UCPS. However, the increased PMF also increases the chance for electron leak and subsequent ROS production. Therefore, LEAK respiration quantifies oxygen consumption linked to proton and electron leak exclusive of ATP synthesis. (E) Maximal capacity of the electron transfer system (ETS) through the addition of exogenous uncouplers. Uncoupling proteins create pores in the electron transfer system that allow protons to flow through the membrane, ultimately dissipating the PMF. (F) FCE_{RL} efficiency represents the proportion of ROUTINE oxygen consumption linked to ATP synthesis, excluding oxygen consumption due to LEAK respiration. (G) FCE_{EL} efficiency represents the proportion of maximum mitochondrial capacity (ETS) linked to ATP synthesis, excluding oxygen consumption linked to LEAK respiration. (H) FCE_{ER} efficiency represents the remaining reserve capacity during ROUTINE respiration. Panels (F, G, and H) are theoretical models.

field laboratory. Samples were immediately aliquoted for three different assays as follows:

1. The whole blood samples were centrifuged at $420 \times g$ for 5 min at 4°C to separate the plasma from the blood cells. Microscopy revealed that the vast majority of the cells in the sample were erythrocytes, with a very small number of white blood cells present; however, for simplicity, we will refer to these samples as red blood cells (RBC) going forward. The plasma was immediately removed and stored at -20°C until corticosterone measurement at Bucknell University.
2. After removing the plasma from the samples, the RBC were washed in 1 mL of cold MiRO5 respiratory buffer (Oroboros Instruments, Innsbruck, Austria) and centrifuged again at $588 \times g$ for 5 min at 4°C to remove the wash buffer (Stier et al. 2017). $25 \mu\text{L}$ of RBC were taken to prepare a cell suspension for mitochondrial measurement.
3. The remaining RBC ($20 \mu\text{L}$) were used for a DNA extraction protocol for oxidative damage measurement.

2.4 | Corticosterone Measurement

We measured plasma corticosterone concentrations using a commercially available enzyme immunoassay (EIA) kit (cat. K003-H Arbor Assays, Ann Arbor, MI, USA), according to the manufacturer's instructions. We validated this assay kit in two ways. First, we assayed ten unique samples both in this EIA and in a corticosterone radioimmunoassay run in our laboratory for the past decade (McCollum et al. 2024; Majer et al. 2023), revealing a strong correlation between the two assays (Pearson $r = 0.94$). Second, we assessed sample parallelism by comparing serial dilutions of storm-petrel samples (1:20, 1:40, and 1:80) to the standard curve using an analysis of covariance, and the results showed no significant interaction between sample and dilution factor, confirming parallel slopes ($F_{2,2} = 0.5$, $p = 0.6$). For the study samples, $4 \mu\text{L}$ of storm-petrel plasma were added to an equal volume of dissociation reagent (1:1), mixed, diluted in the provided assay buffer (1:40), and stored overnight at -20°C . Standards were run in duplicate, and samples were run in triplicate. Sample order was randomized within the microplate and blind to the person performing the assay. Two internal controls were run in triplicate on the plate, and the average intra-assay coefficient of variation was 6.5%.

2.5 | Mitochondrial Respiration

In the field, we used intact RBC to perform high-resolution respirometry using an Oroboros NextGen-O2k (Oroboros Instruments, Innsbruck, Austria). Before the study, we validated our methodology, inhibitor concentrations, and uncoupler concentrations on RBC in Leach's storm-petrel chicks (Supporting Information: Figures S1A,B and S2A,B). To control for user variation, all mitochondrial measurements were performed by the same individual (Walsh et al. 2023). Samples were immediately processed in the lab within 2 h of blood collection (1.4 ± 0.2 [SD] hours). To create a cell suspension,

$25 \mu\text{L}$ of intact RBC were added to $175 \mu\text{L}$ of 39°C MiRO5 buffer to mimic the internal body temperature of a Leach's storm-petrel chick (Ricklefs and Travis 1980). After adding the cell suspension to the Oroboros, we waited for signal stabilization (~ 10 min) and recorded mitochondrial baseline respiration (ROUTINE; Figure 1A). We performed 2–3 titrations of 0.00125 mM oligomycin (Sigma-Aldrich) to quantify mitochondrial leak respiration (LEAK; Figure 1B). Following this, we added 4–5 titrations of 0.25 mM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, Sigma-Aldrich) to stimulate maximal mitochondrial respiration (ETS; Figure 1C). The number of oligomycin and FCCP titrations were sample-specific as samples may respond differently to oligomycin inhibitor and FCCP uncoupler. Lastly, we added 5 mM antimycin A to capture residual oxygen consumption (ROX) of non-electron transport system origin (Gnaiger 2020). ROX was subtracted from all reported mitochondrial variables (ROUTINE, LEAK, ETS) to exclude respiration of non-electron transfer system origin. Because we performed respirometry on intact cells, all measures are respective of endogenous substrates (Gnaiger 2020). Mitochondrial respiration rates were normalized for mitochondrial density by dividing ROUTINE, LEAK, and ETS by citrate synthase activity reaction rates (Eigentler et al. 2020). Both the baseline and post-handling samples from the same individual were measured simultaneously, and a representative trace can be seen in the Electronic Supplemental Material.

Three flux control efficiencies (FCE) were calculated to determine mitochondrial efficiency: FCE_{RL} , FCE_{EL} , and FCE_{ER} (Gnaiger 2020). As described in Figure 1, FCE_{RL} represents oxygen consumption associated with ATP production compared to electron and proton leak (or the ATP:Leak ratio during routine activity). Individuals with relatively higher FCE_{RL} use more oxygen consumption for ATP production and less for leak (Figure 1F). FCE_{EL} is similar to FCE_{RL} , but instead looks at the ratio of ATP produced to leak during maximal electron transfer system activity. Individuals with relatively higher FCE_{EL} use more oxygen consumption for ATP production and less for leak when their electron transfer systems are working at peak capacity (Figure 1G). Lastly, FCE_{ER} represents the ratio of mitochondrial reserve capacity to routine mitochondrial respiration. Individuals with relatively higher FCE_{ER} have a higher proportion of their total mitochondrial capacity held in reserve (Figure 1H).

2.6 | Citrate Synthase Activity—Mitochondrial Respiration Per Mitochondria

Citrate synthase is an enzyme found in the mitochondrial matrix that participates in the Citric Acid Cycle of aerobic respiration, and its activity can be used as a proxy for mitochondrial density (Eigentler et al. 2020). Following mitochondrial analysis, blood samples were recovered from the Oroboros oxygen chambers and centrifuged at $588 \times g$ for 5 min at 4°C to precipitate the MiRO5. Samples were then stored at -20°C until measured at Bucknell University. Citrate synthase activity was measured using a kinetic assay (cat. 701040, Cayman Chemical, Ann Arbor, MI, USA). Briefly, samples were centrifuged at

588 × g for 5 min at 4°C, and the packed RBC were diluted 1:250. Data was collected from regions of linear enzyme activity during the range of 4–14 min during the reaction, and reaction rates were calculated according to the manufacturer's guidelines.

2.7 | DNA Extraction and Digestion for Oxidative Damage (8-OHdG)

The oxidized guanosine 8-Hydroxy-2'-deoxyguanosine (8-OHdG) is an established biomarker for DNA oxidative damage (Valavanidis et al. 2009; Kasai 1997). For DNA extraction, 20 μL of RBC were homogenized in DNazol (ThermoFisher) lysis reagent and incubated on ice for 90 min. After incubation, the samples were centrifuged at 13,000 × g for 10 min at 4°C to precipitate protein contamination. The supernatant was added to 500 μL of absolute ethanol to precipitate a DNA pellet. Following precipitation, the DNA pellet was washed and stored in ethanol (75% and 95%). Based on Huang et al. (2001), we developed a digestion protocol in which DNA pellets were digested using DNase (Zymo Research), phosphodiesterase (0.1 U/μL, Sigma-Aldrich), and alkaline phosphatase (1 U/μL, Sigma-Aldrich). Initially, the DNA pellets were added to DNase, DNA digestion buffer, and 1.25 mM deferoxamine mesylate (DFO, Sigma-Aldrich). After a 90 min incubation at 37°C, phosphodiesterase, Tris-HCl (0.8 M, pH 9), and DFO were added. Following a 90 min incubation at 37°C, alkaline phosphatase was added before a final 90 min incubation at 37°C. The digested samples were quantified before 8-OHdG detection, and all DNA yields exceeded 1000 ng/μL. To standardize DNA oxidative damage levels among individuals, we report 8-OHdG as a ratio to control for the amount of unoxidized guanosine (dG) in the sample (8-OHdG:dG). Ratios are presented 1:1000000.

2.8 | Oxidative Damage (8-OHdG/10⁶dG) Quantification by HPLC-MS/MS and HPLC-UV

8-Hydroxy-2'-deoxyguanosine (8-OHdG) and 2'-Deoxyguanosine monohydrate (dG) standards were purchased from Sigma-Aldrich (cat. H5653 and D7145). A ¹⁵N₅-8-OHdG standard was purchased from Cambridge Isotope Laboratories Inc. (cat. NLM-6715-1.2, Tewksbury, MA). Formic acid was purchased from J.T. Baker (New Jersey, USA). Optima LC-MS grade water, acetonitrile, methanol, and other chemicals were purchased from Fisher Scientific (New Jersey, USA). 8-OHdG standard stock solution (1 mg/mL) and internal standard ¹⁵N₅-8-OHdG stock solution (25 μg/mL) were prepared in water. All the stock solutions and the working solutions were kept at −20°C before use. Standard working solutions were freshly prepared by serial dilution of the stock with methanol, resulting in concentrations from 10 to 10,000 ng/mL. The internal standard working solution was prepared by dilution of the stock solution to make the concentration at 1000 ng/mL. Standard samples were prepared by spiking 5 μL standard working solution and 5 μL internal standard working solution into 40 μL 20% methanol. Standard curves were constructed by plotting the ratio of the peak area of the analyte to the peak area of the internal standard versus analyte concentration.

Samples (50–100 μL) were spiked with 5 μL internal standard ¹⁵N₅-8-OHdG working solution and then mixed with 500 μL

H₂O and vortexed before loading onto a Phenomenex Strata-X cartridge for solid phase extraction (SPE). The SPE cartridge was preconditioned by 1 mL methanol and then was equilibrated by 1 mL H₂O. After loading the samples, the SPE cartridge was washed with 1 mL H₂O twice. The cartridge was dried before the analytes were eluted out with 1 mL of 100% methanol. The eluent was then dried by Speed Vac and followed by reconstitution with 50 μL 20% methanol. The reconstituted sample was centrifuged at 4°C, 4000 rpm for 5 min before injection into the HPLC-MS/MS system. The calculated concentrations from the standard curves were adjusted by a pre-concentrated factor to reflect the original 8-OHdG level in DNA samples.

HPLC-MS/MS analysis. 8-OHdG was analyzed using a Sciex QTRAP 6500+ mass spectrometry coupled with a SciexEXion HPLC separation system. A 1.7 μmAcquity UPLC BEH C18 analytical column (2.1 × 100 mm, Waters, Ireland) was used to separate analytes with impurities. The gradient elution was conducted using a flow rate of 0.3 mL/min with the following conditions: initial at 5% mobile phase B (0.1% formic acid in acetonitrile) and 95% mobile phase A (0.1% formic acid in water), and kept the initial condition for 2.5 min, followed by a linear gradient to 90% mobile phase B in 0.8 min, and kept at 90% mobile phase B for an additional 2 min to flush the column before returning back to initial conditions to equilibrate the column. The SciexQTrap 6500+ mass spectrometer was equipped with an electrospray ionization probe operated in positive mode. The decluster potential (DP) was 16 V; the entrance potential (EP) was 10 V; the collision energy (CE) was 23 V; the collision cell exit potential (CXP) was 10 V. The curtain gas (CUR) was 35 L/h, the collision gas (CAD) was medium. The ionspray voltage was 5500 V, the temperature was 400°C, gas 1 was 20 L/h, and gas 2 was 15 L/h. The multiple reaction monitoring mode (MRM) was used to analyze and quantify 8-OHdG and the internal standards ¹⁵N₅-8-OHdG, with the transitions of *m/z* 284 > 168 for 8-OHdG, 289 > 173 for ¹⁵N₅-8-OHdG. All peaks were integrated and quantified by Sciex OS 3.0 software.

dG analysis via HPLC-UV. Samples were diluted 5-10-fold in ultrapure water and 5-10 μL were injected onto a HPLC equipped with a variable wavelength detector. The system consisted of two Shimadzu (Kyoto, Japan) LC-10AD pumps, a Shimadzu SIL-10AD autosampler, and a Shimadzu SPD-10A UV-Vis detector. The dG was separated by a Phenomenex C18 Bondclone (10 micron, 3.9 × 300 mm; Torrance, CA, USA) running an isocratic solvent system of 93% 0.1% formic acid in water and 7% methanol. The flow was 1 mL/min for 20 min with a wash of 95% methanol for 5 min before returning to initial conditions. dG was detected at 254 nm and peaks recorded and integrated using a Hitachi (Tokyo, Japan) D-2500 Integrator. All measurements were carried out at room temperature (22 ± 1°C). Calibration curves based on a dG standard was used to determine the concentration of the peak of interest.

2.9 | Statistical Analysis

The goal of this study was to understand how acute restraint stress impacts broad physiological parameters. In addition, we wanted to investigate the interconnection among our variables.

To accomplish this, our statistical analysis consisted of three steps, conducted using SPSS 29.0 and JMP Pro 17.2.0.

Step 1: General Estimating Equations (GEE) to assess physiological responses to acute stress.

Justification. To start, we wanted to explore population-level effects of acute handling stress on corticosterone levels, mitochondrial respiration rates, and DNA oxidative damage. We used Generalized Estimating Equations (GEE), following Stier et al. (2019) because they focus on estimating population-averaged (marginal) effects, making them ideal for assessing broad biological trends rather than individual-specific variability. GEE are also robust to misspecification of the correlation structure (e.g., independent, exchangeable, autoregressive), providing reliable estimates of population-level effects even when the exact nature of within-subject correlations is uncertain. Furthermore, by accounting for the correlated nature of repeated measures, GEE produces valid standard errors and helps mitigate the risk of Type I errors (false positives) as the number of statistical tests increases.

Analytical procedure. We used repeated-measures GEE and specified a Gaussian distribution with an identity link function to examine the effects of acute handling stress on corticosterone levels, three mitochondrial measures, three flux control efficiencies, and DNA oxidative damage. Bird ID was included as the individual factor, stress condition (baseline vs. acute stress) was treated as the within-subject repeated measure, and time of day was included as a covariate. We had relatively small sample sizes due to both ethical (minimizing colony disturbance) and practical constraints, and so we chose to report standardized effect sizes d and their 95% confidence intervals to convey the magnitude of observed biological effects (Stier et al. 2019; Nakagawa and Cuthill 2007). The eight separate analyses we performed come at the cost of increasing the likelihood of Type I statistical errors (i.e. false positives). To control for this, we applied false discovery rate (FDR) to p -values and used the two-stage sharpened method for FDR (Benjamini and Hochberg 1995).

Step 2: General Linear Model (GLM) to assess the relationships between corticosterone or mitochondrial respiration measures and oxidative damage (8-OHdG) over an acute stress response.

Justification. Here, we shifted our focus to understanding how changes in the significant predictors identified in Step 1 influence changes in 8-OHdG over the stress response. We did this by focusing on the changes (Δ) between the baseline and post-handling samples as that allows us to focus on the dynamic changes during acute stress rather than absolute levels, aligning with our interest in understanding how acute stress affects physiological processes. We used GLM because it allows for flexible modeling of the relationships between these continuous predictors and the outcome variable, and it accommodates interaction terms. Unlike in Step 1, we are now modeling individual-level variation in the changes over time, focusing on the biological significance of acute stress-induced changes rather than repeated measures. GLM provides the flexibility needed for these comparisons without requiring the inclusion of random effects, as we are now modeling differences rather than

nested structures. It is important to note that absolute stress induced glucocorticoid concentrations are more informative than changes between baseline and post-handling because glucocorticoids interact with two distinct receptors with different binding affinities (Romero 2004). Because of this, for any analysis using Δ Corticosterone we also ran models using stress induced corticosterone instead and found no difference between these two sets of analyses (analyses not shown). Based on this, we chose to use Δ Corticosterone in any analyses that also included mitochondrial changes (Δ) between baseline and post-handling.

Analytical procedure. We calculated the change (Δ) in each significant predictor from Step 1, and in SPSS, included all of those predictors and their interactions in a GLM in the initial model. We iteratively simplified the model by removing the nonsignificant terms, starting by dropping the term with the greatest p -value. We then compared models using the Bayesian Information Criterion (BIC). BIC is a model selection criterion that penalizes model complexity to prevent overfitting. Lower BIC values indicate a better-fitting model, as they reflect both a good fit to the data and a minimal number of predictors. In general, differences in BIC values of less than 2 points suggest little evidence for one model over another, 2–6 points suggest positive improvement, and differences greater than 10 points indicate strong evidence in favor of the model with the lower BIC (Kass and Raftery 1995).

Step 3: Path Analysis to Explore Causal Pathways.

Justification. In the final step, we aimed to investigate the causal pathways underlying the significant acute stress-induced changes identified in the most parsimonious GLM from Step 2. Path analysis, while similar to structural equation modeling, exclusively works with observed variables and does not incorporate latent variables. This makes it more suitable for studies with low sample sizes. Importantly, both types of analysis allow for the simultaneous examination of multiple dependent and independent variables, providing insights into the underlying causal structure of our data. These analyses also enable the simultaneous testing of direct, indirect, and mediated effects within a unified framework. For example, imagine we have an independent variable (X), a dependent variable (Y), and a mediator (M). X could directly affect Y ($X \rightarrow Y$). In addition, X could affect M which in turn affects Y ($X \rightarrow M \rightarrow Y$). Finally, M could mediate the relationship between X and Y . In this case, if ($X \rightarrow M$) and ($M \rightarrow Y$), then we can test whether the direct effect of ($X \rightarrow Y$) diminishes or becomes nonsignificant when the mediator is included. This would suggest that the relationship between X and Y is largely explained by the mediator.

Analytical Procedure. In JMP Pro, we specified an initial model that included the variables from the best model in Step 2. We tested all hypothesized relationships and iteratively refined the model by removing nonsignificant paths and simplifying the structure (Gómez and Zamora 2000). We evaluated alternative models involving several approaches:

- χ^2 goodness of fit test statistic, in which a smaller value indicates better consistency with observed data (Streiner 2006).

- **Bentler's Comparative Fit Index (CFI)** is an incremental fit index that compares the fit of a hypothesized model with that of an independent model, which has the worst fit (Xia and Yang 2019). This is in contrast to RMSEA which is an absolute fit index, in that it assesses how far a hypothesized model is from a perfect model. In the past, a CFI above 0.90 was used as a cutoff for good fitting models, but more recent consensus suggests that this value should be increased to approximately above 0.95 (Hu and Bentler 1999).
- **Root Mean Square Error of Approximation (RMSEA)** estimates the amount by which estimated values differ from actual values and was used for model comparison. Values over 0.10 are considered to be a bad fit, those less than 0.08 reflect a reasonable fit, and values less than 0.05 indicate a good fit (Streiner 2006).
- **Universal Bayesian information criterion (BICu)** distinguishes between models derived from the maximum likelihood estimation, with the most parsimonious model being associated with the smallest BICu value. A model is considered substantially better if its BICu is more than two units lower than competing models. BICu balances model fit with complexity, favoring simpler models that adequately explain the data. BICu is generally robust across varying sample sizes compared to AICc.

3 | Results

Physiological responses during acute stress. Plasma stress-induced corticosterone was significantly higher in response to our standardized chick processing protocol compared to baseline levels (Figure 2 and 3A; Table 1). Two out of six mitochondrial respiration measures were significantly affected by our handling protocol (Figure 2). Both FCE_{RL} and FCE_{EL} decreased in response to acute stress (Figure 3B, C; Table 2). We found no significant effect of the handling protocol on oxidative damage (Figure 2, Table 1). Likewise, the time of day that blood samples were taken had no effect on any of the physiological responses (Table 1).

Effects of corticosterone and mitochondrial respiration measures on oxidative damage (8-OHdG) over an acute stress response. Based on our first analysis, we evaluated the effect of stress-induced changes in corticosterone (Δ Corticosterone) and two flux control ratios (ΔFCE_{RL} and ΔFCE_{EL}) on stress-induced changes in oxidative damage (Δ 8-OHdG). The most parsimonious model included only Δ Corticosterone and ΔFCE_{RL} as predictors which both had significant effects on Δ 8-OHdG ($F_{2,8} = 9.7$, $p = 0.007$; Table 2). Δ Corticosterone was inversely correlated with Δ 8-OHdG (estimate = -0.09 ± 0.03 , $t_{2,8} = -3.3$, $p = 0.01$), so those individuals who had the greatest increase in corticosterone during the acute stress response tended to have a reduction in 8-OHdG (Figure 4A). ΔFCE_{RL} was positively correlated with Δ 8-OHdG (estimate = 683.09 ± 232.22 , $t_{2,8} = 2.9$, $p = 0.02$), meaning that those individuals who had the largest decrease in mitochondrial efficiency had reduced ATP production and reduced levels of 8-OHdG in response to the acute handling stressor (Figure 4B).

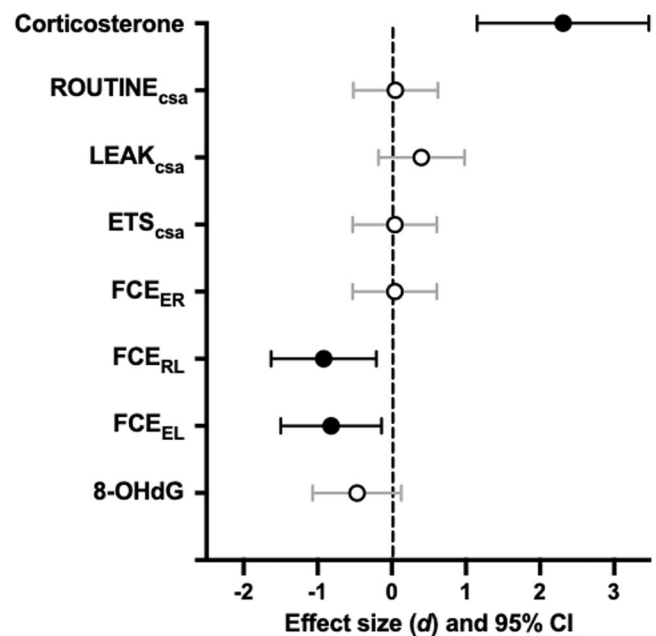


FIGURE 2 | Standardized effects of acute stress on corticosterone, mitochondrial respiration measures, and DNA oxidative damage (8-OHdG). Standardized effect sizes (d) and their 95% confidence intervals are shown. Nonsignificant effects from General Estimating Equations are shown with open circles and light gray 95% CI bars overlapping the zero line. Parameters remaining statistically significant after false discovery rate (FDR) have filled circles and black 95% CI bars.

Exploring causal pathways during stress. To further investigate the causal pathways linking corticosterone, mitochondrial respiration, and oxidative damage, we conducted structural equation modeling (SEM). Four models were compared (Figure 5). Based on the fit indices (Table 3), the Independent Effects Model provided the best fit. This model indicates that both Δ Corticosterone and ΔFCE_{RL} independently predict changes in Δ 8-OHdG, with no evidence supporting a mediation pathway.

4 | Discussion

Organismal responses to challenges are likely optimized by evolution to yield the best fitness outcomes (Monaghan 2014). We investigated the relationship between corticosterone, mitochondrial function, and DNA oxidative damage in response to an acute stressor in Leach's storm-petrels, a long-lived marine bird. While the influence of chronically elevated glucocorticoids on oxidative damage (reviewed in Monaghan and Spencer 2014; Costantini et al. 2011) and mitochondrial function (reviewed in Picard et al. 2018; Spiers et al. 2015) have received considerable attention, less is known about how acute challenges affect these physiological parameters. This knowledge gap is particularly critical given the potential for these physiological responses to serve as biomarkers of stress in ecologically relevant contexts (MacDougall-Shackleton et al. 2019). Notably, this study revealed that the changes in corticosterone levels and mitochondrial efficiency during the acute handling challenge had two independent effects on DNA oxidative damage. First, individuals who exhibited the largest increase in corticosterone also accumulated the least amount of oxidative damage. Second,

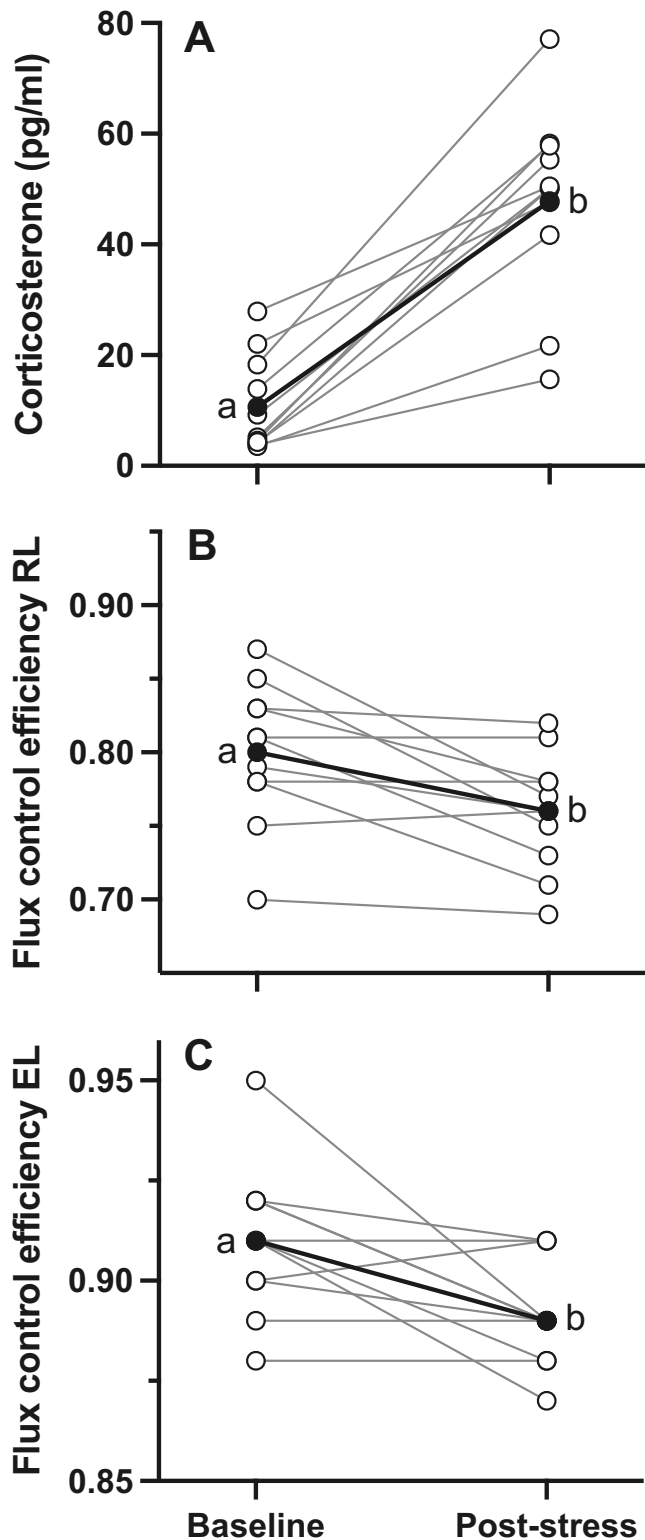


FIGURE 3 | Corticosterone, and mitochondrial flux control efficiency ratios (FCE) at baseline (< 3 min of capture) and after the chick handling protocol (stress-induced at 20 min). (A) Corticosterone, (B) FCE_{RL} which measures the proportion of ROUTINE respiration used for ATP production by excluding the portion attributed to LEAK respiration, and (C) FCE_{EL} measures the proportion of maximal ETS capacity available for productive ATP synthesis after accounting for proton leak. Individual responses are shown with open circles and gray lines and the mean population response is shown with black circles and lines. Different letters indicate significant differences between the two sampling times (i.e. population means) according to statistical models presented (Table 1).

individuals who prioritized ATP production during the acute challenge showed higher levels of oxidative damage.

Corticosterone's relationship with oxidative damage presents a paradox, particularly in the context of acute stress. The traditional view links elevated glucocorticoids to increased oxidative damage, especially in the case of chronic glucocorticoid exposure (Jimeno et al. 2023; Costantini 2019; Gormally et al. 2019; Costantini et al. 2011). However, acute stressors are also often linked to elevated oxidative damage (Majer et al. 2023; Haussmann et al. 2012; Costantini et al. 2008; Flint et al. 2007; Lin et al. 2004), and a causal role of the acute rise in glucocorticoids in this context has been demonstrated (Majer et al. 2019). However, our results revealed an unexpected inverse relationship between the magnitude of increase in corticosterone and the change in oxidative damage during the acute handling challenge. Chicks who experienced the highest increase in corticosterone showed a reduction in oxidative damage, suggesting that glucocorticoids can sometimes mitigate, rather than exacerbate, oxidative damage. Recent research supports this more complex relationship in the context of acute stressors, where an increase in corticosterone may actually reduce oxidative damage. Stier et al. (2019) demonstrated that king penguins (*Aptenodytes patagonicus*) with a more pronounced corticosterone response to an acute restraint stress presented lower levels of a number of oxidative damage markers (Stier et al. 2019). Further, while not during an acute stressor, Vágási et al. (2018) reported that experimental increases in baseline corticosterone in house sparrows (*Passer domesticus*) led to a reduction in oxidative damage. These effects of glucocorticoids may be due to their acute stimulatory effects on antioxidant activity (Costantini et al. 2011). Taken together, these findings highlight the need to view glucocorticoids in a more nuanced way, distinguishing them from the broader concept of stress itself (MacDougall-Shackleton et al. 2019).

We found a positive correlation between ΔFCR_{RL} and $\Delta 8\text{-OHdG}$. FCR_{RL} represents the proportion of oxygen consumption channeled toward ATP synthesis as opposed to leak respiration. On average, during the acute stressor, storm-petrel chicks increased leak. While this change decreased ATP synthesis, it also may have resulted in the decreased DNA oxidative damage. We did not measure the type of leak respiration, and so we cannot specify with certainty the relative contribution of proton leak vs. Electron leak, which leads to ROS production. However, because we also saw a reduction in oxidative damage during the acute stressor, we can speculate that birds may have increased the amount of proton leak, reducing the PMF and in turn reducing electron leak and ROS production. Casagrande et al. (2020) found that despite not exhibiting reduced oxidative damage, corticosterone-treated great tit nestlings displayed higher proton leak, which led to decreased ATP production efficiency. This finding, together with our observations in storm-petrels, supports the hypothesis that proton leak, though seemingly inefficient in the context of ATP production, may serve a protective role against oxidative damage, particularly during periods of heightened stress. Similarly, the work of Stier et al. (2019) in king penguins further strengthens this connection, showing that penguins with higher corticosterone responses to acute stress also exhibited increased proton leak,

TABLE 1 | Summary of the statistical models (GEEs) examining the impact of the acute stressor and time of day on corticosterone, mitochondrial respiration parameters, and DNA oxidative damage.

	Acute stressor		Time of day	
	Estimate \pm SE	<i>p</i> value (χ^2)	Estimate \pm SE	<i>p</i> value (χ^2)
Corticosterone	<u>936.147 \pm 107.35</u>	<u>< 0.001 (76.05)*</u>	-0.32 \pm 0.55	0.56 (0.34)
ROUTINE _{csa}	0.004 \pm 0.03	0.890 (0.19)	0.52 \pm -0.75	0.49 (0.48)
LEAK _{csa}	0.008 \pm 0.01	0.168 (1.89)	0.04 \pm 0.15	0.84 (0.04)
ETS _{csa}	0.005 \pm 0.05	0.924 (0.009)	0.91 \pm 1.0	0.54 (0.38)
FCE _{ER}	0.004 \pm 0.03	0.892 (0.18)	-0.03 \pm 1.00	0.99 (0.01)
FCE _{RL}	<u>-0.042 \pm 0.01</u>	<u>< 0.001 (11.96)*</u>	0.77 \pm -0.77	0.32 (0.99)
FCE _{EL}	<u>-0.019 \pm 0.01</u>	<u>0.003 (8.98)*</u>	0.25 \pm -0.24	0.30 (1.05)
8-OHdG	27.607 \pm 15.05	0.067 (3.36)	0.07 \pm -0.08	0.35 (0.87)

Note: Significant results ($p \leq 0.05$) are highlighted in bold and underlined. Parameters that remain significant after false discovery rate (FDR) correction are underlined and marked with an asterisk (*). Deviation of the dependent variable or covariate (time of day) is shown. Estimates are provided for acute stress and time of day, and mitochondrial time of day estimates were multiplied by 10,000 for ease of interpretation.

TABLE 2 | Model comparison and significant effects from Generalized Linear Models (GLMs) predicting Δ 8-OHdG.

Model	Predictors	Interactions	BICc	Δ BICc
1	<u>Cort, RL</u>		117.1	0.0
2	<u>Cort, RL, EL</u>		117.2	0.1
3	<u>Cort, RL, EL</u>	<u>Cort*RL, Cort*EL</u>	122.8	5.7
4	<u>Cort, RL, EL</u>	Cort*EL	122.8	5.7
5	<u>Cort, RL, EL</u>	Cort*RL, <u>Cort*EL</u> , RL*EL	140.2	23.1
6	<u>Cort, RL, EL</u>	Cort*RL, Cort*EL, RL*EL, Cort*RL*EL	174.7	57.6

Note: Each model includes different combinations of predictors (Δ Cort, Δ FCE_{RL}, and Δ FCE_{EL}) and their interactions, but for simplicity, these are referred to as Cort, RL, and EL, respectively. Bayesian Information Criterion corrected for small sample size (BICc) was used to evaluate model fit, with lower values indicating better fit. Δ BICc represents the difference in BICc values relative to the initial model. Significant effects ($p \leq 0.05$) for each model are bolded. The final model is highlighted as the most parsimonious, balancing goodness of fit and model simplicity.

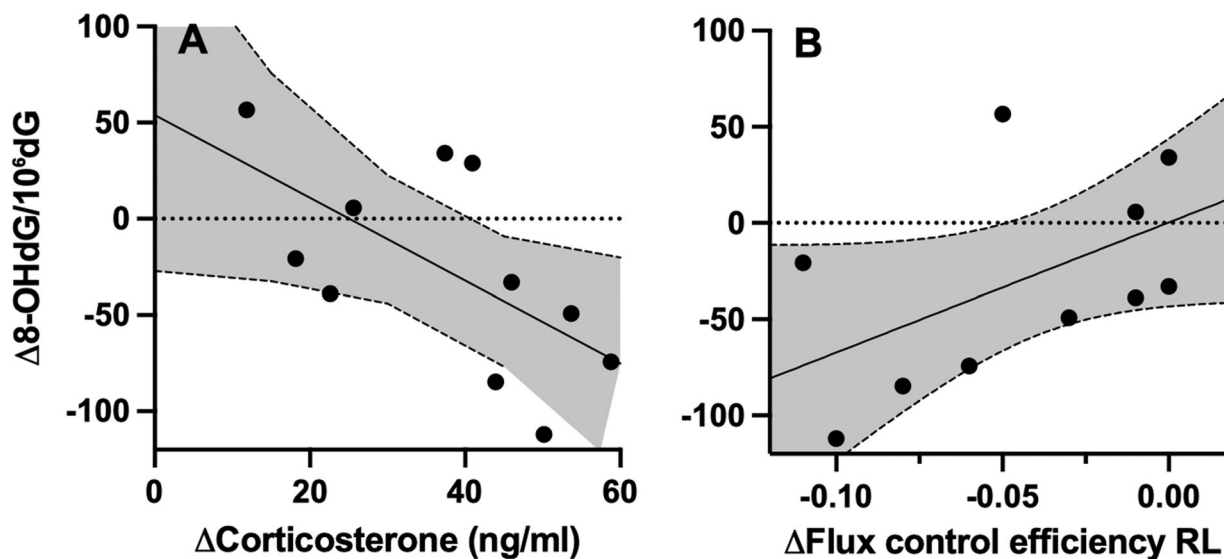


FIGURE 4 | Relationships between stress-induced changes in corticosterone (Δ Corticosterone) or FCE_{RL} (Δ Flux control efficiency RL) on stress-induced changes in oxidative damage (Δ 8-OHdG). (A) The negative relationship between Δ Corticosterone and Δ 8-OHdG suggests that individuals with greater increases in corticosterone during acute stress exhibit reductions in oxidative damage. (B) The positive relationship between Δ Flux control efficiency RL (proportion of ROUTINE respiration dedicated to ATP production minus proton leak) and Δ 8-OHdG indicates that individuals decreasing mitochondrial efficiency, and thus ATP production, also have lower levels of oxidative damage. Data points represent individual responses, with shaded regions indicating the 95% confidence intervals of the regression lines.

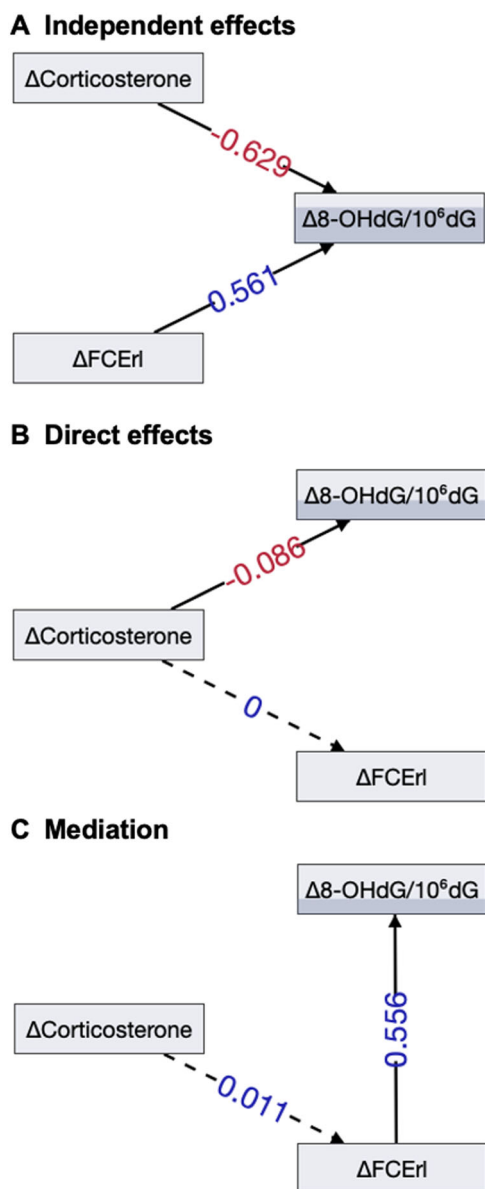


FIGURE 5 | Path diagrams exploring the relationships between Δ Corticosterone, Δ Flux Control Efficiency RL (Δ FCE_{RL}), and Δ 8-OHdG/10⁶dG. (A) Independent Effects Model tests whether both Δ Corticosterone and Δ FCE_{RL} independently predict Δ 8-OHdG/10⁶dG, and this model has the greatest support (Table 3). (B) Direct Effects Model tests only the direct effect of Δ Corticosterone on Δ 8-OHdG/10⁶dG, with no relationship between Δ Corticosterone and Δ FCE_{RL}, and (C) Mediation Model tests whether Δ FCE_{RL} mediates the relationship between Δ Corticosterone and Δ 8-OHdG. Solid arrows represent significant paths, while dashed arrows indicate nonsignificant paths. Blue numbers show positive standardized path coefficients, and red numbers show negative standardized path coefficients. Path coefficients and R² values (indicated by box shading) are provided for each model.

suggesting a potential mechanism for mitigating oxidative stress during a challenge. Taken together, these studies suggest a consistent pattern across bird species: some individuals facing acute stressors may prioritize preventing or reducing oxidative damage over maximizing ATP production. The concept of mitochondrial uncoupling provides a useful framework for understanding this trade-off. Increased proton leak, facilitated

by uncoupling proteins or the adenine nucleotide translocase, reduces the proton motive force across the mitochondrial membrane, thus limiting ROS production at the electron transport system (Zhao et al. 2019; Berry et al. 2018). While this process reduces ATP production efficiency, it offers a mechanism to mitigate oxidative damage. Our findings in storm-petrels, in conjunction with the research of Casagrande et al. (2020) and Stier et al. (2019), contribute to a growing body of evidence suggesting that birds exhibit remarkable plasticity in mitochondrial function, enabling them to fine-tune metabolic strategies in response to ecological pressures (Speakman et al. 2015; Wiersma et al. 2004).

Path analysis found that both changes in corticosterone (Δ Corticosterone) and mitochondrial respiration (Δ FCE_{RL}) independently predict changes in DNA oxidative damage (Δ 8-OHdG). Glucocorticoids can influence oxidative stress through both direct and indirect pathways (Spiers et al. 2015). Directly, glucocorticoids can affect mitochondrial function by altering gene expression which ultimately impacts ATP and ROS production (Picard et al. 2018; Dröge 2002). Indirectly, glucocorticoids can influence oxidative stress by modulating other physiological systems, such as the immune system, which can also generate ROS and contribute to oxidative damage (Wada 2019; Sapolsky 2000). This intricate interplay between direct and indirect effects makes it challenging to disentangle the specific role of glucocorticoids in oxidative stress regulation. For example, while a study in reptiles showed reduced ROS production in response to glucocorticoid treatments, there was no apparent effect on mitochondrial respiration or efficiency (Voituron et al. 2017). In contrast, our results along with Stier et al. (2019), suggest that glucocorticoids may play a role in regulating mitochondrial uncoupling and thereby decrease oxidative damage. The relationships among glucocorticoids, mitochondrial function, and oxidative damage during stressors are complex and are likely to vary depending on factors such as the duration and intensity of stress exposure (Majer et al. 2019), the type of stressor (Breuner et al. 2013), the specific marker of oxidative damage being measured (Stier et al. 2019; Monaghan et al. 2009), and species-specific adaptations (Sapolsky 2021).

Our study measures acute changes in DNA oxidative damage, but future work should also include measures of ROS. Directly measuring ROS could provide valuable insights into the mechanisms driving changes in oxidative damage, particularly in the context of mitochondrial uncoupling (Stier et al. 2017). Additionally, investigating antioxidant capacity is crucial for constructing a more comprehensive picture of redox balance during acute stress. This could involve integrating multiple markers related to oxidative stress, such as those reflecting damage, repair, and antioxidant defenses, which would deepen our understanding of these complex processes (Speakman et al. 2015; Monaghan et al. 2009). Another important avenue for exploration is tissue-specific responses to oxidative stress. Differences in how corticosterone levels interact with mitochondrial respiration and oxidative damage across tissues warrant closer investigation (Beattie et al. 2024). Mitochondrial respiration rates vary considerably among tissue types (Koch et al. 2021; Salin et al. 2016), and while there is limited evidence that some tissue types correlate (Stier et al. 2017), a multi-tissue approach is advantageous (Koch et al. 2021). While avian

TABLE 3 | Comparison of Path Analysis models testing the relationships between Δ Corticosterone, Δ FCE_{RL}, and Δ 8-OHdG).

Model	Number of parameters	X2 (p value)	CFI	RMSEA	BICu
Independent effects	8	0.001 (0.972)	1	0	-2.4
Direct effects	8	8.07 (0.005)	0.32	0.8	5.7
Mediation effects	8	9.44 (0.002)	0.19	0.88	7
<i>Independence</i>	6	13.52 (0.004)	0	0.56	6.3

Note: The independence model assumes no relationships between variables and serves as a baseline for comparison. The other models test various hypothesized relationships among the variables. Fit indices include Chi-Square (χ^2), Comparative Fit Index (CFI), Root Mean Square Error of Approximation (RMSEA), and Bayesian Information Criterion (BICu). Lower BICu values indicate better model fit while penalizing complexity. The best-fitting model is bolded.

erythrocytes provide a useful model because they require minimally invasive sampling, normalization of respiration rates between samples can be challenging (Gnaiger 2020). However, because blood samples can be collected repeatedly from the same individual, longitudinal studies that follow mitochondrial function over an individual's lifespan or in response to experimental manipulations are possible. Finally, longitudinal work can capture the broad impacts that long-term environmental challenges can have on oxidative stress (Beattie et al. 2024; Majer et al. 2023; Wada 2019).

5 | Conclusion

In conclusion, this study sheds light on the intricate relationships among corticosterone, mitochondrial function, and oxidative damage in response to acute stress in Leach's storm-petrel chicks. Notably, our findings reveal two key insights: (1) individuals with the highest corticosterone responses exhibited reduced oxidative damage, challenging traditional views linking glucocorticoids solely to increased oxidative stress, and (2) those prioritizing ATP production during acute stress experienced higher DNA oxidative damage, supporting the protective role of proton leak and mitochondrial uncoupling. These results align with emerging evidence across avian species that glucocorticoids and mitochondrial function exhibit remarkable and rapid plasticity, allowing organisms to fine-tune metabolic strategies during periods of acute stress (Casagrande et al. 2020; Stier et al. 2019).

Despite these advancements, the complexity of the stress response underscores the need for further research. Future studies should explore the mechanisms underlying glucocorticoid-induced changes in mitochondrial function, particularly the roles of antioxidant defenses and ROS production, as well as tissue-specific responses to oxidative stress (see Koch et al. 2021). Integrative approaches that encompass multiple levels of biological organization—from cellular processes to organismal and ecological dynamics—will be critical to unravel the interplay between corticosterone, mitochondrial function, and oxidative damage under different stress scenarios.

This study provides novel insights into the trade-offs between ATP production and oxidative damage during acute challenges in wild animals. The nuanced roles of glucocorticoids and mitochondrial plasticity described here add to other recent papers (MacDougall-Shackleton et al. 2019), emphasizing the importance of moving beyond simplistic interpretations of stress markers to a more integrative framework. Such

approaches are vital for assessing the ecological and evolutionary consequences of stressors.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets used for the study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.