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RESEARCH ARTICLE

Flying on empty: reduced mitochondrial function and flight capacity in food-deprived monarch butterflies

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ABSTRACT

Mitochondrial function is fundamental to organismal performance, health and fitness – especially during energetically challenging events, such as migration. With this investigation, we evaluated mitochondrial sensitivity to ecologically relevant stressors. We focused on an iconic migrant, the North American monarch butterfly (*Danaus plexippus*), and examined the effects of two stressors: 7 days of food deprivation and infection by the protozoan parasite *Ophryocystis elektroscirrha* (known to reduce survival and flight performance). We measured whole-animal resting metabolic rate (RMR) and peak flight metabolic rate, and mitochondrial respiration of isolated mitochondria from the flight muscles. Food deprivation reduced mass-independent RMR and peak flight metabolic rate, whereas infection did not. Fed monarchs used mainly lipids in flight (respiratory quotient 0.73), but the respiratory quotient dropped in food-deprived individuals, possibly indicating switching to alternative energy sources, such as ketone bodies. Food deprivation decreased mitochondrial maximum oxygen consumption but not basal respiration, resulting in lower respiratory control ratio (RCR). Furthermore, food deprivation decreased mitochondrial complex III activity, but increased complex IV activity. Infection did not result in any changes in these mitochondrial variables. Mitochondrial maximum respiration rate correlated positively with mass-independent RMR and flight metabolic rate, suggesting a link between mitochondria and whole-animal performance. In conclusion, low food availability negatively affects mitochondrial function and flight performance, with potential implications for migration, fitness and population dynamics. Although previous studies have reported poor flight performance in infected monarchs, we found no differences in physiological performance, suggesting that reduced flight capacity may be due to structural differences or low energy stores.

KEY WORDS: Dietary restriction, Infection, Metabolic rate, Migration, Oxidative stress

INTRODUCTION

Mitochondrial function is key to organismal performance and health (Brand and Nicholls, 2011; Heine and Hood, 2020; Koch et al., 2021; Melvin and Ballard, 2006). Events that disrupt mitochondrial function may therefore affect individual fitness and, ultimately, population dynamics. Individuals balance investing finite resources in their own survival and maintenance versus current and future reproduction, while also engaging in a number of energetically expensive activities, such as movement. Of all types of movement, flapping flight requires the highest mass-specific energy consumption rates, and flying insects in particular expend energy at extremely high rates (Suarez, 2000). Even though flight is costly, the rewards of flight are nevertheless high, and flight is crucial for survival and fitness in many insects. Flight also enables long-distance migration, a critical and challenging life-history event for many winged animals (Chapman et al., 2015; Dingle, 1996).

The North American monarch butterfly, *Danaus plexippus* (Linnaeus 1758) is well known for its multi-stage seasonal migration: the annual life cycle of eastern monarchs includes a southward migration in the autumn and overwintering in central Mexico. From there, the same individuals fly to southern USA to reproduce, and the next generations continue the northward migration. The last generation produced each summer will ultimately reinitiate the migratory cycle, turning south in autumn (Brower, 1996; Flockhart et al., 2013). Monarchs rely on flower nectar that they convert to stored lipids to fuel migration (Alonso-Mejía et al., 1997; Brower et al., 2006). The disappearance of natural habitats and agricultural intensification are among factors that have been identified as likely causes for the apparently global trend of insect loss (Bell et al., 2020; Dirzo et al., 2014; Hallmann et al., 2017; Raven and Wagner, 2021). Annual censuses suggest that eastern and western North American monarch butterflies, too, have significantly declined in the last decades (Pelton et al., 2019; Semmens et al., 2016), with loss of larval and adult food sources as likely drivers of the decline (Flockhart et al., 2015; Thogmartin et al., 2017). Different levels of dietary restriction and low food quality have been shown to directly reduce reproductive output in insects (Bauerfeind et al., 2007; Boggs and Ross, 1993; Maklakov et al., 2008), and to have negative effects on flight capacity in some insects (Kaufmann and Briegel, 2004; Reim et al., 2019). While moderate dietary restriction has been shown to extend lifespan in some species (Speakman and Mitchell, 2011), prolonged and severe periods of food restriction can lead to diminished animal performance and even death (McCue, 2010). The lack of nectar sources as a result of habitat destruction and droughts (Thogmartin et al., 2017) could thus have severe negative consequences for migrating monarchs.

In addition to the challenges of finding enough food, animals may also be faced with the physiological and energetic demands of combating pathogens, which may have direct effects on health and survival. The monarch butterfly is host to a specialist pathogen, the neogregarine protozoan parasite *Ophryocystis elektroscirrha*, which infects larvae, replicates asexually in the hypoderm of the growing larva, and is transmitted as spores produced during the pupal

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stage, and carried by adult butterflies (Barriga et al., 2016). The occurrence of the parasite varies in space and time, but the proportion of heavily infected adults during the autumn migration may exceed 10% (Bartel et al., 2011). Heavily infected individuals display higher mortality during development, are smaller at emergence, live shorter lives as adults and have lower fecundity than uninfected individuals (Altizer and Oberhauser, 1999; De Roode et al., 2008). Experiments on flight mills have revealed that infected monarchs fly shorter distances and at slower speeds than healthy individuals (Bradley and Altizer, 2005). Further evidence for the cost of infection comes from field sampling studies that have found signs of migratory culling, i.e. the proportion of infected individuals decreases as the travelled distance increases (Altizer et al., 2015; Bartel et al., 2011; Majewska et al., 2022). The mechanism explaining reduced flight performance may be smaller size or structurally weaker wings (Davis and de Roode, 2018), but could also reflect lower flight fuel stores or compromised physiological performance. Here, we focused on the latter. Mitochondria consume the majority of an organism's oxygen. Specifically, oxygen is consumed by complex IV of the electron transport chain, as complex IV, like complex I and III, transfers protons into the intermembrane space, contributing to a proton gradient that forms across the mitochondrial inner membrane. The proton gradient powers ATP synthesis in the final step of the oxidative phosphorylation pathway. If any of these steps are disrupted, compromised mitochondrial function may result in cascading effects that ultimately manifest as reduced whole-animal performance (Brand and Nicholls, 2011). Neogregarine parasites have been shown to interact with host mitochondria in complex ways (Blank et al., 2018; Blume and Seeber, 2018; Lentini et al., 2018; Pernas et al., 2014). Infection by *Toxoplasma gondii* led to impaired mitochondrial function in host mitochondria as demonstrated by reduced mitochondrial complex I, II and IV activity in infected cells (Syn et al., 2017).

To address the question 'does mitochondrial dysfunction underlie reduced whole-animal performance under stress?', we subjected monarch butterflies to two simultaneous stressors: approximately 7 days of food deprivation and parasitism by the specialist parasite *O. elektroscirrha*. Assessing the effects of two stressors at the same time offers a biologically realistic framework and allowed us to investigate the effects of each stressor individually and in combination with the other. To further understand the basis of health and physiological performance, we measured metabolic rate at two levels: the whole-animal level and the mitochondrial level. Simultaneous measurement of CO_2 production and O_2 consumption during flight allowed us to quantify the respiratory quotient (RQ) which indicates which energy substrates are used. In addition, we measured the activity of complexes III and IV of the mitochondrial electron transport chain. We hypothesised that, as mitochondrial function is fundamental to organismal performance, there would be a positive correlation between mitochondrial respiration rate and whole-animal metabolic rate.

MATERIALS AND METHODS

Rearing of butterflies and inoculation with parasite spores

The monarchs used in this investigation were offspring of outbred crosses of butterflies collected in GA, USA. Eggs were collected on milkweed (*Asclepias incarnata*). Rearing took place at Emory University. Two days after hatching, caterpillars were either infected with the parasite *O. elektroscirrha* or left uninfected: caterpillars were placed in a 10 cm Petri dish with a wet filter paper and a small milkweed leaf disk (8 mm diameter), to which 10 parasite spores were manually added (uninfected caterpillars received a leaf disk without parasites). The parasite used was a revived isolate of the parasite clone E10, originally obtained from the wild in eastern USA (De Roode et al., 2008). After finishing their leaf disk in the next 48 h, caterpillars were transferred to a live potted milkweed plant, which was covered with a mesh bag (13×57 cm). The larvae were maintained in a greenhouse with natural light at an average temperature of 26°C. Following pupation, the pupae were transported to Auburn University where they were glued onto the lid of individual 500 ml solo cups and maintained at a daylight cycle of 12 h:12 h light:dark and a 28°C:24°C temperature cycle. Upon eclosion, individuals were weighed, sexed and marked, then allocated into four treatment groups: uninfected, fed (n=9), infected, fed (n=6), uninfected, food deprived (n=9), and infected, food deprived (n=7).

Food deprivation treatment

We focused on females only in this study. In both feeding treatments, females were kept in large indoor cages (90 cm×90 cm×210 cm) under a 12 h:12 h light:dark cycle and 28°C:24°C temperature cycle. Infected and uninfected females were kept in the same cages, as parasite transmission occurs from adult to larval offspring, and adults cannot become infected. Control females had constant access to 10% honey water provided from saturated sponges placed in small plastic cups. Females in the food deprivation treatment were provided with water only, served from similar sponges in cups.

Whole-animal metabolic rate

We measured resting metabolic rate (RMR) and flight metabolic rate using flow-through respirometry following similar procedures to those described for other Lepidoptera (Niitepõld and Boggs, 2015) and monarch butterflies (Zhan et al., 2014). In short, on the evening before the measurements, monarchs were individually placed in covered plastic cups and kept at room temperature overnight. The next day, post-absorptive monarchs were provided with water before being placed in a cylindrical 1-litre respirometry chamber that was covered with a black cloth ca. 20 min before the measurement of RMR. The chamber was continuously flushed with dry, CO_2-free air, obtained from a Whatman purge-gas generator (Whatman, Haverhill, MA, USA) at a flow rate of 1.5 l min^{-1} and subsampled at a flow rate of 0.75 l min^{-1} using two Sable Systems Mass Flow Systems (Sable Systems, Las Vegas, NV, USA). We pulled the excurrent air through a column filled with magnesium perchlorate to remove traces of humidity and then through a Li-Cor 6262 CO_2 analyser (Li-Cor, Lincoln, NE, USA) and, finally, an Oxzilla II O_2 analyser (Sable Systems). Individuals remained calm and motionless when kept in the dark, but if an individual became restless, this could immediately be seen in the CO_2 production curve. In these rare cases, we allowed the individual to settle before continuing with the experiment. We took the average of 1.5 min of stable CO_2 production to represent RMR. Flight metabolic rate was measured when the cover was removed, and the butterfly was stimulated to fly by gently shaking the chamber each time the butterfly attempted to land. The stimulation continued for 7 min, after which the chamber was covered again. We used standard respirometry equations (Lighton, 2008), and converted CO_2 and O_2 concentrations to metabolic rate expressed in millilitres per hour. We used peak metabolic rate, the highest rate of CO_2 production and O_2 consumption, to represent flight capacity. Peak metabolic rate was typically recorded during the first minutes of the flight trial. We calculated RQ by dividing peak CO_2 production rate by O_2

consumption rate from the same time point. As the respirometry setup was optimised for flight measurements, we do not report O_2 consumption rate and RQ for RMR.

We aimed at a balanced age distribution among the treatment groups, although this was challenging because of the limited material and the number of individuals that could be processed each day. The mean±s.d. ages for the groups were: uninfected, fed 5.8±1.1 days; uninfected, food deprived 6.6±1.1 days; infected, fed 6.0±2 days; and infected, food deprived 6.7±2.9 days. After measurement, the butterflies were weighed, and individuals in the feeding treatment were given honey water solution, food-deprived individuals were given water, and all butterflies were placed in individual glassine envelopes and stored at room temperature overnight. The next day, butterflies were killed and used for mitochondrial assays.

Isolation of mitochondria

We first decapitated the individual with scissors, then quickly separated the body parts, and weighed the thorax without the legs and wings. Half of the thorax was placed in 10 ml of mitochondrial isolation solution [100 mmol l^{-1} KCl, 40 mmol l^{-1} Tris HCl, 10 mmol l^{-1} Tris base, 1 mmol l^{-1} $MgSO_4$, 0.1 mmol l^{-1} EDTA, 0.2 mmol l^{-1} ATP and 0.15% (w/v) free fatty acid bovine serum albumin (BSA)] and was minced with scissors, after which we removed remnants of the exoskeleton. We homogenised the muscle tissue with a VirTis homogeniser for 5 s. We then added trypsin (5 mg g^{-1} of muscle) and repeatedly mixed the solution for 7 min and, finally, added isolation solution (10 ml) to terminate the reaction. The solution was then centrifuged at 500 ***g*** for 10 min, transferred through a cheesecloth filter into a new centrifuge tube and centrifuged at 3500 ***g*** for 10 min. The supernatant was discarded, and we resuspended the mitochondrial pellet in isolation solution (10 ml) and centrifuged the sample for a second time at 3500 ***g*** for 10 min. The supernatant was again discarded, and the mitochondrial pellet was resuspended in isolation solution, as above, but with no BSA (10 ml), and the sample was centrifuged for a third time at 3500 ***g*** for 10 min. The final mitochondrial pellet was resuspended in mannitol/sucrose solution (220 mmol l^{-1} mannitol, 70 mmol l^{-1} sucrose, 10 mmol l^{-1} Tris HCl and 1 mmol l^{-1} EGTA) with a Dounce homogeniser.

Mitochondrial respiration

Mitochondrial respiration was measured polarographically (Oxytherm, Hansatech Instruments, Norfolk, UK) following procedures (with minor modifications) described by Hyatt et al. (2017). Isolated mitochondria (20 µl) were incubated in a final volume of 1 ml of respiration buffer (100 mmol l^{-1} KCl, 50 mmol l^{-1} Mops, 10 mmol l^{-1} KH_2PO_4, 20 mmol l^{-1} glucose, 10 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EGTA and 0.2% fatty acid free BSA, pH 7.0; adapted from Wanders et al., 1984) at 37°C with continuous stirring. The measurement temperature corresponds to the upper end of the range of thoracic temperatures measured in monarchs under field conditions (Masters et al., 1988). We used the complex I substrates 2 mmol l^{-1} malate, 10 mmol l^{-1} glutamate and 2 mmol l^{-1} pyruvate (final concentrations). We measured state 3 respiration (maximal respiration) after adding 5.0 µl of 50 mmol l^{-1} solution of ADP to the chamber, and state 4 (basal) respiration was recorded following the phosphorylation of the added ADP as described by Estabrook (1967). State 3 and state 4 respiration were normalised to total protein content that was measured by the Bradford assay (Bradford, 1976). We calculated the respiratory control ratio (RCR) by dividing state 3 by state 4.

Complex activity

Microplate spectrophotometric enzymatic assays using isolated mitochondria were performed using the protocols (with minor modifications) described by Trounce et al. (1996). Because of limited tissue material and working with a novel study system, only the activity of complexes III and IV was successfully quantified for all individuals.

Briefly, complex III activity (EC 1.10.2.2) was obtained (30°C) by recording the reduction of cytochrome *c* at 550 nm catalysed by the presence of reduced decylubiquinone. The reaction mixture (250 µl) contained 250 mmol l^{-1} sucrose, 1 mmol l^{-1} EDTA, 50 mmol l^{-1} Tris HCl (pH 7.4), 50 µmol l^{-1} cytochrome *c* and 2 mmol l^{-1} KCN, and the reaction was initiated by adding 1.4 µl of reduced decylubiquinone (10 mmol l^{-1}) to each well.

Briefly, complex IV activity (EC 1.9.3.1) was obtained (∼22°C) by recording the oxidation of reduced cytochrome *c* at 550 nm. The reaction mixture (200 µl) contained 10 mmol l^{-1} potassium phosphate buffer (pH 7.4), 20 µmol l^{-1} reduced cytochrome *c* and 0.025% lauryl maltoside, and the reaction was initiated by adding mitochondria to each well.

Complex III and IV activity was normalised to citrate synthase activity (EC 4.1.3.7) that was obtained (30°C) by monitoring the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) at 412 nm. The reaction mixture (250 µl) contained 125 mmol l^{-1} Tris HCl (pH 8.0), 0.3 mmol l^{-1} acetyl-CoA and 1 mmol l^{-1} DTNB. The reaction was initiated by adding 5 µl of oxaloacetate (50 mmol l^{-1}) to each well.

Confirmation of infection success

We quantified the spore load of infected females based on the abdomen. The abdomen was placed in a 5 ml scintillation vial, and vortexed at maximum speed with a Vortex Genie. We counted parasite spores on a haemocytometer slide and calculated the original number of spores in the 5 ml vial.

Statistical analysis

ANCOVA was used to examine the effects of food deprivation, infection and their interaction on morphology, mitochondrial respiration and whole-animal metabolic rate. We took into account the effect of body mass on metabolic rate by adding body mass as a covariate in models on whole-animal metabolic rate and mitochondrial respiration rate. This statistical approach is preferred over dividing metabolic rate by body mass (mass-specific metabolic rate), which does not remove the effect of body mass as metabolic rate does not scale linearly with body mass (Lighton, 2008). In analyses examining the relationship between mitochondrial respiration rate and whole-animal metabolic rate, we accounted for variation in body mass by using mass-independent metabolic rate, i.e. the residuals extracted from a linear regression between metabolic rate and body mass. Mass-independent metabolic rate can be positive (data point lies above the regression line) or negative (data point lies below the regression line). All statistical analyses were done with SAS 9.3 and SAS Studio using Proc Mixed with an alpha level of 0.05.

RESULTS

Morphology

There were no differences in pupal mass between infected and uninfected females ($F_{1,26}$=0.07, P=0.79) (Table 1). Butterfly body mass was strongly affected by the food deprivation treatment ($F_{1,27}$=27.9, P<0.0001). At the age of approximately 7 days, food deprivation reduced body mass on average by 18% among

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Table 1. Summary table of morphological values

	Fed		Food deprived	
Trait	Uninfected	Infected	Uninfected	Infected
Pupal mass (g)	1.31±0.10^{ns}	1.33±0.13^{ns}	1.38±0.21^{ns}	1.33±0.19^{ns}
Adult body mass (g)	0.531±0.061^{a}	0.498±0.075^{a,b}	0.438±0.093^{a,b}	0.408±0.073^{b}
Abdomen mass (g)	0.232±0.055^{a}	0.201±0.047^{a}	0.122±0.032^{b}	0.111±0.029^{b}
Thorax mass (g)	0.213±0.022^{a}	0.199±0.014^{a,b}	0.186±0.034^{a,b}	0.163±0.037^{b}

Mean±s.d. values are shown. Superscript letters indicate significant differences among groups according to a *post hoc* Tukey's test. Body mass was measured after the measurement of metabolic rate at approximately 7 days of age. Thorax and abdomen mass were measured the following day. n=9 uninfected, fed individuals; n=6 infected, fed individuals; n=9 uninfected, food-deprived individuals; and n=7 infected, food-deprived individuals.

uninfected females (Table 1). Infected, fed females weighed 6% less than uninfected, fed females, whereas infected, food-deprived females weighed 23% less than uninfected, fed females. There was a non-significant trend towards lower body mass in infected females compared with uninfected females ($F_{1,27}$=3.59, P=0.07). The interaction between food deprivation and infection was not significant ($F_{1,27}$=0.06, P=0.80).

The decreases in body mass due to food deprivation were largely explained by reduced abdomen mass. Among uninfected females, food deprivation reduced abdomen mass on average by 47% (Table 1). The abdomens of food-deprived, infected females weighed 52% less than those of fed, uninfected females. The abdomens of fed, infected females weighed 7% less than those of fed, uninfected females. The overall effect of food deprivation was highly significant ($F_{1,27}$=41.0, P<0.0001). The effect of infection was not statistically significant ($F_{1,27}$=1.90, P=0.18), and the interaction between food deprivation and infection was also not significant ($F_{1,27}$=0.41, P=0.53).

Thorax mass decreased with food deprivation on average by 13% among uninfected females (Table 1). Fed, infected females had thoraces that were 7% lighter than those of fed, uninfected females. Thorax mass was 23% lower in food-deprived, infected females compared with fed, uninfected females. The effect of food deprivation was statistically significant ($F_{1,27}$=8.83, P=0.006). Infection resulted in a statistically non-significant trend towards lower thorax mass ($F_{1,27}$=3.05, P=0.09). The food deprivation by infection interaction was not statistically significant ($F_{1,27}$=0.15, P=0.70).

Whole-animal RMR

RMR was positively affected by body mass ($F_{1,24}$=7.97, P=0.009) (Fig. 1A). Food deprivation had a strong negative effect on RMR ($F_{1,24}$=50.4, P<0.0001) (Fig. 1B). Infection had no significant effect on RMR ($F_{1,24}$=0.23, P=0.63), and there was no significant food deprivation by infection interaction ($F_{1,24}$=0.75, P=0.40).

Whole-animal flight metabolic rate

Peak CO_2 production rate was positively affected by body mass ($F_{1,26}$=5.56, P=0.03) (Fig. 1C). Food deprivation reduced peak metabolic rate ($F_{1,26}$=28.2, P<0.0001), but there was no effect of infection ($F_{1,26}$=0.04, P=0.84) (Fig. 1D). The interaction between food deprivation and infection was not significant ($F_{1,26}$=1.43, P=0.24).

Peak O_2 consumption rate was positively affected by body mass ($F_{1,26}$=12.75, P=0.001). The effect of food deprivation was significant ($F_{1,26}$=12.49, P=0.002). There was no significant effect of parasitic infection ($F_{1,26}$=0.0, P=0.95), and the food deprivation by infection interaction was also non-significant ($F_{1,26}$=1.26, P=0.27).

The RQ differed significantly between flying females in the control and food deprivation treatment ($F_{1,27}$=11.73, P=0.002). The mean RQ for females in the control treatment was 0.73 and the mean for food-deprived females was 0.64 (Fig. 1E,F). Infection had no significant effect on RQ ($F_{1,27}$=0.00, P=0.99), and the interaction between food deprivation and infection was not significant ($F_{1,27}$=0.04, P=0.84).

Mitochondrial respiration

Food deprivation reduced mitochondrial state 3 respiration (maximum respiration rate) on average by 38% ($F_{1,26}$=13.1, P=0.001). In contrast, there was no statistically significant effect of infection ($F_{1,26}$=0.60, P=0.45), and the interaction between food deprivation and infection was also not statistically significant ($F_{1,26}$=2.53, P=0.12) (Fig. 2A). The effect of body mass was not statistically significant ($F_{1,26}$=1.2, P=0.28).

State 4 (basal) respiration was not affected by food deprivation ($F_{1,26}$=0.91, P=0.35). Infection had no effect on state 4 respiration ($F_{1,26}$=0.18, P=0.89), and the interaction between food deprivation and infection was not statistically significant ($F_{1,26}$=1.49, P=0.23) (Fig. 2B). Body mass had a negative effect on state 4 respiration ($F_{1,26}$=4.45, P=0.05).

The RCR was strongly reduced as a result of food deprivation ($F_{1,26}$=9.61, P=0.005). Infection had no statistically significant effect on RCR ($F_{1,26}$=0.06, P=0.81). The interaction between food deprivation and infection was not significant ($F_{1,26}$=0.79, P=0.38) (Fig. 2C). RCR was not affected by body mass ($F_{1,26}$=2.15, P=0.16).

Mitochondrial complex activity

Food deprivation reduced mitochondrial complex III activity ($F_{1,24}$=12.5, P=0.002). The effect of infection was not significant ($F_{1,24}$=1.02, P=0.32). There was no significant food deprivation by infection interaction ($F_{1,24}$=0.71, P=0.41) (Fig. 3A).

In contrast, mitochondrial complex IV activity increased with food deprivation ($F_{1,24}$=5.66, P=0.03). There was no effect of infection ($F_{1,24}$=0.03, P=0.85) and the food deprivation by infection interaction was non-significant ($F_{1,24}$=0.43, P=0.52) (Fig. 3B).

Correlation between mitochondrial respiration and whole-animal metabolic rate

Mitochondrial state 3 respiration rate and mass-independent whole-animal RMR showed a positive correlation (r=0.67) (Fig. 4A). A linear regression model showed that state 3 respiration significantly predicted mass-independent RMR (β=0.67, P<0.0001, r^2=0.45). In contrast, mitochondrial state 4 respiration rate and mass-independent whole-animal RMR were not correlated (r=−0.04, P=0.82).

Mitochondrial state 3 respiration rate significantly predicted mass-independent peak CO_2 production rate (β=0.46, P=0.009, r^2=0.215). The correlation coefficient r was 0.46 (Fig. 4B). Mitochondrial state 3 respiration rate also significantly predicted

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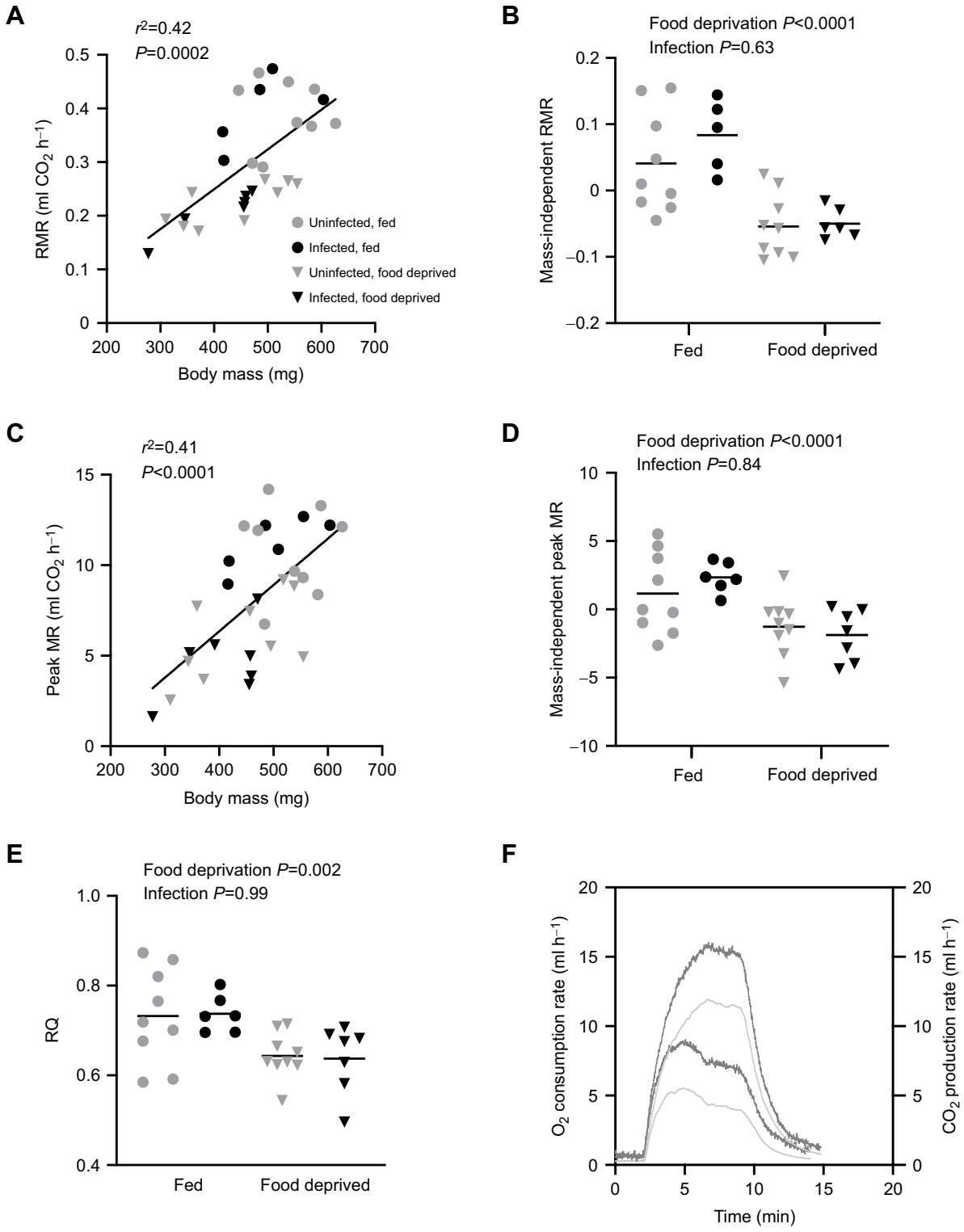

Fig. 1. Whole-animal metabolic rate of monarch butterflies. (A) Whole-animal CO_2 production of butterflies in the four groups (uninfected, fed; uninfected, food deprived; infected, fed; infected, food deprived) measured at rest. Body mass had a significant effect on resting metabolic rate (RMR). (B) Statistically adjusted mass-independent RMR showing a significant decrease in whole-animal CO_2 production due to food deprivation. Infection had no effect on RMR. (C) Whole-animal peak metabolic rate (MR). (D) Mass-independent peak MR, showing an effect of food deprivation, but no effect of infection. (E) The respiratory quotient (RQ) differed significantly between fed and food-deprived females. (F) Examples of CO_2 production rate (light grey) and O_2 consumption rate (dark grey) data in two uninfected females. The female with higher values was fed, the one with lower values was food deprived. Note that the data in this plot have not been mass adjusted. The first 2 min represent RMR; after that, the individuals were stimulated to fly for exactly 7 min. Statistics from a linear regression model. For statistics, see Results. *n*=9 uninfected, fed individuals; *n*=6 infected, fed individuals; *n*=9 uninfected, food-deprived individuals; and *n*=7 infected, food-deprived individuals.

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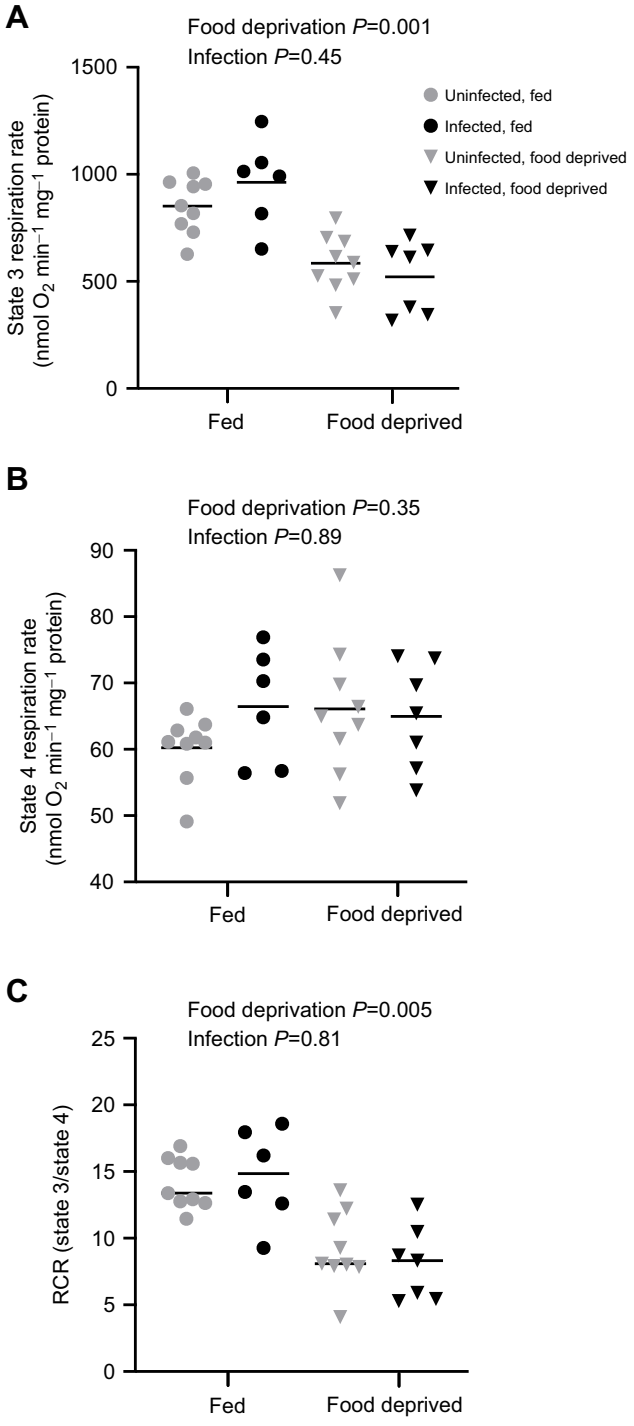

Fig. 2. Mitochondrial respiration of monarch butterflies. (A) State 3 respiration (maximum rate) after the addition of metabolic substrates and ADP. (B) State 4 respiration (basal rate) after the added ADP had been consumed. Mitochondrial respiration did not differ between uninfected and infected individuals. (C) The respiratory control ratio (RCR) differed between fed and food-deprived individuals, but not between infected and uninfected individuals. *n*=9 uninfected, fed individuals; *n*=6 infected, fed individuals; *n*=9 uninfected, food-deprived individuals; and *n*=7 infected, food-deprived individuals.

mass-independent peak O_2 consumption rate (β=0.40, P=0.03, r^2=0.161). The correlation coefficient was 0.40. State 4 respiration rate was not correlated with peak CO_2 production rate (r=0.08, P=0.67) or O_2 consumption rate (r=0.12, P=0.53).

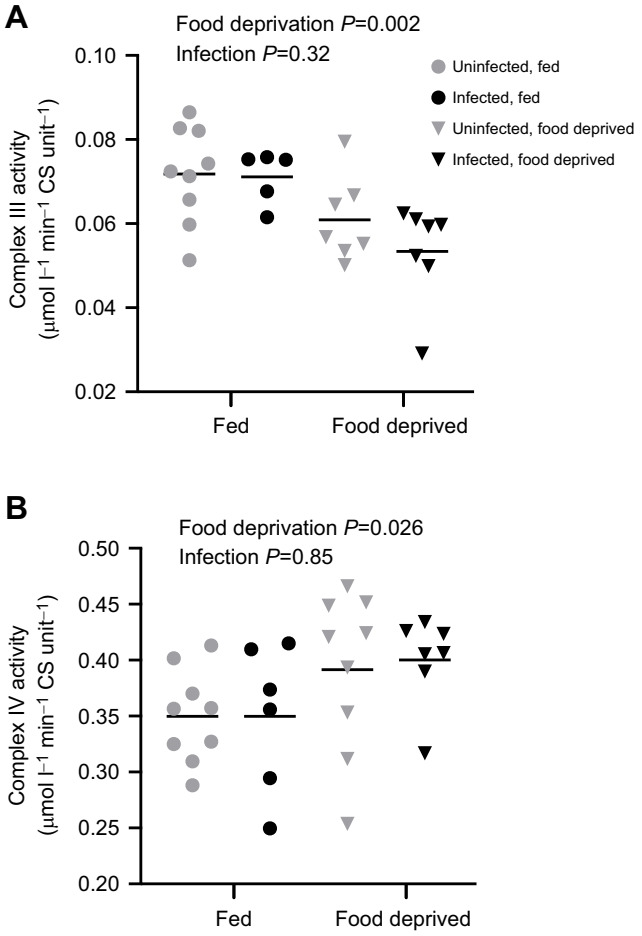

Fig. 3. Mitochondrial complex activity of monarch butterflies. (A) Mitochondrial complex III activity decreased with food deprivation. (B) Mitochondrial complex IV activity increased with food deprivation. *n*=9 uninfected, fed individuals; *n*=6 infected, fed individuals; *n*=9 uninfected, food-deprived individuals; and *n*=7 infected, food-deprived individuals.

DISCUSSION

Organisms battle multiple stressors throughout their lives. We asked whether food deprivation and parasitic infection have physiological effects that could affect the performance of the monarch butterfly, a species highly dependent on its capacity to fly long distances. We found that food deprivation reduced body mass, flight muscle mitochondrial maximum respiration, whole-animal RMR and peak flight metabolic rate in the monarch. The RQ of fed females was close to 0.7 which indicates that they used lipids as a flight fuel, as expected for migratory butterflies. Yet, many food-deprived females displayed a RQ below 0.7, which may suggest that they were using alternative energy sources such as ketone bodies. Limited access to food can thus impair flight capacity, which has a strong negative impact on the capacity of volent animals to forage, find mates and migrate (Southwood and Johnson, 1971). Also, food deprivation affected the mitochondrial electron transport chain, as food deprivation resulted in reduced mitochondrial complex III activity, whereas complex IV activity increased. In contrast to food deprivation, parasitic infection did not affect metabolism at the mitochondrial or whole-animal level. Previous studies have found effects of parasitic infection on monarch lean body mass and distance flown in a flight mill (Altizer and Oberhauser, 1999; Bradley and Altizer, 2005) as well as migration distance in the field

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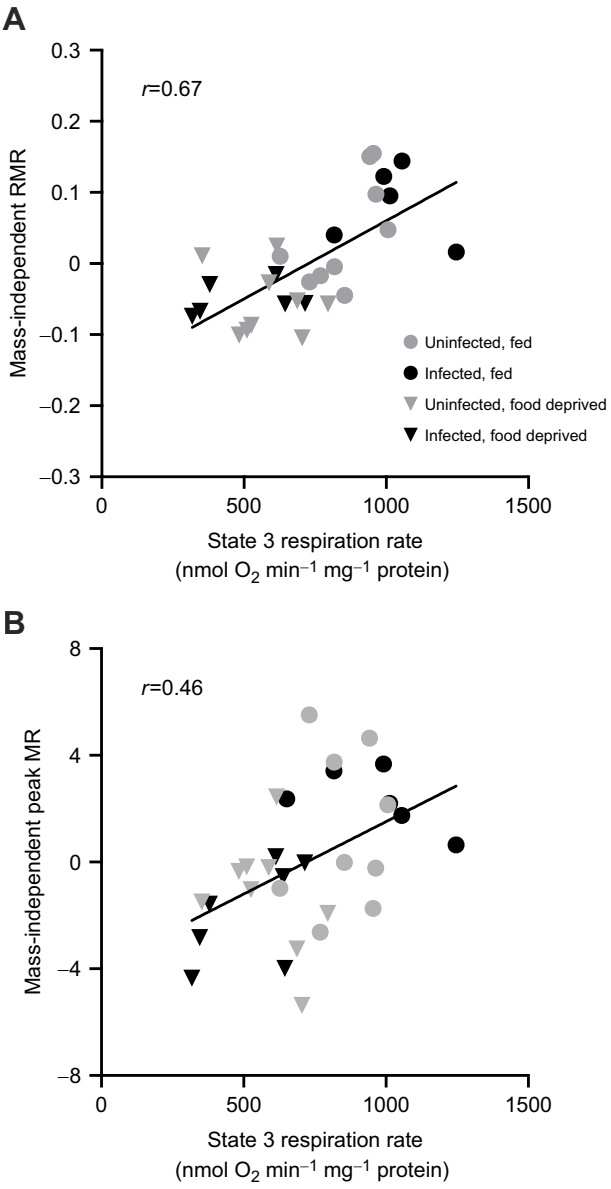

Fig. 4. Correlation between mitochondrial respiration and whole-animal metabolic rate of monarch butterflies. (A) Positive correlation between protein mass-corrected mitochondrial state 3 respiration and mass-independent RMR (CO_2 production) in monarch butterflies. (B) Positive correlation between protein mass-corrected mitochondrial state 3 respiration and mass-independent peak flight MR (CO_2 production). Mass-independent whole-animal metabolic rate was obtained by extracting the residuals of linear regressions between metabolic rate and body mass. *n*=9 uninfected, fed individuals; *n*=6 infected, fed individuals; *n*=9 uninfected, food-deprived individuals; and *n*=7 infected, food-deprived individuals.

(Altizer et al., 2015). Our results suggest that negative impacts of infection on migration success in previous studies are caused by insufficient energy reserves, possibly due to competition for the same resources between the host and parasite (Pernas et al., 2018), or structurally weaker flight muscles and wings (Davis and de Roode, 2018), rather than direct negative effects of infection on mitochondrial performance and metabolic flight capacity. We found positive correlations between mitochondrial maximum respiration and whole-animal RMR and peak metabolic rate, after accounting for body mass, which suggests that variation in mitochondrial performance may be linked to variation in animal performance and fitness.

Effects of food deprivation and infection on whole-animal metabolic rate

Monarchs lost between 18% and 23% of their mass as a result of food deprivation. The mass loss was strongest in the abdomen, where the majority of the fat reserves are located, but thorax mass was also reduced. To account for differences in body mass among individuals, all measurements of whole-animal metabolic rate were statistically corrected for body mass. Mass-independent RMR and flight metabolic rate decreased with food deprivation, indicating that individuals entered a state of low physiological activity and reduced flight capacity. This result was consistent with findings in other insects where food deprivation had a negative impact on flight performance. For example, food deprivation led to shortened flight distances in a flight mill in mosquitoes (Kaufmann and Briegel, 2004), and food-deprived *Lycaena tityrus* butterflies flew shorter durations in a shaking test compared with fed controls (Reim et al., 2019). In contrast, studies where butterflies have been subjected to dietary restriction by 50% have shown that when sugar is available, albeit in reduced quantities, butterflies tend to conserve their flight metabolic rate and thus flight capacity (Niitepõld, 2019; Niitepõld et al., 2014). Depending on life-history strategies, food-stressed butterflies may rather compromise their fecundity than flight capacity (Niitepõld et al., 2014), which highlights the crucial role flight has in the lives of butterflies and many other flying insects.

The average RQ for fed females was 0.73, suggesting that flight was mostly fuelled by lipids and to a much smaller degree by carbohydrates. Although simultaneous measurements of O_2 consumption and CO_2 production of flying monarchs have been lacking until the present study, it has been assumed that monarchs rely on lipids during migration and overwintering based on the dynamics of lipid stores (Alonso-Mejía et al., 1997; Brower et al., 2006). Evidence also points to other migrant butterflies using lipids to power flight (Dudley and Srygley, 2008), and lipids are the main energy source for most overwintering insects (Sinclair et al., 2018). Our direct measurements of RQ in flight confirm the important role of lipids for monarch butterflies. Interestingly, food-deprived females had a mean RQ of 0.64, lower than the RQ would be if lipids were the only source of energy. RQ lower than 0.7 have been recorded in several other insects (Arnqvist et al., 2017; Bosch et al., 2010; DeVries et al., 2013), and may indicate the oxidation of ketone bodies that have been formed from lipid stores (Matarese, 1997; Schutz and Ravussin, 1980). There is evidence of the presence of acetoacetate and D-3-hydroxybutyrate in desert locusts (*Schistocerca gregaria*) when exposed to flight or starvation when fat body lipid reserves are being depleted (Bailey et al., 1972). Although our understanding of ketosis in insects is highly limited, it seems plausible that the food-deprived monarchs in this study would have entered a state of ketosis as food-deprived individuals did not receive any sugar during their adult life. Insects store carbohydrates mainly as glycogen in the fat body (Arrese and Soulages, 2010) and after approximately 7 days of food deprivation, circulating trehalose levels and glucose stores may have been strongly depleted. Food-deprived monarchs lost approximately 50% of their abdomen mass, most probably as a result of consumption of lipids in the fat body, suggesting a considerable reduction of energy stores.

Monarchs infected by *O. elektroscirrha* have previously been shown to fly shorter distances in a flight mill than uninfected individuals (Bradley and Altizer, 2005). Field studies have shown

that average parasite loads decrease as the distance from the overwintering sites increases, suggesting that heavily infected individuals do not fly far (Altizer et al., 2000, 2015; Bartel et al., 2011; Majewska et al., 2022). Here, we found no significant effect of infection on mitochondrial performance, RMR or flight metabolic rate, which suggests that flight capacity was not directly negatively affected by parasitic infection. As infection by *O. elektroscirrha* has been shown to result in lower lean body mass (Altizer and Oberhauser, 1999; Satterfield et al., 2013), the reduced flight endurance found in previous studies on the effects of infection could be a result of structural changes to flight muscles or wings due to disrupted development (Davis and de Roode, 2018). As our results clearly demonstrate that food deprivation had a negative effect on flight metabolism, low energy stores could also be a plausible mechanism for reduced flight endurance in infected monarchs in poor condition.

Effects of food deprivation and infection on mitochondrial performance

Mitochondrial activity varies greatly among tissues (Hullbert et al., 2006; Salin et al., 2016), and responses to fasting may differ between different tissue types (Monternier et al., 2015). Measurements on mitochondrial function should therefore focus on tissue types that are best suited for the study question. As flight is the main mode of locomotion in many insects, and the majority of oxygen consumption in flight occurs in the flight muscles (Suarez, 2000), we focused on mitochondria isolated from flight muscle in this study. Food deprivation significantly reduced mitochondrial maximum respiration rate (state 3), whereas basal respiration did not change. Food deprivation thus significantly reduced the RCR, indicating less mitochondrial coupling and increased mitochondrial dysfunction (Brand and Nicholls, 2011). Our result differs from effects of fasting in birds that may be adapted to periods of food shortage. For example, fasting king penguin (*Aptenodytes patagonicus*) chicks showed a decrease in both state 3 and state 4 respiration in skeletal muscle mitochondria, and no significant change in RCR (Monternier et al., 2014). Long-term fasting in penguin chicks resulted in an even greater reduction in mitochondrial respiration than short-term fasting (Bourguignon et al., 2017). Fasted Muscovy ducklings (*Cairina moschata*) have also been shown to exhibit lower state 3 and state 4 respiration with no effect on RCR (Monternier et al., 2015), although when respiring on succinate, a reduction in RCR was observed (Roussel et al., 2018).

To better understand changes in mitochondrial respiration, we measured the activity of mitochondrial complexes III and IV of the electron transport chain in the inner mitochondrial membrane, which maintains the protonmotive force to power ATP synthesis. As we were working with a novel study system and had limited amounts of tissue available, we had to focus on the two final complexes in the electron transport chain, instead of all mitochondrial complexes. Nevertheless, these two complexes, III and IV, are of special interest as complex III (along with complex I) is a major site of reactive oxygen species (ROS) production in the mitochondrion (Bleier and Dröse, 2013), and complex IV, as the last complex in the electron transport chain, is responsible for the majority of oxygen consumption in the mitochondrion. Complexes III and IV carry out different functions, and, interestingly, these two complexes appeared to respond in different ways to food deprivation: complex III showed lower activity in food-deprived females, whereas the activity of complex IV was higher. Reduced complex III activity was in line with our finding of reduced mitochondrial state 3 respiration rate, which can be interpreted as a sign of mitochondrial dysfunction. In rats (*Rattus norvegicus*) subjected to starvation, the activity of all complexes decreased (with the exception of succinate dehydrogenase, complex II) (Bhardwaj et al., 1998). In humans on a low-calorie diet, genes related particularly to complex III were downregulated in the adipose tissue (van der Kolk et al., 2021). In contrast, in *Drosophila melanogaster*, dietary restriction led to a marked increase in cytochrome *c* oxidase (complex IV) activity in pooled whole-animal samples (Zid et al., 2009). An increase in complex IV activity due to starvation was also reported in the liver of the gilthead sea bream fish (*Sparus aurata*) as indicated by genetic upregulation of several subunits of cytochrome *c* oxidase (Silva-Marrero et al., 2017). Similar complex-specific changes have also been found in the mitochondria of mice: cytochrome *c* oxidase activity increased in the liver tissue of fasted mice, whereas complex II and citrate synthase activity did not change (Casas et al., 2000). Increased complex IV activity could indicate compensation for electron leak prior to complex IV in the electron transport chain. Our measurements of RCR indicated lower mitochondrial coupling in food-deprived monarchs, which may be linked to increased electron leak, but elucidating the mechanisms behind the findings would require further studies. Future studies on monarch butterflies should also take advantage of the relatively large size of this insect and include samples of fat body tissue in analyses of complex activity. Overall, the literature on mitochondria is biased towards liver mitochondria because the liver is a highly dynamic organ, and this applies to the fat body as well. Starving insects deplete lipids stored in the fat body (Smith and Appel, 1996), and as the fat body carries out similar functions to the liver in vertebrates, including mobilisation of stored energy substrates, mitochondria in the fat body should therefore be expected to respond to food deprivation.

Correlation between mitochondrial respiration and whole-animal metabolic rate

Linking mitochondrial variation to organismal performance is one of the big challenges in the emerging fields of mitochondrial and mitonuclear ecology (Ballard and Melvin, 2010; Heine and Hood, 2020; Hill, 2019; Koch et al., 2021). Our study allowed us to measure metabolic rate in the same individuals at the mitochondrial and whole-animal level, and to examine correlations between the two levels. We found positive correlations between mitochondrial maximum respiration rate (state 3) and resting metabolic rate (r=0.67), and between state 3 respiration rate and peak flight metabolic rate (r=0.46). These findings indicate that measurements of mitochondrial performance have predictive power on whole-animal metabolic performance. It is noteworthy that mitochondrial state 4 respiration did not correlate with whole-animal metabolic rate. Here, our results differ from those obtained on brown trout (*Salmo trutta*) where state 4 respiration of mitochondria isolated from liver tissue correlated with RMR (Salin et al., 2016). The use of fat body tissue might have yielded different results in our study. Nevertheless, whether variation in cell-level metabolism underpins variation in whole-animal metabolic rate is not inevitable and may be particularly difficult to demonstrate in interspecific studies where the proportional sizes of different organs vary (Jimenez et al., 2019). It is noteworthy that the RCRs expressed by monarchs in the current study were relatively high, indicating a high level of mitochondrial coupling, improving the tight connection between mitochondrial respiration and whole-animal peak metabolic rate. Our result agrees with studies that have found connections between cellular metabolism and evolutionary life history strategies such as the case of the slower pace of life in tropical birds compared with the faster life histories of temperate birds (Jimenez et al., 2014). Taken

together, our findings demonstrate that variation in mitochondrial respiration can be linked to whole-animal performance, suggesting that mitochondrial function forms the basis of the complex physiological and biomechanical machinery that manifests as organismal performance, a critical component of individual fitness.

The monarch butterfly has reached an iconic status as a result of its unique, and now potentially endangered migration. Our work shows that the monarch study system has ample potential for the study of energetics all the way from the mitochondrial level to the whole-animal level. The current study has also revealed a striking gap in the literature on mitochondrial function, as very few studies on insects exist. Future studies may further explore the many ways mitochondria interact with their symbiont under changing environmental conditions. Here, one potential question is the connection between wing coloration and flight performance: studies have found that monarchs with redder wings tend to fly longer distances in the laboratory than individuals with less red pigment (Davis et al., 2012), and migratory monarchs in the autumn are redder than residents in the summer (Davis, 2009). Migration distances of monarchs are correlated with the level of wing melanisation, and in the case of males, also with the amount of orange pigmentation in wings (Hanley et al., 2013). Mitochondrial function may have a role to play in this relationship, too, in a similar way to that in the house finch (*Haemorhous mexicanus*), where condition-dependent carotenoid-based plumage colour has been shown to correlate with mitochondrial function (Hill et al., 2019). Given that the ommochrome-based wing colour in the monarch also displays hints of condition dependency (Figon and Casas, 2019), it would be interesting to evaluate the relationship between butterfly colour and mitochondrial performance in future studies.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.N., A.G.A., J.C.d.R., A.N.K., W.R.H.; Methodology: K.N., H.A.P., A.G.A., J.C.d.R., A.N.K., W.R.H.; Software: A.G.A.; Validation: K.N., H.A.P., A.G.A., J.C.d.R., A.N.K.; Formal analysis: K.N., N.R.H.; Investigation: K.N., H.A.P., A.G.A., J.C.d.R., A.N.K.; Resources: A.G.A., J.C.d.R., A.N.K., W.R.H.; Data curation: K.N., H.A.P., N.R.H., A.G.A., J.C.d.R., A.N.K.; Writing - original draft: K.N.; Writing - review & editing: K.N., H.A.P., N.R.H., A.G.A., J.C.d.R., A.N.K., W.R.H.; Visualization: K.N., W.R.H.; Supervision: W.R.H.; Project administration: K.N., W.R.H.; Funding acquisition: J.C.d.R., W.R.H.

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Data availability
The data are available from Dryad (Niitepõld et al., 2022): https://doi.org/10.5061/dryad.d2547d84x.

References
Alonso-Mejía, A., Rendon-Salinas, E., Montesinos-Patiño, E. and Brower, L. P. (1997). Use of lipid reserves by monarch butterflies overwintering in Mexico: implications for conservation. *Ecol. Appl.* **7**, 934-947. doi:10.1890/1051-0761(1997)007[0934:UOLRBM]2.0.CO;2

Altizer, S. M. and Oberhauser, K. S. (1999). Effects of the protozoan parasite *Ophryocystis elektroscirrha* on the fitness of monarch butterflies (*Danaus plexippus*). *J. Invertebr. Pathol.* **74**, 76-88. doi:10.1006/jipa.1999.4853

Altizer, S. M., Oberhauser, K. S. and Brower, L. P. (2000). Associations between host migration and the prevalence of a protozoan parasite in natural populations of adult monarch butterflies. *Ecol. Entomol.* **25**, 125-139. doi:10.1046/j.1365-2311.2000.00246.x

Altizer, S., Hobson, K. A., Davis, A. K., De Roode, J. C. and Wassenaar, L. I. (2015). Do healthy monarchs migrate farther? Tracking natal origins of parasitized vs. Uninfected monarch butterflies overwintering in Mexico. *PLoS One* **10**, e0141371-e0141371. doi:10.1371/journal.pone.0141371

Arnqvist, G., Stojković, B., Rönn, J. L. and Immonen, E. (2017). The pace-of-life: a sex-specific link between metabolic rate and life history in bean beetles. *Funct. Ecol.* **31**, 2299-2309. doi:10.1111/1365-2435.12927

Arrese, E. L. and Soulages, J. L. (2010). Insect fat body: energy, metabolism, and regulation. *Annu. Rev. Entomol.* **55**, 207-225. doi:10.1146/annurev-ento-112408-085356

Bailey, E., Horne, J. A., Izatt, M. E. G. and Hill, L. (1972). Role of ketone bodies in the metabolism of the adult desert locust. *Biochem. J.* **128**, 79. doi:10.1042/bj1280079Pa

Ballard, J. W. O. and Melvin, R. G. (2010). Linking the mitochondrial genotype to the organismal phenotype. *Mol. Ecol.* **19**, 1523-1539. doi:10.1111/j.1365-294X.2010.04594.x

Barriga, P. A., Sternberg, E. D., Lefèvre, T., de Roode, J. C. and Altizer, S. (2016). Occurrence and host specificity of a neogregarine protozoan in four milkweed butterfly hosts (*Danaus* spp.). *J. Invertebr. Pathol.* **140**, 75-82. doi:10.1016/j.jip.2016.09.003

Bartel, R. A., Oberhauser, K. S., De Roode, J. C. and Altizer, S. M. (2011). Monarch butterfly migration and parasite transmission in eastern North America. *Ecology* **92**, 342-351. doi:10.1890/10-0489.1

Bauerfeind, S. S., Fischer, K., Hartstein, S., Janowitz, S. and Martin-Creuzburg, D. (2007). Effects of adult nutrition on female reproduction in a fruit-feeding butterfly: the role of fruit decay and dietary lipids. *J. Insect Physiol.* **53**, 964-973. doi:10.1016/j.jinsphys.2007.01.013

Bell, J. R., Blumgart, D. and Shortall, C. R. (2020). Are insects declining and at what rate? An analysis of standardised, systematic catches of aphid and moth abundances across Great Britain. *Insect Conserv. Divers.* **13**, 115-126. doi:10.1111/icad.12412

Bhardwaj, S. K., Sharma, M. L., Gulati, G., Chhabra, A., Kaushik, R., Sharma, P. and Kaur, G. (1998). Effect of starvation and insulin-induced hypoglycemia on oxidative stress scavenger system and electron transport chain complexes from rat brain, liver, and kidney. *Mol. Chem. Neuropathol.* **34**, 157-168. doi:10.1007/BF02815077

Blank, M. L., Parker, M. L., Ramaswamy, R., Powell, C. J., English, E. D., Adomako-Ankomah, Y., Pernas, L. F., Workman, S. D., Boothroyd, J. C., Boulanger, M. J. et al. (2018). A *Toxoplasma gondii* locus required for the direct manipulation of host mitochondria has maintained multiple ancestral functions. *Mol. Microbiol.* **108**, 519-535. doi:10.1111/mmi.13947

Bleier, L. Dröse, S. (2013). Superoxide generation by complex III: from mechanistic rationales to functional consequences. *Biochim. Biophys. Acta Bioenerg.* **1827**, 1320-1331. doi:10.1016/j.bbabio.2012.12.002

Blume, M. and Seeber, F. (2018). Metabolic interactions between *Toxoplasma gondii* and its host. *F1000Res.* **7**, 1719. doi:10.12688/f1000research.16021.1

Boggs, C. L. and Ross, C. L. (1993). The effect of adult food limitation on life history traits in *Speyeria mormonia* (Lepidoptera: Nymphalidae). *Ecology* **74**, 433-441. doi:10.2307/1939305

Bosch, J., Sgolastra, F. and Kemp, W. P. (2010). Timing of eclosion affects diapause development, fat body consumption and longevity in *Osmia lignaria*, a univoltine, adult-wintering solitary bee. *J. Insect Physiol.* **56**, 1949-1957. doi:10.1016/j.jinsphys.2010.08.017

Bourguignon, A., Rameau, A., Toullec, G., Romestaing, C. and Roussel, D. (2017). Increased mitochondrial energy efficiency in skeletal muscle after long-term fasting: its relevance to animal performance. *J. Exp. Biol.* **220**, 2445-2451. doi:10.1242/jeb.159087

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254. doi:10.1016/0003-2697(76)90527-3

Bradley, C. A. and Altizer, S. (2005). Parasites hinder monarch butterfly flight: implications for disease spread in migratory hosts. *Ecol. Lett.* **8**, 290-300. doi:10.1111/j.1461-0248.2005.00722.x

Brand, M. D. and Nicholls, D. G. (2011). Assessing mitochondrial dysfunction in cells. *Biochem. J.* **435**, 297-312. doi:10.1042/BJ20110162

Brower, L. P. (1996). Monarch butterfly orientation: missing pieces of a magnificent puzzle. *J. Exp. Biol.* **199**, 93-103. doi:10.1242/jeb.199.1.93

Brower, L. P., Fink, L. S. and Walford, P. (2006). Fueling the fall migration of the monarch butterfly. *Integr. Comp. Biol.* **46**, 1123-1142. doi:10.1093/icb/icl029

Casas, F., Pineau, T., Rochard, P., Rodier, A., Daury, L., Dauça, M., Cabello, G. and Wrutniak-Cabello, C. (2000). New molecular aspects of regulation of mitochondrial activity by fenofibrate and fasting. *FEBS Lett.* **482**, 71-74. doi:10.1016/S0014-5793(00)02023-8

Chapman, J. W., Reynolds, D. R. and Wilson, K. (2015). Long-range seasonal migration in insects: mechanisms, evolutionary drivers and ecological consequences. *Ecol. Lett.* **18**, 287-302. doi:10.1111/ele.12407

Davis, A. K. (2009). Wing color of monarch butterflies (*Danaus plexippus*) in Eastern North America across life stages: migrants are “redder” than breeding and overwintering stages. *Psyche* **2009**, 705780. doi:10.1155/2009/705780
Davis, A. K. and de Roode, J. C. (2018). Effects of the parasite, *Ophryocystis elektroscirrha*, on wing characteristics important for migration in the monarch butterfly. *Anim. Migr.* **5**, 84-93. doi:10.1515/ami-2018-0008
Davis, A. K., Chi, J., Bradley, C. and Altizer, S. (2012). The redder the better: wing color predicts flight performance in monarch butterflies. *PLoS One* **7**, e41323. doi:10.1371/journal.pone.0041323
De Roode, J. C., Yates, A. J. and Altizer, S. (2008). Virulence-transmission trade-offs and population divergence in virulence in a naturally occurring butterfly parasite. *Proc. Natl. Acad. Sci. USA* **105**, 7489-7494. doi:10.1073/pnas.0710909105
DeVries, Z. C., Kells, A. K. and Appel, G. A. (2013). Standard metabolic rate of the bed bug, *Cimex lectularius*: effects of temperature, mass, and life stage. *J. Insect Physiol.* **59**, 1133-1139. doi:10.1016/j.jinsphys.2013.08.012
Dingle, H. (1996). *Migration: The Biology of Life on the Move*. New York: Oxford University Press.
Dirzo, R., Young, H. S., Galetti, M., Ceballos, G., Isaac, N. J. B. and Collen, B. (2014). Defaunation in the Anthropocene. *Science* **345**, 401-406. doi:10.1126/science.1251817
Dudley, R. and Srygley, R. B. (2008). Airspeed adjustment and lipid reserves in migratory Neotropical butterflies. *Funct. Ecol.* **22**, 264-270. doi:10.1111/j.1365-2435.2007.01364.x
Estabrook, R. W. (1967). [7] Mitochondrial respiratory control and the polarographic measurement of ADP: O ratios. *Meth. Enzymol.* **10**, 41-47. doi:10.1016/0076-6879(67)10010-4
Figon, F. and Casas, J. (2019). Ommochromes in invertebrates: biochemistry and cell biology. *Biol. Rev.* **94**, 156-183. doi:10.1111/brv.12441
Flockhart, D. T. T., Wassenaar, L. I., Martin, T. G., Hobson, K. A., Wunder, M. B. and Norris, D. R. (2013). Tracking multi-generational colonization of the breeding grounds by monarch butterflies in eastern North America. *Proc. R. Soc. B Biol. Sci.* **280**, 20131087. doi:10.1098/rspb.2013.1087
Flockhart, D. T. T., Pichancourt, J. B., Norris, D. R. and Martin, T. G. (2015). Unravelling the annual cycle in a migratory animal: breeding-season habitat loss drives population declines of monarch butterflies. *J. Anim. Ecol.* **84**, 155-165. doi:10.1111/1365-2656.12253
Hallmann, C. A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T. et al. (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLoS One* **12**, e0185809. doi:10.1371/journal.pone.0185809
Hanley, D., Miller, N. G., Flockhart, D. T. T. and Norris, D. R. (2013). Forewing pigmentation predicts migration distance in wild-caught migratory monarch butterflies. *Behav. Ecol.* **24**, 1108-1113. doi:10.1093/beheco/art037
Heine, K. B. and Hood, W. R. (2020). Mitochondrial behaviour, morphology, and animal performance. *Biol. Rev.* **95**, 730-737. doi:10.1111/brv.12584
Hill, G. E. (2019). *Mitonuclear Ecology*. Oxford, UK: Oxford University Press.
Hill, G. E., Hood, W. R., Ge, Z., Grinter, R., Greening, C., Johnson, J. D., Park, N. R., Taylor, H. A., Andreasen, V. A., Powers, M. J. et al. (2019). Plumage redness signals mitochondrial function in the house finch. *Proc. R. Soc. B Biol. Sci.* **286**, 20191354. doi:10.1098/rspb.2019.1354
Hulbert, A. J., Turner, N., Hinde, J., Else, P. and Guderley, H. (2006). How might you compare mitochondria from different tissues and different species? *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **176**, 93-105. doi:10.1007/s00360-005-0025-z
Hyatt, H. W., Zhang, Y., Hood, W. R. and Kavazis, A. N. (2017). Lactation has persistent effects on a mother’s metabolism and mitochondrial function. *Sci. Rep.* **7**, 17118. doi:10.1038/s41598-017-17418-7
Jimenez, A. G., Van Brocklyn, J., Wortman, M. and Williams, J. B. (2014). Cellular metabolic rate is influenced by life-history traits in tropical and temperate birds. *PLoS One* **9**, e87349. doi:10.1371/journal.pone.0087349
Jimenez, A. G., O’Connor, E. S., Tobin, K. J., Anderson, K. N., Winward, J. D., Fleming, A., Winner, C., Chinchilli, E., Maya, A., Carlson, K. et al. (2019). Does cellular metabolism from primary fibroblasts and oxidative stress in blood differ between mammals and birds? The (lack-thereof) scaling of oxidative stress. *Integr. Comp. Biol.* **59**, 953-969. doi:10.1093/icb/icz017
Kaufmann, C. and Briegel, H. (2004). Flight performance of the malaria vectors *Anopheles gambiae* and *Anopheles atroparvus*. *J. Vector Ecol.* **29**, 140-153.
Koch, R. E., Buchanan, K. L., Casagrande, S., Crino, O., Dowling, D. K., Hill, G. E., Hood, W. R., McKenzie, M., Mariette, M. M., Noble, D. W. A. et al. (2021). Integrating mitochondrial aerobic metabolism into ecology and evolution. *Trends Ecol. Evol.* **36**, 321-332. doi:10.1016/j.tree.2020.12.006
Lighton, J. R. B. (2008). *Measuring Metabolic Rates: A Manual for Scientists*. New York: Oxford University Press.
Lentini, G., Dos Santos Pacheco, N. and Burleigh, B. A. (2018). Targeting host mitochondria: a role for the *Trypanosoma cruzi* amastigote flagellum. *Cell. Microbiol.* **20**, e12807. doi:10.1111/cmi.12807
Majewska, A. A., Davis, A. K., Altizer, S. and de Roode, J. C. (2022). Parasite dynamics in North American monarchs predicted by host density and seasonal migratory culling. *J. Anim. Ecol.* **91**, 780-793. doi:10.1111/1365-2656.13678
Maklakov, A. A., Simpson, S. J., Zajitschek, F., Hall, M. D., Dessmann, J., Clissold, F., Raubenheimer, D., Bonduriansky, R. and Brooks, R. C. (2008). Sex-specific fitness effects of nutrient intake on reproduction and lifespan. *Curr. Biol.* **18**, 1062-1066. doi:10.1016/j.cub.2008.06.059
Masters, A. R., Malcolm, S. B. and Brower, L. P. (1988). Monarch butterfly (*Danaus Plexippus*) thermoregulatory behavior and adaptations for overwintering in Mexico. *Ecology* **69**, 458-467. doi:10.2307/1940444
Matarese, L. E. (1997). Indirect calorimetry: technical aspects. *J. Am. Diet. Assoc.* **97**, S154-S160. doi:10.1016/S0002-8223(97)00754-2
McCue, M. D. (2010). Starvation physiology: reviewing the different strategies animals use to survive a common challenge. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **156**, 1-18. doi:10.1016/j.cbpa.2010.01.002
Melvin, R. G. and Ballard, J. W. O. (2006). Intraspecific variation in survival and mitochondrial oxidative phosphorylation in wild-caught *Drosophila simulans*. *Aging Cell* **5**, 225-233. doi:10.1111/j.1474-9726.2006.00211.x
Monternier, P. A., Marmillot, V., Rouanet, J. L. and Roussel, D. (2014). Mitochondrial phenotypic flexibility enhances energy savings during winter fast in king penguin chicks. *J. Exp. Biol.* **217**, 2691-2697. doi:10.1242/jeb.104505
Monternier, P. A., Fongy, A., Hervant, F., Drai, J., Collin-Chavagnac, D., Rouanet, J. L. and Roussel, D. (2015). Skeletal muscle phenotype affects fasting-induced mitochondrial oxidative phosphorylation flexibility in cold-acclimated ducklings. *J. Exp. Biol.* **218**, 2427-2434. doi:10.1242/jeb.122671
Niitepõld, K. (2019). Effects of flight and food stress on energetics, reproduction, and lifespan in the butterfly *Melitaea cinxia*. *Oecologia* **191**, 271-283. doi:10.1007/s00442-019-04489-8
Niitepõld, K. and Boggs, C. L. (2015). Effects of increased flight on the energetics and life history of the butterfly *Speyeria mormonia*. *PLoS One* **10**, e0140104. doi:10.1371/journal.pone.0140104
Niitepõld, K., Perez, A. and Boggs, C. L. (2014). Aging, life span, and energetics under adult dietary restriction in Lepidoptera. *Physiol. Biochem. Zool.* **87**, 684-694. doi:10.1086/677570
Niitepõld, K., Parry, H., Harris, N., Appel, A., de Roode, J., Kavazis, A. and Hood, W. (2022). Flying on empty: reduced mitochondrial function and flight capacity in food-deprived monarch butterflies. *Dryad, Dataset*. doi:10.5061/dryad.d2547d84x
Pelton, E. M., Schultz, C. B., Jepsen, S. J., Black, S. H. and Crone, E. E. (2019). Western monarch population plummets: status, probable causes, and recommended conservation actions. *Front. Ecol. Evol.* **7**, 258. doi:10.3389/fevo.2019.00258
Pernas, L., Adomako-Ankomah, Y., Shastri, A. J., Ewald, S. E., Treeck, M., Boyle, J. P. and Boothroyd, J. C. (2014). Toxoplasma effector MAF1 mediates recruitment of host mitochondria and impacts the host response. *PLoS Biol.* **12**, e1001845. doi:10.1371/journal.pbio.1001845
Pernas, L., Bean, C., Boothroyd, J. C. and Scorrano, L. (2018). Mitochondria restrict growth of the intracellular parasite *Toxoplasma gondii* by limiting its uptake of fatty acids. *Cell Metab.* **27**, 886-897.e4. doi:10.1016/j.cmet.2018.02.018
Raven, P. H. and Wagner, D. L. (2021). Agricultural intensification and climate change are rapidly decreasing insect biodiversity. *Proc. Natl. Acad. Sci. USA* **118**, e2002548117. doi:10.1073/pnas.2002548117
Reim, E., Eichhorn, D., Roy, J. D., Steinhoff, P. O. M. and Fischer, K. (2019). Nutritional stress reduces flight performance and exploratory behavior in a butterfly. *Insect Sci.* **26**, 897-910. doi:10.1111/1744-7917.12596
Roussel, D., Boël, M. and Romestaing, C. (2018). Fasting enhances mitochondrial efficiency in duckling skeletal muscle by acting on the substrate oxidation system. *J. Exp. Biol.* **221**, jeb172213. doi:10.1242/jeb.172213
Salin, K., Auer, S. K., Rudolf, A. M., Anderson, G. J., Selman, C. and Metcalfe, N. B. (2016). Variation in metabolic rate among individuals is related to tissue-specific differences in mitochondrial leak respiration. *Physiol. Biochem. Zool.* **89**, 511-523. doi:10.1086/688769
Satterfield, D. A., Wright, A. E. and Altizer, S. (2013). Lipid reserves and immune defense in healthy and diseased migrating monarchs *Danaus plexippus*. *Curr. Zool.* **59**, 393-402. doi:10.1093/czoolo/59.3.393
Schutz, Y. and Ravussin, E. (1980). Respiratory quotients lower than 0.70 in ketogenic diets. *Am. J. Clin. Nutr.* **33**, 1317-1319. doi:10.1093/ajcn/33.6.1317
Semmens, B. X., Semmens, D. J., Thogmartin, W. E., Wiederholt, R., López-Hoffman, L., Diffendorfer, J. E., Pleasants, J. M., Oberhauser, K. S. and Taylor, O. R. (2016). Quasi-extinction risk and population targets for the Eastern, migratory population of monarch butterflies (*Danaus plexippus*). *Sci. Rep.* **6**, 23265. doi:10.1038/srep23265
Silva-Marrero, J. I., Sáez, A., Caballero-Solares, A., Viegas, I., Almajano, M. P., Fernández, F., Baanante, I. V. and Metón, I. (2017). A transcriptomic approach to study the effect of long-term starvation and diet composition on the expression of mitochondrial oxidative phosphorylation genes in gilthead sea bream (*Sparus aurata*). *BMC Genomics* **18**, 768. doi:10.1186/s12864-017-4148-x
Sinclair, B. J., Marshall, K. E., Suarez, R. K. and Hoppeler, H. H. (2018). The many roles of fats in overwintering insects. *J. Exp. Biol.* **221**, jeb161836. doi:10.1242/jeb.161836
Smith, L. M. and Appel, A. G. (1996). Toxicity, repellence, and effects of starvation compared among insecticidal baits in the laboratory for control of American and

smokybrown cockroaches (Dictyoptera: Blattidae). *J. Econ. Entomol.* **89**, 402-410. doi:10.1093/jee/89.2.402

Southwood, T. R. E. and Johnson, C. G. (1971). *Migration and Dispersal of Insects by Flight*. London: Methuen.

Speakman, J. R. and Mitchell, S. E. (2011). Caloric restriction. *Mol. Aspects Med.* **32**, 159-221. doi:10.1016/j.mam.2011.07.001

Suarez, R. K. (2000). Energy metabolism during insect flight: biochemical design and physiological performance. *Physiol. Biochem. Zool.* **73**, 765-771. doi:10.1086/318112

Syn, G., Anderson, D., Blackwell, J. M. and Jamieson, S. E. (2017). *Toxoplasma gondii* infection is associated with mitochondrial dysfunction *in-vitro*. *Front. Cell. Infect. Microbiol.* **7**, 512. doi:10.3389/fcimb.2017.00512

Thogmartin, W. E., Wiederholt, R., Oberhauser, K., Drum, R. G., Diffendorfer, J. E., Altizer, S., Taylor, O. R., Pleasants, J., Semmens, D., Semmens, B. et al. (2017). Monarch butterfly population decline in north America: identifying the threatening processes. *R. Soc. Open Sci.* **4**, 170760. doi:10.1098/rsos.170760

Trounce, I. A., Kim, Y. L., Jun, A. S. and Wallace, D. C. (1996). Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines. *Methods Enzymol.* **264**, 484-509. doi:10.1016/S0076-6879(96)64044-0

van der Kolk, B. W., Muniandy, M., Kaminska, D., Alvarez, M., Ko, A., Miao, Z., Valsesia, A., Langin, D., Vaittinen, M., Pääkkönen, M. et al. (2021). Differential mitochondrial gene expression in adipose tissue following weight loss induced by diet or bariatric surgery. *J. Clin. Endocrinol. Metab.* **106**, 1312-1324. doi:10.1210/clinem/dgab072

Wanders, R. J., Groen, A. K., Van Roermund, C. W. and Tager, J. M. (1984). Factors determining the relative contribution of the adenine-nucleotide translocator and the ADP-regenerating system to the control of oxidative phosphorylation in isolated rat-liver mitochondria. *Eur. J. Biochem.* **142**, 417-424. doi:10.1111/j.1432-1033.1984.tb08303.x

Zhan, S., Zhang, W., Niitepõld, K., Hsu, J., Haeger, J. F., Zalucki, M. P., Altizer, S., De Roode, J. C., Reppert, S. M. and Kronforst, M. R. (2014). The genetics of monarch butterfly migration and warning colouration. *Nature* **514**, 317-321. doi:10.1038/nature13812

Zid, B. M., Rogers, A. N., Katewa, S. D., Vargas, M. A., Kolipinski, M. C., Lu, T. A., Benzer, S. and Kapahi, P. (2009). 4E-BP extends lifespan upon dietary restriction by enhancing mitochondrial activity in *Drosophila*. *Cell* **139**, 149-160. doi:10.1016/j.cell.2009.07.034