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RESEARCH ARTICLE

Enzyme polymorphism, oxygen and injury: a lipidomic analysis of flight-induced oxidative damage in a *succinate dehydrogenase d* (*Sdhd*)-polymorphic insect

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ABSTRACT

When active tissues receive insufficient oxygen to meet metabolic demand, succinate accumulates and has two fundamental effects: it causes ischemia–reperfusion injury while also activating the hypoxia-inducible factor pathway (HIF). The Glanville fritillary butterfly (*Melitaea cinxia*) possesses a balanced polymorphism in *Sdhd*, shown previously to affect HIF pathway activation and tracheal morphology and used here to experimentally test the hypothesis that variation in succinate dehydrogenase affects oxidative injury. We stimulated butterflies to fly continuously in a respirometer (3 min duration), which typically caused episodes of exhaustion and recovery, suggesting a potential for cellular injury from hypoxia and reoxygenation in flight muscles. Indeed, flight muscle from butterflies flown on consecutive days had lipidome profiles similar to those of rested paraquat-injected butterflies, but distinct from those of rested untreated butterflies. Many butterflies showed a decline in flight metabolic rate (FMR) on day 2, and there was a strong inverse relationship between the ratio of day 2 to day 1 FMR and the abundance of sodiated adducts of phosphatidylcholines and co-enzyme Q (CoQ). This result is consistent with elevation of sodiated lipids caused by disrupted intracellular ion homeostasis in mammalian tissues after hypoxia–reperfusion. Butterflies carrying the *Sdhd M* allele had a higher abundance of lipid markers of cellular damage, but the association was reversed in field-collected butterflies, where focal individuals typically flew for seconds at a time rather than continuously. These results indicate that Glanville fritillary flight muscles can be injured by episodes of high exertion, but injury severity appears to be determined by an interaction between SDH genotype and behavior (prolonged versus intermittent flight).

KEY WORDS: Succinate dehydrogenase, Complex II, Sodiated lipid, Oxidative damage, Hypoxia, Muscle

INTRODUCTION

Aerobic metabolism requires a balance between oxygen supply and demand, with demand changing by factors of 10 (endotherms: Bennett and Ruben, 1979; Hinds et al., 1993) to ∼100 (ecotherms: Bartholomew and Casey, 1978) depending on activity level. When the rate of oxygen delivery to mitochondria matches consumption rate, reactive oxygen species (ROS) are generated slowly and buffered by antioxidants (Turrens, 2003), thereby minimizing oxidative damage. Activity rates that exceed oxygen delivery rate cause elevated ROS generation and damage to cellular components such as lipids (Rubbo et al., 1994), proteins (Stadtman and Levine, 2000) and nucleic acids (Evans and Cooke, 2004). Extensive ROS generation and injury occur when oxygen delivery is blocked (ischemia) and then returns (reperfusion), with most of the damage occurring during reperfusion. The recently described mechanism underlying ischemia reperfusion injury is re-oxidation of accumulated succinate by succinate dehydrogenase (SDH; also known as mitochondrial complex II), followed by reverse electron transport at mitochondrial complex I, causing superoxide formation (Chouchani et al., 2014).

Succinate plays an additional physiological role in the regulation of oxygen delivery to tissues. During development and physiological adjustments throughout life, oxygen supply to the tissues is regulated by the hypoxia-inducible factor (HIF) pathway, a feedback loop that uses accumulation of succinate as a signal of insufficient oxygen supply (Pollard et al., 2005; Selak et al., 2005). Succinate is therefore a key metabolite in the regulation of both oxygen delivery and ROS generation, and variation in SDH function has the potential to affect development of the oxygen delivery network, metabolic performance and oxidative damage, in ways that may be interrelated. Here, we tested that hypothesis using a species that possesses a polymorphism in *succinate dehydrogenase d* (*Sdhd*) (Wheat et al., 2011; Marden et al., 2013), one of four subunits that comprise the SDH enzyme.

The Glanville fritillary butterfly, a model species for ecology and population biology (Ehrlich and Hanski, 2004), has provided extraordinary resolution of links between genetic variation and eco-evolutionary dynamics (Hanski et al., 2017; Hanski, 2011), supported by insights about the way genetic variation affects individual physiology and life history (Niitepõld and Saastamoinen, 2017; Niitepõld et al., 2009; Saastamoinen et al., 2009). One of the ecologically interesting genetic polymorphisms in Glanville fritillaries involves the tricarboxylic acid (TCA) cycle enzyme SDH (Wheat et al., 2011). Individuals carrying the *Sdhd M* allele have a 25% reduction in SDH enzyme catalytic rate and nearly twofold greater cross-sectional area of tracheae and tracheoles in their flight muscles, likely caused by constitutive pseudohypoxia signaling that stimulates elaborated tracheal development (Marden et al., 2013). Peak and sustained flight metabolism of *Sdhd M* allele butterflies is less affected by reduced atmospheric oxygen, and they have less post-flight activation of HIF signaling, consistent with their elaborated tracheal development. Experimental increases in succinate cause accumulation of HIF-1a protein (Marden et al., 2013). Flight metabolism of Glanville fritillaries is reduced in a 10% oxygen atmosphere, except under starvation conditions (Fountain et al., 2016). These lines of evidence suggest that

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variation in SDH enzyme catalytic rate affects activation of the HIF pathway, which in turn affects tracheal development, oxygen conductivity to the flight muscles and flight metabolic capacity.

Based on this background, we aimed to determine how *Sdhd* genotype and flight affect susceptibility to oxidative damage. Specifically, we tested the hypothesis that biomarkers of oxidative damage in flight muscles vary according to treatment (flown, rested, paraquat injected), flight metabolic rate, *Sdhd* genotype and behavioral context (continuous versus intermittent flight). Prior studies of oxidative damage to insect tissues have commonly used protocols that measure thiobarbituric acid reactive substances (TBARS) to quantify lipid peroxidation products such as aldehydes (Liu et al., 1997; Magwere et al., 2006). However, that reaction is non-specific and can be affected by a number of other biological molecules. Glanville fritillaries contain iridoid glycosides obtained from larval host plants, comprising up to 3.5% of adult dry mass (Suomi et al., 2003). To avoid the possibility of artifacts affecting the assay and to gain insights about specific molecules, we opted to profile the entire flight muscle lipidome using mass spectrometry (LC-MS/MS).

Lipidomic profiling to examine oxidative injury has previously been performed using mammalian models. Following stroke in the rat brain, certain phosphatidylcholines show higher levels of sodiation in affected cells (Wang et al., 2010), likely caused by the influx of sodium into cells and organelles during ischemia–reperfusion injury (Sanada et al., 2011). Hence, we looked for associations between such molecular signatures, oxidative damage and sustainability of flight metabolic performance.

MATERIALS AND METHODS

Butterflies

For laboratory experiments, we captured Glanville fritillary butterflies, *Melitaea cinxia* (Linnaeus 1758), at the beginning of the flying season (N=46) from three scattered populations in the Catalunya region of northeast Spain, near the villages of El Brull, Viladrau and Rupit. All individuals appeared to be recently emerged adults, characterized by wings with fresh colors, intact scales and no substantial wear. They were housed indoors in mesh cages in a cool shaded area where they did not fly and were fed a honey water solution twice daily. After 1 day of rest following capture, these butterflies were used for experiments involving continuously stimulated flight in a respirometer.

We separately obtained tissue from free-living butterflies of a minimum known age using a mark–recapture experiment. At the Rupit site, we captured recently emerged adults, wrote numbers on their hindwings, then released and recaptured them (sometimes multiple times) 2–7 days later. All butterflies recaptured on the last day were kept in cool dark containers for 4 h prior to freezing in liquid nitrogen.

Treatment

To examine the energetics and physiology of prolonged, high-exertion flight, we stimulated butterflies (N=30) to fly as continuously as possible over a 3 min period in a 1 liter respirometer at ∼30°C (for methodological details, see Haag et al., 2005). The atmosphere, either normoxic (21%) or mildly hypoxic (14% oxygen-over-nitrogen), was passed through the respirometer at 0.95 l min^{-1}. We flew each butterfly again on the following day in the alternative gas concentration; the order of these gas treatments was randomized. Shortly after the second flight, each individual was flash frozen in liquid nitrogen. We used a Z-transformation (Bartholomew et al., 1981) to obtain approximations of instantaneous metabolic rate.

From the same initial sample of butterflies, we randomly selected another group (N=16) to rest for 44 h indoors in cool shade, where they did not fly. As a positive control for oxidative damage, six of these individuals were injected in their lateral thorax with 2 µl of insect saline containing 30 mmol l^{-1} paraquat, which undergoes redox cycling to generate superoxide and cause oxidative damage in living tissue (Bus and Gibson, 1984; Hosamani and Muralidhara, 2013). Those butterflies were flash frozen 4 h later. We flash froze the remaining rested individuals (N=10; negative controls) at the end of the 48 h period. All sampled tissues were maintained in liquid nitrogen during transfer and storage prior to extraction of lipids.

Genotyping

To determine *Sdhd* genotypes (Marden et al., 2013; Wheat et al., 2011), we extracted total RNA using Trizol (Life Technologies, Carlsbad, CA, USA) from individual flash-frozen heads, followed by RNA column purification (RNeasy, Qiagen, Hilden, Germany) and cDNA synthesis with T7-(dT)24 primers. We amplified a portion of the 3′-UTR containing the *Sdhd* indel polymorphism using fluorescently labeled primers and used capillary electrophoresis (ABI 3730XL, Waltham, MA, USA) and GeneMapper software to determine sizes of the labeled amplicons and assign genotypes (deletion, D; mini deletion, M; insertion, I; or extra deletion, E). The primers and 3′ indel genotype sequences have been described previously (Marden et al., 2013).

Sample preparation

Butterfly material was removed from liquid nitrogen storage and immediately placed on dry ice for dissection. First, we sliced the thorax into two halves using a sagittal section along the dorsal longitudinal axis. Using the dorsal anterior half of one of these sections, comprising primarily the dorsal longitudinal flight muscle, we prepared tissue for lipidome processing using an established protocol that optimizes sample quality (Sarafian et al., 2014). The tissue sample was weighed to the nearest 0.1 mg, placed in a round-bottomed 2 ml Eppendorf Safe-Lock microcentrifuge tube and homogenized to a dry powder with a steel bead for 45 s at 20 Hz on a Retsch MM301 (Haan, Germany) mixer mill in pre-chilled hardware (−20°C) in a cold room at 5°C. To this still-frozen powder, we added 2 ml of cold 3:3:2 isopropanol:acetonitrile:water solution containing 1 µmol l^{-1} chloropropamide (an internal standard not present in nature), then homogenized the mixtures again for 2 min at 20 Hz on the mixer mill.

Homogenized samples were shaken at 4°C on a multi-therm shaker (Benchmark Scientific H5000-HC Multi-Therm Shaker, Edison, NJ, USA) for 20 min. Solids were removed by centrifugation at 12,000 ***g*** for 20 min in a refrigerated centrifuge (4°C), then the supernatant was transferred to clean, labeled 2 ml Eppendorf tubes and stored at 4°C prior to further processing. Protein pellets from the homogenized samples were stored at −80°C. Supernatants were dried in a vacuum centrifuge at room temperature, then dissolved in 300 µl of 50:50 acetonitrile:water solution. A 100 µl sample was transferred to autosampler vials; tubes were crimped and stored at −20°C.

Lipidomics data processing

At the Metabolomics core facility in the Huck Institutes of the Life Sciences, we used liquid chromatography-mass spectrometry (LC-MS) to create a lipid profile for each individual, utilizing a protocol

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previously employed to examine the lipid composition of insect tissues (de Bekker et al., 2013). Samples (5 µl) were run using the HPLC-QTOFMS (Shimadzu Prominence 20-UFLC XR, Kyoto, Japan; and AB Sciex 5600 TripleTOF, Framingham, MA, USA) platform equipped with a Waters Acquity CSH C18 column (100 mm×2.1 mm with a particle size of 1.7 µm; Waters Corporation, Milford, MA, USA). The composition of mobile phase A was 60% acetonitrile and 40% water with 10 mmol l^{-1} ammonium formate and 0.1% formic acid. Mobile phase B was 90% isopropanol and 10% acetonitrile with 10 mmol l^{-1} ammonium formate and 0.1% formic acid. Flow rate and temperature were maintained at 0.225 ml min^{-1} and 55°C, respectively. Positive ion electrospray ionization mass spectra were acquired over the mass range 100–1200 Da. We aligned the LC-MS traces using MarkerView (AB Sciex).

Lipidomics data analyses

LC-MS generated 22,515 features in positive ion mode, from which we analyzed the 3485 monoisotopic peaks with retention times between 1 and 20 min. Peaks were normalized first by the chloropropamide standard to correct for machine drift over the course of the numerous samples. Peak height was then normalized by wet tissue mass (g) to yield a measure of lipid abundance per amount of tissue sampled from each individual.

We used Metaboanalyst 3.0 software (www.metaboanalyst.ca) to examine the dataset in its entirety and determine whether there was a difference in the global lipid profile between treatments. We used a mass tolerance of 0.01 (*m*/*z*) and visual inspection to determine a retention time tolerance that robustly produced correct alignments. Peak abundance was log transformed and filtered based on interquartile range (IQR).

To identify individual lipid species affected by oxidative damage, we performed Wilcoxon tests (a non-parametric alternative to *t*-tests, necessitated by non-normality of peak heights) to compare the log-transformed (value+1, to retain zero values) normalized monoisotopic peak heights between the rested and paraquat-injected butterflies. For the flown butterflies, we performed linear regression analyses of the log-transformed monoisotopic peak heights versus the ratio of day 2 to day 1 peak flight metabolic rate (day 2/day 1 FMR, a quantitative measure of the sustainability of metabolic performance). To assess how false discovery arising from thousands of individual statistical tests may have affected the results, we first examined the distribution of *P*-values in both experiments to see whether there was an overabundance of low *P*-values, then performed a Fisher's exact test to determine whether there was a higher than expected number of lipid species that were significantly associated with both paraquat treatment and day 2/day 1 FMR.

During preliminary exploration of these data, we found that a large number of lipid species were more abundant in butterflies that had the greatest decline in peak FMR, but two individuals had large increases in their day 2 metabolic performance unrelated to their lipid profiles. These two outliers may have been stunned at the time of capture in the field and performed poorly on day 1, but recovered by day 2. In the remaining 28 individuals, the correlation between the abundance of many lipids and day 2/day 1 FMR was consistent and robust, suggesting that exclusion of the two outliers was justified and required in order to observe major features of the relationship between lipid profiles and the sustainability of flight performance.

Generating product ion spectra

Where possible, we used MS/MS product ion spectra to identify the individual lipid species most associated with treatments and flight muscle injury. The lipidomics data were initially acquired in information-dependent acquisition (IDA) mode, wherein abundant lipid species detected in a survey scan triggered up to 20 MS/MS product ion spectra before the next survey scan began. Analyses of those data revealed that many of the lipids most associated with paraquat and day 2/day 1 FMR existed in low abundance, often insufficient to trigger a product ion scan in IDA. For that reason, the unused portion of frozen samples of flight muscle from six individuals with the highest abundance of those lipids of primary interest were prepared for lipidomics using the same methods as the original samples and processed using LC-MS parameters as before, but this time we included the MS/MS product ion scan in the duty cycle so that even low-abundance lipids of interest would generate fragment spectra. Those data were further explored using LipidView to identify lipid species of particular interest. For one of the identified lipid species, we compared samples with a co-enzyme Q9 (CoQ9) standard (item 16866, Cayman Chemical Company, Ann Arbor, MI, USA).

RESULTS

Flight performance

The total CO_2 emitted and peak FMR of butterflies continuously stimulated to fly in a respirometer were affected primarily by gas treatment (lower total CO_2 emitted in a 14% O_2 atmosphere compared with normoxia) and the presence of an *Sdhd M* allele (as previously reported for this sample of Spanish butterflies; Marden et al., 2013), but there were no significant main effects of day (day 1 versus day 2), body mass, sex or the interaction between day and gas treatment (Table 1). Most butterflies periodically became exhausted during the 3 min of continuously stimulated flight in either O_2 atmosphere and lay immobile and unresponsive, despite the short duration of the experiment and their well-fed status. These periodic episodes of immobility, followed by recovery, likely involved tissue-level hypoxia and reoxygenation, similar to conditions that cause ischemia–reperfusion injury in vertebrates.

Flown butterflies varied in their ability to achieve a comparable peak FMR across two consecutive days. Some individuals increased their peak FMR by as much as 70% on the second day, whereas others achieved less than half of their day 1 peak FMR. There was no

Table 1. Flight metabolic performance (measured as total and peak CO_2 emission) of Glanville fritillaries

Source	d.f.	*F*-ratio	*P*
Total CO_2 emitted (ml)			
Day (day 1 or day 2)	1	0.11	0.741
Gas (14% or 21% O_2)	1	4.23	0.049
Day×gas	1	3.02	0.095
Sex	1	0.80	0.380
Body mass	1	1.88	0.183
Sdhd M versus no *M*	1	7.39	0.012
Peak CO_2 emission rate (ml CO_2 h^{-1})			
Day (day 1 or day 2)	1	1.31	0.263
Gas (14% or 21% O_2)	1	2.03	0.165
Day×gas	1	0.56	0.461
Sex	1	1.22	0.280
Body mass	1	0.56	0.462
Sdhd M versus no *M*	1	3.94	0.058

Flight metabolic performance is shown as a function of day (first or second; one flight per day), gas environment (14% or 21% oxygen), sex, body mass, presence/absence of the *Sdhd M* allele, and the interaction between day and gas environment. *N*=30 butterflies during 3 min of continuously stimulated flight. Butterfly was included as a random factor to account for repeated measurement of individuals (once each on day 1, day 2).

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overall trend to increase or decrease performance over consecutive days (Table 1; absence of a day effect), and mean day 2/day 1 FMR did not differ from unity (P=0.58).

Treatment effects on the flight muscle lipidome

We tested the hypothesis that the global lipid composition of flight muscles was affected by the treatments (rested, paraquat and flown). The order of gas treatment was dropped from analyses because there were no significant interactions between day and gas treatment (Table 1), and all of the flown butterflies experienced both the 14% and 21% O_2 environments.

We used hierarchical clustering (heatmaps; Metaboanalyst 3.0) to identify lipids most different between treatments (discriminant analysis based on partial least squares and variable importance of projection; Xia and Wishart, 2016). Rested and paraquat-injected butterflies clustered separately on two basal branches (Fig. 1). Two of the flown individuals grouped with rested butterflies; the remainder (N=26) clustered in the same basal group as paraquat-injected butterflies. Nearly all (N=24) of the flown butterflies formed a distinct subgroup with some lipid abundances characteristic of the paraquat group and others more similar to those of rested butterflies (blocks of similar color in Fig. 1).

Flight decline and oxidative damage

To further explore the lipidome similarity of flown and paraquat-treated butterflies, we focused on lipid species (N=3485 monoisotopic peaks) associated with both paraquat effects and the decline in flight metabolic performance (day 2/day 1 FMR). False discovery must be considered, as chance associations unavoidably arise when performing a large number of comparisons. To test the null hypothesis that associations between lipid abundances and paraquat treatment or day 2/day 1 FMR were a collection of false positives, we determined whether the frequency distribution of P-values departed from uniform (i.e. 5% with P<0.05, 50% with P<0.50, and so forth). There was a large enrichment of small P-values for lipid abundances correlated with either day 2/day 1 FMR (Fig. 2A) or paraquat treatment (Fig. 2B). From this result, we can infer that a large portion of the flight muscle lipidome (many hundreds of lipid species) varied with paraquat treatment and the decline of flight metabolic performance.

Certain lipid species showed significant associations with both paraquat treatment and day 2/day 1 FMR, but here again we must consider the null hypothesis that these overlaps occurred by chance. There were 390 lipid species associated significantly (P<0.05) with paraquat treatment, 517 associated significantly with day 2/day 1

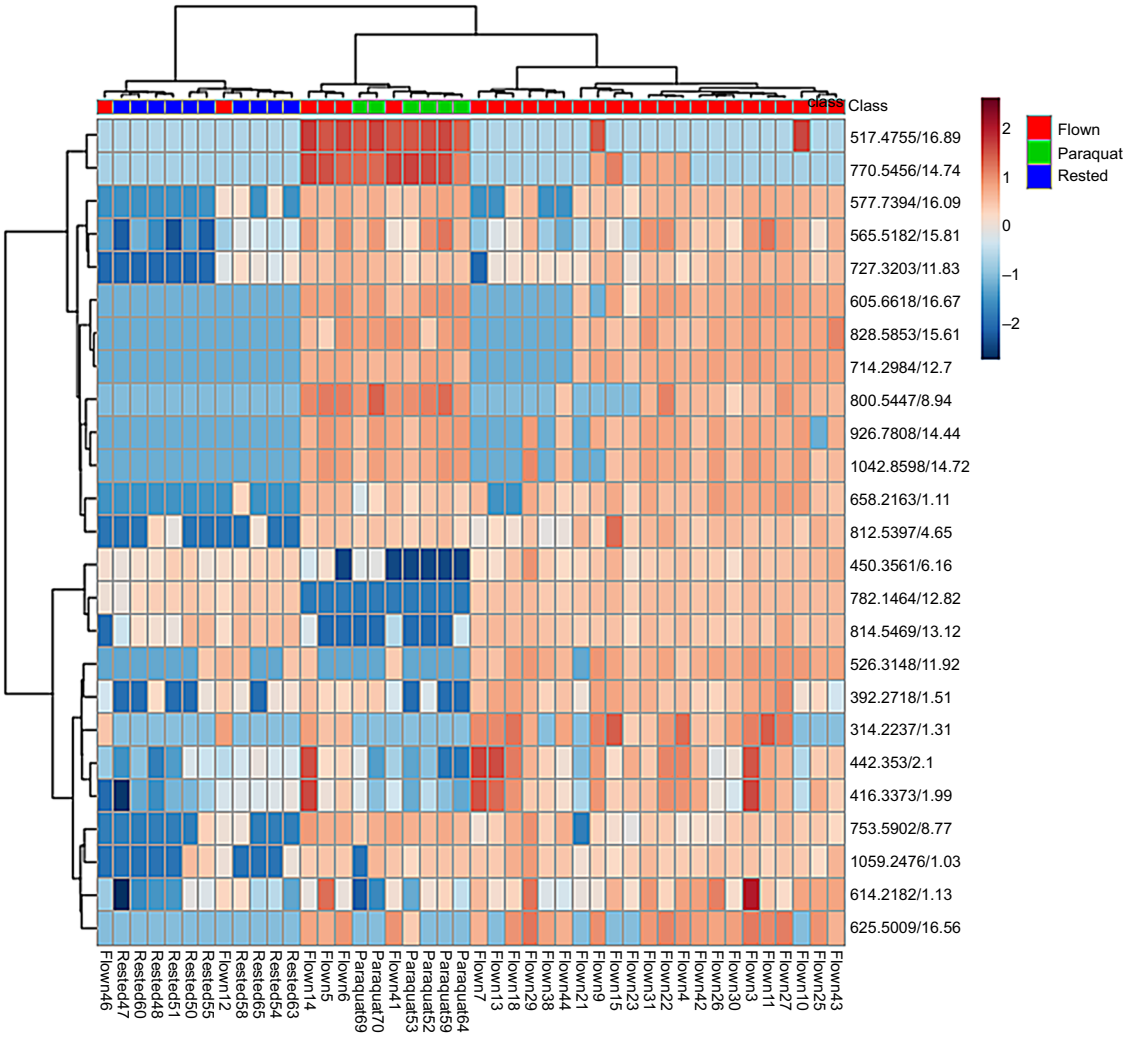

Fig. 1. Hierarchical cluster analysis of lipids most associated with treatments. Hierarchical clustering for the 25 most significantly different lipids between flown (N=30), paraquat-injected (N=16) and rested butterflies (N=10). Data are Metaboanalyst 3.0 PLS-DA VIP scores.

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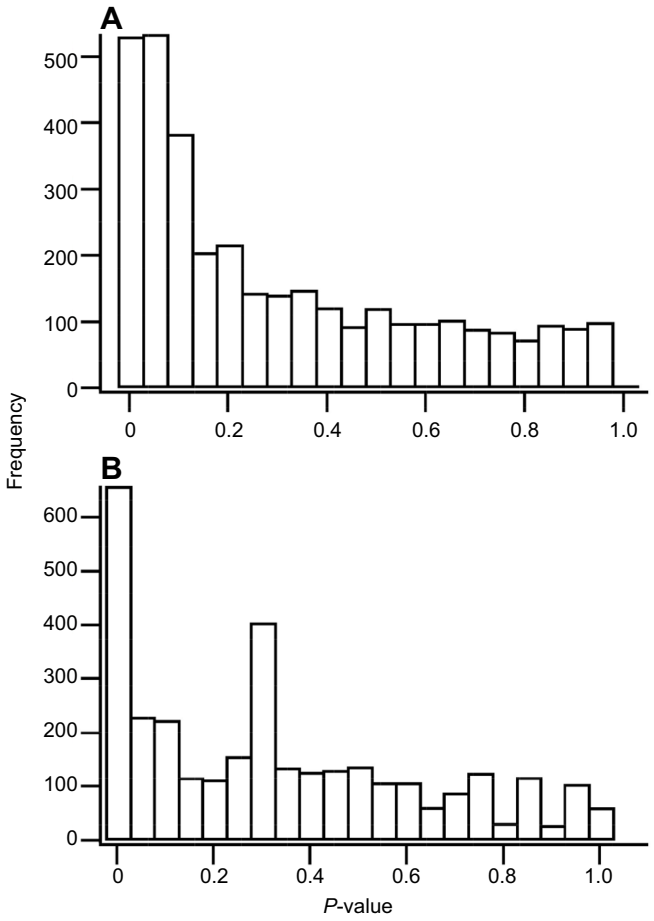

Fig. 2. Distribution of *P*-values from tests (*N*=3485) relating individual lipid abundance to phenotype. *P*-values are from (A) linear regressions where the response variable was day 2/day 1 flight metabolic rate (FMR) of flown butterflies (*N*=28), and (B) Wilcoxon ranked-sum tests comparing rested (*N*=10) and paraquat-treated (*N*=16) unflown butterflies.

FMR, and 139 associated with both. The probability of this large an overlap occurring by chance is very small (*P*=0.0002, Fisher's exact test). Furthermore, 99 of the top 100 correlations between lipid abundances and day 2/day 1 FMR were negative (higher abundance in butterflies with greater decline in metabolic performance), and 94 of those were also higher in paraquat-treated versus rested butterflies, whereas random false positives would yield an approximately even mix of positive and negative associations. The overlap of lipids associated with both paraquat and day 2/day 1 FMR and the nearly uniform directionality of these associations indicate that many of the same lipid species, perhaps those most indicative of oxidative damage, were markers for both paraquat effects and flight-induced decline of metabolic performance.

Association between lipid markers of oxidative damage and peak FMR on consecutive days

There were 20 lipid features (Table S1) strongly associated (*P*<0.01) with both paraquat treatment and day 2/day 1 FMR; we treated these as biomarkers of oxidative damage and used them in additional analyses. To attain equal statistical weight, we normalized the abundance of these 20 lipids to the grand mean of each, across individuals. The mean of these normalized abundances was positively related to peak FMR on day 1 (*P*=0.02), and negatively related to peak FMR on day 2 (*P*=0.002; Fig. 3). These opposite associations suggest that high FMR on day 1 tended to cause oxidative damage, followed by a reduction of FMR on day 2. The mean of these normalized abundances was negatively related to day 2/day 1 FMR and was increased by paraquat treatment (*P*<0.0001 for both; Fig. 4A,D).

MS/MS identification of particular lipid species associated with both paraquat treatment and declining flight metabolic performance

In certain cases we were able to identify the composition of lipids of interest from their product ion spectra. The two lipid species most strongly associated with both paraquat and day 2/day 1 FMR (Fig. 4B,C,E,F) were a *m*/*z*=836.58 peak and a related peak at *m*/*z*=838.56. Secondary ion spectra identified these as glycerol phosphatidylcholines (PCs; 16:0/18:2 and 16:0/18:3, respectively) with adducts of acetonitrile (the extraction solvent) and sodium ($[M+Na+C_2H_3N+NH_4]^+$) (Fig. S1A), similar to an increase in sodiated lipids in mammalian tissues after ischemia–reperfusion injury (Sanada et al., 2011), likely caused by an influx of sodium into cells and organelles (Wang et al., 2010). Informative fragment ion spectra underlying these two lipid identifications (Fig. S1B) included $[PC\ Na\text{-}59]^+$, $[PC\ Na\text{-}183]^+$ and $[PC\ Na\text{-}205]^+$, representing losses of trimethylamine (TMA), non-sodiated choline phosphate and sodiated choline phosphate, respectively (Al-Saad et al., 2003; Domingues et al., 2001). The parent PCs had the following masses: $[M+H]^+$=756.55 and $[M+H]^+$=758.55, respectively; these were the 4th and 5th most abundant lipid features (means across individuals). It is unclear whether these lipids were more susceptible to sodiation or whether their high abundance simply made their sodiated adducts more detectable.

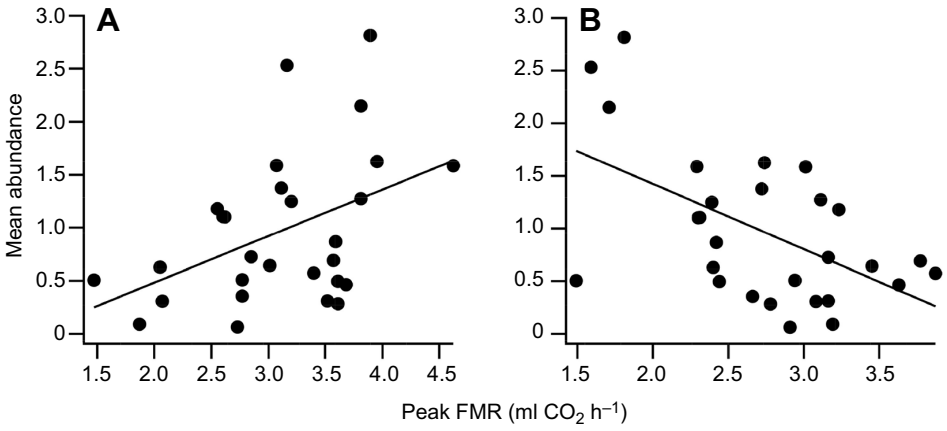

Fig. 3. Mean normalized abundance of 20 lipids associated with oxidative damage compared with peak FMR on two consecutive days (*N*=28). (A) Day 1; (B) day 2.

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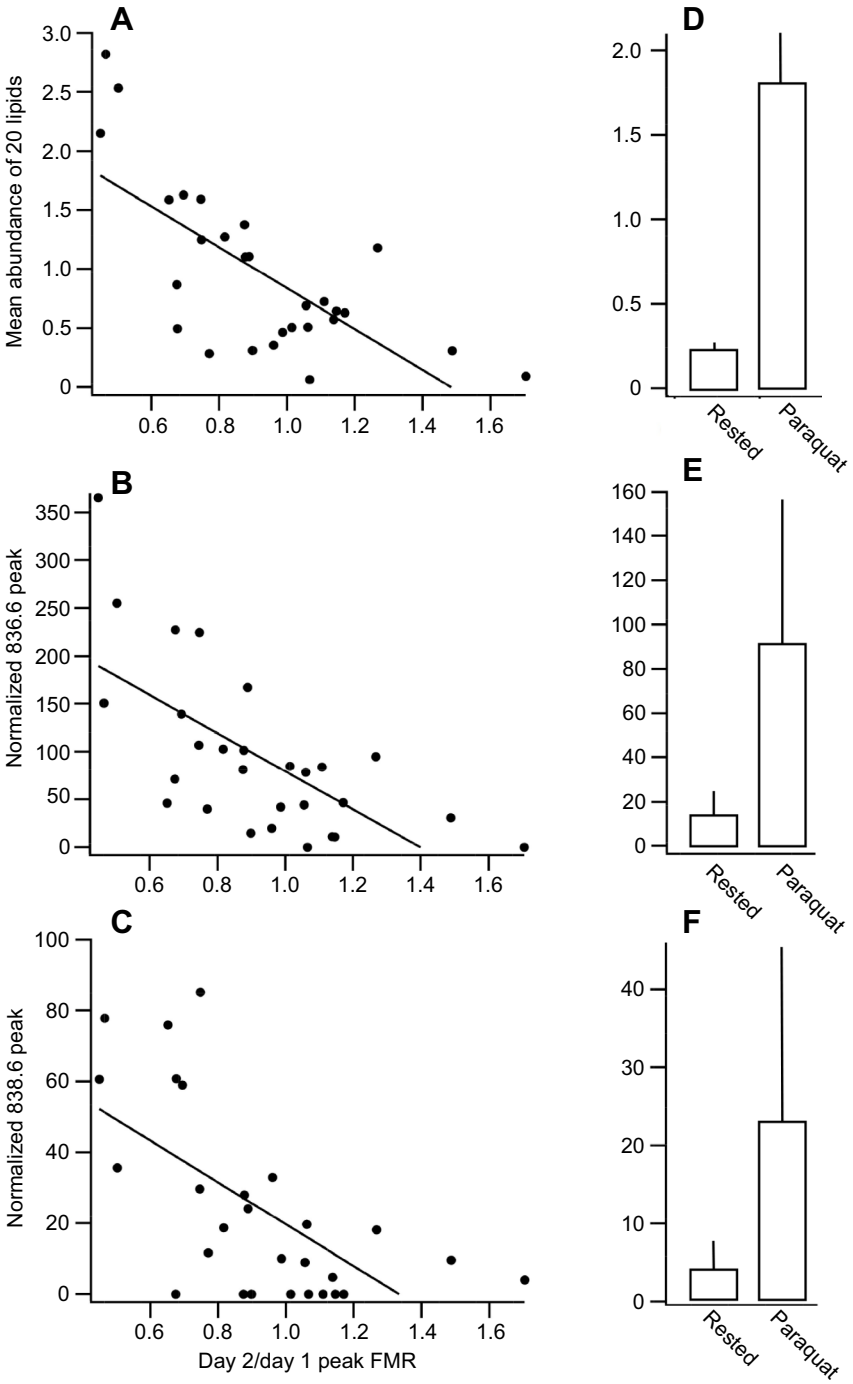

Fig. 4. Relationship between lipid abundance and decline of peak FMR (left) and treatments in unflown butterflies (right). (A,D) Grand mean abundance of 20 lipid species associated with oxidative damage plotted against peak FMR (A) and in rested versus paraquat-treated butterflies (D). (B,E) As for A and D, with lipid *m*/*z*=836.58 (a sodiated adduct of a 16:0/18:2 phosphatidylcholine; P=0.0008 for day 2/day 1 FMR, P=0.0003 for paraquat). (C,F) As for A and D, with lipid *m*/*z*=838.56 (a sodiated adduct of a 16:0/18:3 phosphatidylcholine; P<0.0001 for day 2/day 1 FMR, P=0.009 for paraquat). Bars show means±s.e.m. A–C, N=28; D–F, N=10, 16, respectively.

Ubiquinone (CoQ) and its sodium/potassium adducts

A group of lipid features (N=110) co-eluted at 16.9 min and was remarkable for having many significant associations (N=69) with day 2/day 1 FMR (Fig. 5). Peak heights in that group tended to be elevated in butterflies that had the greatest decline in FMR (median correlation coefficient between those normalized peak heights and day 2/day 1 FMR=−0.49; median P=0.008), and one (*m*/*z*=517.48) was among the top-ranked contributors to the hierarchical clustering that distinguished flown, rested and paraquat-treated butterflies (Fig. 1). Exact mass, product ion spectra and comparison with a standard (Fig. S2) identified this elution group as ubiquinol/ubiquinone ($[M+H]^+$=795.63, aka co-enzyme Q; in insects CoQ9), along with fragments and adducts thereof.

Further analysis of CoQ9 peaks revealed strong evidence for the presence of sodium and potassium adducts, the abundance of which had strong associations with treatment and phenotype. Compared with rested butterflies and in relation to the parent molecule, the flown and paraquat-treated butterflies had a higher abundance of sodiated CoQ9 ($[M+Na]^+$=817.61; Fig. 6A; P=0.02) and a lower abundance of potasiated CoQ9 ($[M+K]^+$=833.58; Fig. 6A; P=0.01). The abundance of sodiated CoQ9 was positively correlated with lower day 2/day 1 FMR (Fig. 6B; P=0.02) and with the mean normalized abundance of the aforementioned 20 lipid

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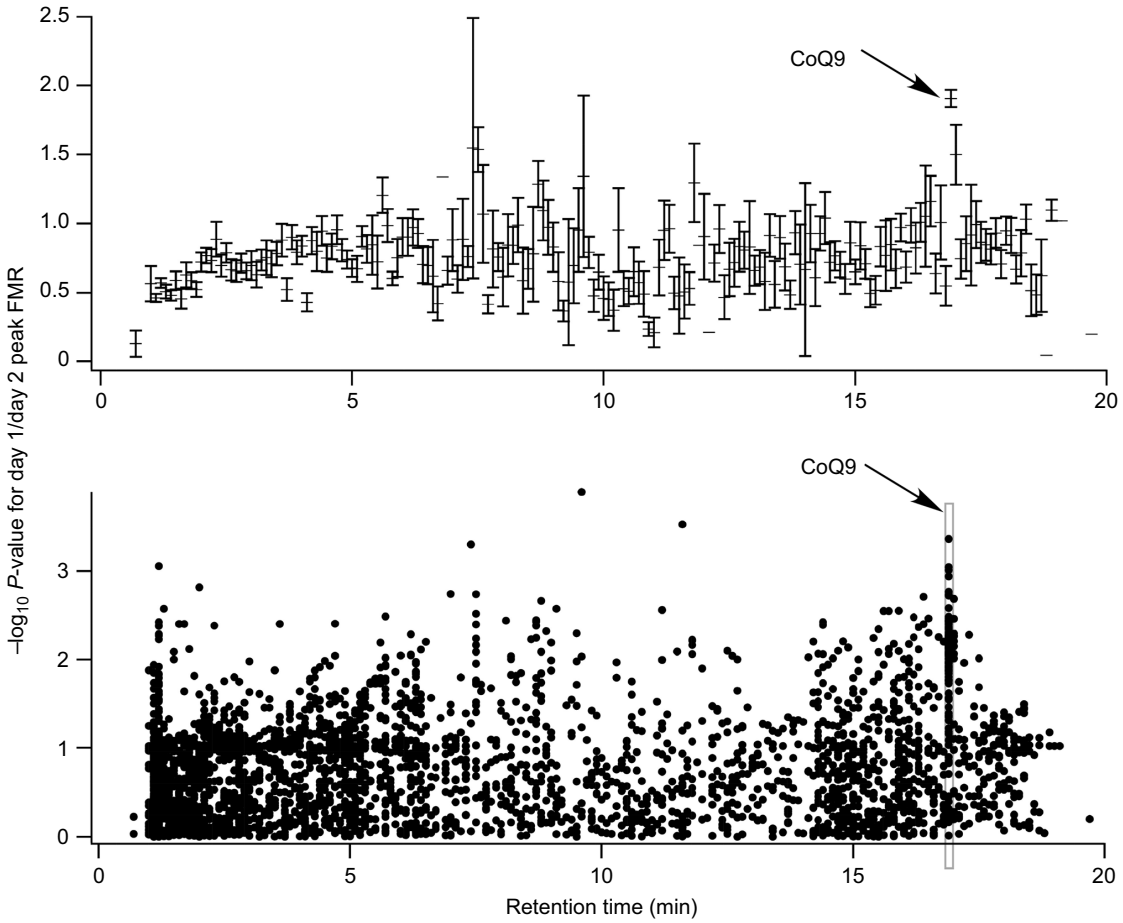

Fig. 5. Plot of $-\log_{10}$ *P*-value relating day 2/day 1 FMR to normalized monoisotopic lipid abundance. Data are grouped by retention time (*N*=28 butterflies). Arrows indicate ubiquinone (co-enzyme Q9, CoQ9) and fragments and adducts thereof (points within box in lower plot). Bars show means±s.e.m.

features most strongly associated with both paraquat and day 2/day 1 FMR (*P*=0.03, not shown). The abundance of the unmodified parent CoQ9 was also strongly associated with lower day 2/day 1 FMR (*P*=0.006). The parent CoQ9 was the 39th most abundant lipid, 13% as abundant as the $[M+H]^+$=756.55 PC described above. The detectability of sodiated adducts of CoQ, despite their relatively low abundance, suggests that CoQ may be particularly vulnerable to modification when mitochondrial ion homeostasis is disrupted.

Sdhd genotype, flight decline and lipid biomarkers of oxidative damage

Butterflies carrying one or more copies of the *Sdhd M* allele had 37% higher mean normalized abundance (*P*=0.02) of the 20 lipids most

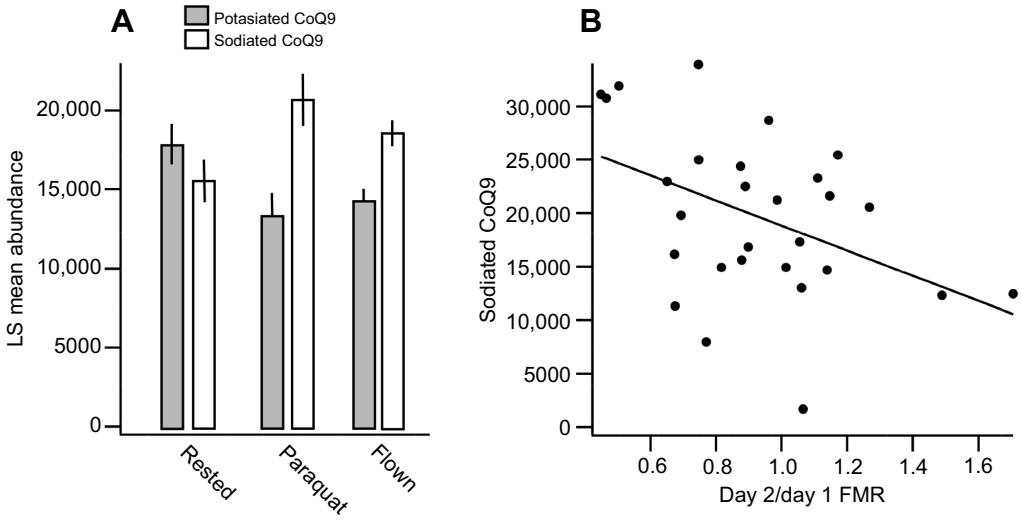

Fig. 6. Abundance of potassium and sodium adducts of CoQ9. (A) Least squares (LS) mean abundance of sodiated CoQ9 in butterflies treated with paraquat (*N*=16) or subjected to continuously stimulated flight (*N*=28); the model accounted for abundance of the parent CoQ9. (B) The abundance of sodiated CoQ9 in relation to the change in peak FMR over consecutive days (*N*=28). Bars show means ±s.e.m.

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Table 2. Mean normalized abundance of 20 lipids associated with oxidative damage (R^2=0.62)

Source	d.f.	*F*-ratio	*P*
Day 1 peak FMR	1	12.5	0.002
Day 2 peak FMR	1	31.4	<0.0001
Sdhd M versus no *M*	1	5.8	0.02

Multivariate model showing how mean normalized abundance of 20 lipids associated with oxidative damage in flight muscles varied in relation to day 1 and day 2 flight metabolic rate (FMR) and *Sdhd* genotype. See Fig. 3 for univariate plots of these data.

strongly associated with both paraquat and day 2/day 1 FMR, independent of the effects of peak FMR on day 1 and day 2 (Table 2). Butterflies with the *Sdhd M* allele did not have a greater abundance of either the CoQ9 parent molecule (P=0.14) or sodiated CoQ9 (P=0.35, accounting for the abundance of the parent molecule).

Sdhd genotype and lipid biomarkers of oxidative damage in free-living butterflies

Three minutes of continuously stimulated flight in a 1 liter jar, including one bout in mildly hypoxic air (14%O_2), may be more physiologically challenging than free flight in nature. To better understand these differences and test for an *Sdhd* allele effect on flight muscle injury in free-living butterflies, we first characterized flight distances and durations of a set of focal individuals (N=35) in the field. These observations were made during times of peak activity (between noon and 16:25 h) on two clear, calm days at ambient temperatures ranging from 17 to 29°C. Median flight duration was 14 s (N=462 flights), with a maximum of 440 s. Median distance was 10 m (N=79 relatively straight flights), with a maximum of 150 m (Fig. 7A,B). Regression of distance on duration indicated an average flight velocity of 1.3 m s^{-1}, slower than any of the velocities reported for 62 free-flying butterfly species (Dudley and Srygley, 1994) and requiring low mechanical power (Dudley, 1991). Hence, free-living Glanville fritillaries typically use a minimally demanding mode of flight to cover short distances, but flights lasting 3 min or longer did sometimes occur (98th percentile of durations). Flights in the field typically comprised a mixture of flapping and gliding, and exhaustion was never observed.

Table 3. Abundance in marked–recaptured butterflies (N=25) of 11 lipids previously associated with oxidative damage and the sodiated adduct of CoQ9

Source	d.f.	*F*-ratio	*P*
Normalized lipid abundance			
Age	1	0.17	0.69
Sdhd M versus no *M*	1	1.86	0.19
Sodiated CoQ9			
Age	1	0.09	0.77
Potasiated CoQ9	1	120.6	<0.0001
Sdhd M versus no *M*	1	7.87	0.01

Independent variables are the presence/absence of the *Sdhd M* allele, and the number of days between initial and final recapture (age; 3–7 days). The analysis of sodiated CoQ9 abundance includes also the abundance of potasiated CoQ9 to control for overall changes in CoQ9. A higher abundance of sodiated CoQ9 in butterflies lacking the *Sdhd M* allele was a robust result, supported also (not shown) in a model that included both potasiated and parent CoQ9 abundance (P=0.02), or when the model included only sodiated CoQ9 abundance (P=0.01).

In a sample of marked and recaptured butterflies (N=58), the median time between initial and final capture was 25 h (maximum of 6 days), median net distance moved was 83 m, and there was a weak positive relationship (P=0.02; R^2=0.09) between the recapture interval and net distance moved. After 6 days, this relationship predicted a net distance moved of ∼188 m. These results are consistent with the focal individual results, indicating primarily short flights by this relatively weak-flying species over a period that comprises a large portion of their expected adult lifespan (∼12 days at a cooler site; Niitepõld and Hanski, 2013).

We performed lipidomics analysis of flight muscle from a separate sample of marked–recaptured butterflies (N=25) and, using exact mass and column retention time, tentatively identified 11 of the 20 lipid peaks previously associated with oxidative damage. The normalized mean abundance of those lipids did not differ according to time since initial capture (3–7 days) or *Sdhd M* allele genotype (P=0.69 and P=0.19, respectively; Table 3). However, butterflies carrying the *Sdhd M* allele (N=10) had a significantly lower level (8% reduction) of sodiated CoQ9 (P=0.01; Table 3, Fig. 7C).

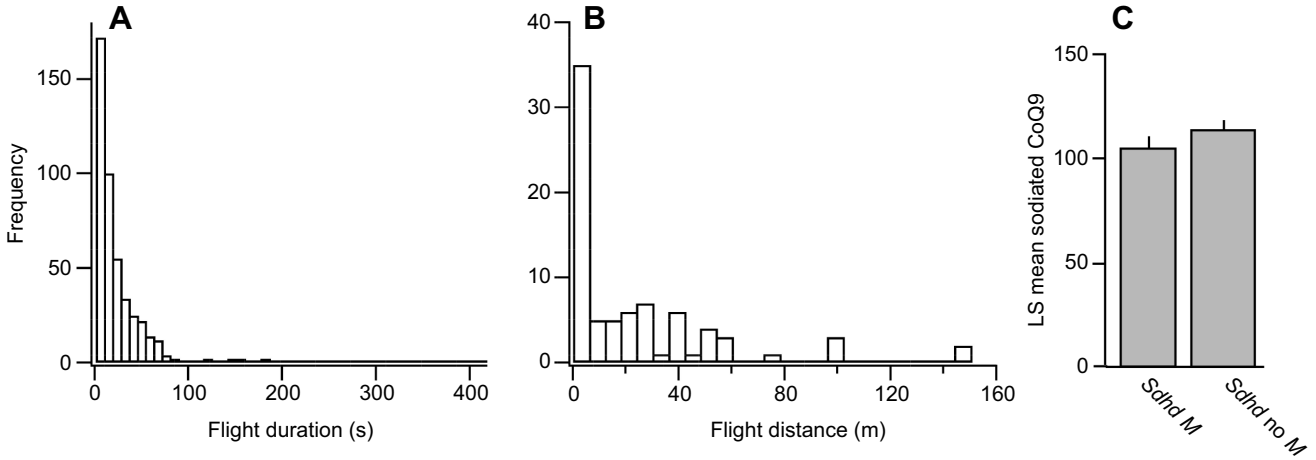

Fig. 7. Flight and CoQ9 data from free-flying butterflies. (A,B) Frequency distributions of flight duration (A) and distance (B) for free-flying Glanville fritillary butterflies in the field. Data are for N=462 flight durations, 79 distances, 35 butterflies. (C) *Succinate dehydrogenase d* (*Sdhd*) genotype-associated abundance of sodiated CoQ9 in marked–recaptured butterflies (N=25 butterflies, 10 with the *Sdhd M* allele; LS means from the model in Table 3, arbitrary units from normalized lipid peak heights). Bars show means±s.e.m.

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DISCUSSION

Insect flight muscles operate at some of the highest metabolic rates of any animal tissues, but in contrast with vertebrate tissues they have not been studied extensively for oxidative damage. Flies repeatedly stimulated to fly show a greater accumulation of oxidative damage compared with rested flies (Magwere et al., 2006; Yan and Sohal, 2000), and flying honeybees produce high levels of ROS in their flight muscles, which, along with age-related decreases in antioxidant activity, may cause senescence and reduced longevity (Williams et al., 2008). Here, we found that flight muscles of Glanville fritillary butterflies had measurable oxidative damage caused by one or two short bouts (3 min) of maximal or near-maximal exertion. Note that this is a relatively weak-flying species (Rauhamaki et al., 2014) and we would not expect similar results from stronger fliers such as monarch butterflies capable of continental-scale migration, or sphinx moths that feed while hovering, cover many kilometers in a single night, and employ novel metabolic physiology that minimizes oxidative damage to their flight muscles (Levin et al., 2017).

The Glanville fritillaries we stimulated to fly appeared to be continuously trying to escape using near-maximal levels of exertion. In this artificial setting, they typically displayed recurring cycles of temporary exhaustion and recovery. Butterflies in our experiment were well fed and rested, so exhaustion was likely caused by exercise-induced hypoxia, after which tissue-level oxygen presumably recovered via tracheal conductance. Hence, the physiological effect should be similar to ischemia–reperfusion injury in vertebrate tissues, which accumulate succinate that drives extensive ROS generation during reperfusion (Chouchani et al., 2014). Evidence supporting oxidative injury in the present sample of butterflies includes lipidomic profiles showing a strong tendency for flown butterflies to cluster with paraquat-treated butterflies in a group distinct from rested butterflies (Fig. 1), and over 100 lipid features that had increased abundance in both paraquat-treated butterflies and butterflies that were least able to sustain their flight metabolic performance on consecutive days.

There was a significant elevation of sodiated in relation to potasiated adducts of two abundant PCs and CoQ9 in experimentally flown butterflies that showed the greatest decline in flight performance over consecutive days. This result is consistent with the increase of sodiated PCs in the mammalian brain (Wang et al., 2010; Shanta et al., 2012) after ischemic stroke and reperfusion. Tissue hypoxia causes depletion of ATP for ion transport, a decrease in intracellular pH, influx of Na^+ into afflicted cells, and opening of the mitochondrial transition pore, ultimately disrupting mitochondrial homeostasis (Sanada et al., 2011). Hence, increases in the abundance of sodiated adducts of lipids in butterfly flight muscles after fatiguing flight matches results from other tissues with a high oxygen demand and may have arisen from both the loss of mitochondrial ion homeostasis during hypoxia and membrane damage following reoxygenation.

The lipid most associated with flight performance decline (Fig. 5), CoQ, interacts directly with SDH (mitochondrial complex II), carrying electrons from the oxidation of succinate to complex III or inappropriately to complex I during reperfusion (Chouchani et al., 2014). CoQ functions also as an antioxidant (Lekli et al., 2008; Tsukahara et al., 1999) that stabilizes membranes, inhibits lipid peroxidation and is effective in treating ischemia–reperfusion injury. Hence, it is interesting that in addition to more sodiated CoQ, butterflies with the greatest decline in flight metabolic performance also had increased abundance of the unmodified parent CoQ. The latter may reflect elevated synthesis to repair mitochondrial membranes and/or provide increased anti-oxidant capacity to protect against further oxidative injury.

Evidence from many taxa indicates that metabolites of energy metabolism have important effects on physiology and fitness (Eanes, 2017; Marden, 2013; Talbert et al., 2015). The succinate-driven nature of oxidative injury (Chouchani et al., 2014; see also Walker et al., 2006) and polymorphism in one of the four subunit genes (*Sdhd*) encoding SDH stimulated us to examine how this locus affects flight physiology of Glanville fritillary butterflies. We found a difference in the abundance of lipids indicative of oxidative damage in the flight muscles of butterflies carrying the *Sdhd M* allele, but the directionality of this difference was context dependent. During continuously stimulated flight in a respirometer, butterflies carrying the *Sdhd M* allele had higher FMR, and their flight muscles contained higher levels of lipids associated with oxidative damage and decreased sustainability of FMR. Greater oxygen conductivity to the flight muscles may have allowed these butterflies to have both a higher FMR and a more rapid return of oxygen during periodic bouts of exhaustion. Accordingly, a more elaborated tracheal network may be a double-edged sword, enhancing peak metabolic capacity but also increasing the rate of oxygen return during episodes of exhaustion and the severity of oxidative damage.

In nature, flights by Glanville fritillaries were mostly only a few seconds in duration and recaptured butterflies showed net movement of only a few hundred meters over many days, comprising a large portion of their expected adult lifespan. Lipid profiles of marked–recaptured butterflies showed a different association with the *Sdhd M* genotype. Free-living butterflies lacking the *Sdhd M* allele had higher levels of sodiated CoQ9. This lab-to-field reversal of an association between the *Sdhd* M allele and markers of oxidative damage may relate to differences in continuous near-maximal flight versus intermittent free flight. During submaximal levels of exertion in a field setting, higher oxygen conductivity of *Sdhd M* allele butterflies should make them less subject to oxidative injury because they operate farther from their maximum oxygen delivery rate ($\dot{V}_{O_2,max}$). In contrast, butterflies lacking the *Sdhd M* allele may in nature perform closer to their $\dot{V}_{O_2,max}$ and, over the course of hundreds to thousands of mostly short flights, experience a greater accumulation or higher equilibrium level (given that we did not observe an age effect) of small oxidative injuries.

Three independent, unbiased and differently focused large-scale screens of Glanville fritillaries have now identified a set of interacting molecules in the HIF pathway that are strongly associated with phenotypes and/or ecological dynamics. First, a transcriptomic analysis compared F1 offspring of the colonizers of isolated patches with average butterflies in the metapopulation and discovered *Sdhd* alleles associated with population dynamics (Wheat et al., 2011) and HIF pathway phenotypes (Marden et al., 2013). A subsequent RNA-Seq study of alleles and gene expression differences between populations occupying different landscape types (patchy versus continuously distributed host plants), and hence with a different need for long-distance dispersal flights, found that allele frequencies and expression phenotypes of *Hif1a* were among the top-ranked differences (Somervuo et al., 2014). Here, we identified CoQ9 as the top biomarker for oxidative injury in flight muscles. These three molecules (SDH enzyme, HIF1a protein and CoQ9) are functionally linked via the regulation of HIF1a protein abundance by succinate and the transfer of electrons from succinate to CoQ. It is striking that these different 'omics' approaches in one species all identified mitochondrial complex II (SDH) and

immediate molecular interactors affecting the HIF pathway and ROS generation as a central axis for variable metabolic phenotypes, with correlated effects on life history and ecology. Alleles of *Sdhd* affect these phenotypes and are apparently maintained by balancing selection, presumably caused by varying fitness in different ecological contexts, including the size (Wheat et al., 2011) and arrangement (Somervuo et al., 2014) of suitable habitat patches in the landscape.

In summary, these results provide new insights regarding damage to mitochondria and lipids by oxidative injury, and supply new details regarding the way in which genetic variation relates to physiological variation in this species that is a model for population biology and ecology. Increased sodiation of membrane lipids appears to be a general and possibly universal biomarker for oxidative damage to membranes. Among mitochondrial lipids, CoQ may be particularly vulnerable to oxidative damage and we observed a strong increase in CoQ abundance in flight muscles from individuals showing oxidative damage and reduced metabolic capacity. Associations between *Sdhd* alleles and oxidative damage support the hypothesis that succinate plays a dual role in both HIF pathway regulation and ischemia–reperfusion injury. These results from an insect may inform our understanding of why humans show evidence for balancing selection at an SDH locus (Baysal et al., 2007), and why alleles affecting the HIF pathway existed in lowland human populations prior to increasing in frequency in populations living at high altitude (Buroker et al., 2012; van Patot and Gassmann, 2011; Yi et al., 2010).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.E.P., J.H.M.; Methodology: J.E.P., P.B.S., J.H.M.; Software: J.H.M.; Validation: J.E.P., P.B.S., J.H.M.; Formal analysis: J.E.P., J.H.M.; Investigation: J.E.P., P.B.S., J.H.M.; Resources: J.H.M.; Data curation: J.E.P., P.B.S., J.H.M.; Writing - original draft: J.E.P., J.H.M.; Writing - review & editing: J.E.P., P.B.S., J.H.M.; Visualization: J.E.P., J.H.M.; Supervision: P.B.S., J.H.M.; Project administration: J.H.M.; Funding acquisition: J.H.M.

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Data availability
Data from this study are available from the Dryad digital repository (Pekny et al., 2018): https://doi.org/10.5061/dryad.p1139.

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