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RESEARCH ARTICLE

Oxygen and energy availability interact to determine flight performance in the Glanville fritillary butterfly

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ABSTRACT

Flying insects have the highest known mass-specific demand for oxygen, which makes it likely that reduced availability of oxygen might limit sustained flight, either instead of or in addition to the limitation due to metabolite resources. The Glanville fritillary butterfly (*Melitaea cinxia*) occurs as a large metapopulation in which adult butterflies frequently disperse between small local populations. Here, we examine how the interaction between oxygen availability and fuel use affects flight performance in the Glanville fritillary. Individuals were flown under either normoxic (21 kPa O_2) or hypoxic (10 kPa O_2) conditions and their flight metabolism was measured. To determine resource use, levels of circulating glucose, trehalose and whole-body triglyceride were recorded after flight. Flight performance was significantly reduced in hypoxic conditions. When flown under normoxic conditions, we observed a positive correlation among individuals between post-flight circulating trehalose levels and flight metabolic rate, suggesting that low levels of circulating trehalose constrains flight metabolism. To test this hypothesis experimentally, we measured the flight metabolic rate of individuals injected with a trehalase inhibitor. In support of the hypothesis, experimental butterflies showed significantly reduced flight metabolic rate, but not resting metabolic rate, in comparison to control individuals. By contrast, under hypoxia there was no relationship between trehalose and flight metabolic rate. Additionally, in this case, flight metabolic rate was reduced in spite of circulating trehalose levels that were high enough to support high flight metabolic rate under normoxic conditions. These results demonstrate a significant interaction between oxygen and energy availability for the control of flight performance.

KEY WORDS: Flight capacity, Glanville fritillary, Hypoxia, Metabolism, Respirometry, Trehalose

INTRODUCTION

Insect flight is energetically costly. Whole-organism metabolism during flight can be up to 200 times higher than that at rest (Kammer and Heinrich, 1978) and the thoracic muscles of insects exhibit the highest mass-specific rates of oxygen consumption known for any locomotory tissue (Dudley, 2000; Suarez, 2000). As flight capacity greatly influences the fitness of individuals in the wild, it is important to understand the factors that limit flight performance of ecological model species.

Insect flight is based on aerobic metabolism (Kammer and Heinrich, 1978) and it has hence been suggested that flight might be limited by oxygen conductance to flight muscles (Dudley, 2000; Marden et al., 2013) as well as by substrates available for the synthesis of adenosine triphosphate (ATP) in mitochondria via the tricarboxylic acid cycle and oxidative phosphorylation. In support of the former hypothesis, flight metabolic rate can be sensitive to ambient oxygen level (Harrison and Lighton, 1998), although many insects are relatively tolerant to hypoxia at rest (Keister and Buck, 1964). However, previous experiments on blow flies have demonstrated that the availability of trehalose – the disaccharide of glucose in insects – is a limiting factor for the duration of flight performance (Clegg and Evans, 1961). Trehalose is the primary blood sugar in insects and it plays a major role in insect flight, acting as the source of instant energy as well as mitigating abiotic stressors (Shukla et al., 2015). Synthesized by the fat body, trehalose is hydrolysed by the trehalase enzyme, releasing glucose into the flight muscle (Becker et al., 1996). Butterflies are thought to use different energy resources to fuel flight. For example, long-distance migrants, such as the Monarch (*Danaus plexippus*) and the red admiral (*Vanessa atalanta*), have been proposed to use fat rather than carbohydrates to fuel flight during migration (Zebe, 1954; Cenedella, 1971; Brown and Chippendale, 1974). By contrast, the vast majority of butterflies fly only short distances at a time and they are likely to use carbohydrates to fuel flight (Becker et al., 1996).

The Glanville fritillary butterfly (*Melitaea cinxia* Linnaeus 1758) is widely distributed across Eurasia, with a range extending from the Iberian Peninsula in the west to China in the east (Hanski, 1999). Whilst some populations in the Alps and the Atlas Mountains can reach an altitude of 2500 m, the majority of populations occur in lowlands. The Glanville fritillary flies short distances at a time: the median distance covered during one flight bout was 25 m (maximum 415 m), based on tracking of free-flying butterflies with harmonic radar (Ovaskainen et al., 2008). The median maximal flight distance in 2 h was 500 m (maximum 3700 m), consisting of several flight bouts. In laboratory flight experiments, in which butterflies are encouraged to fly as continuously as possible, most individuals become exhausted in less than 15 min (Haag et al., 2005; Niitepõld et al., 2009), suggesting that flight metabolism becomes quickly limited by either energy or oxygen, or a combination of both.

Here, we report an experiment that was designed to test whether flight in the Glanville fritillary is limited by the availability of energy in the form of carbohydrates or fat, or by oxygen. We measured the flight metabolic rate of butterflies in the laboratory under normoxia (21 kPa O_2) and hypoxia (10 kPa O_2). Following 10 min of continuous flight, we measured the post-flight concentrations of circulating glucose, trehalose and whole body triglycerides. If oxygen availability below normoxia limits flight then flight metabolism should be lower in hypoxia than in normoxia, although this result in itself would not be sufficient to demonstrate the role of oxygen limitation following prolonged flight in normoxia. If energy limits flight then we would expect the post-

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flight concentration of the limiting metabolite in individual butterflies to be positively correlated with the metabolic rate during the experiment. To experimentally test the hypothesis that circulating trehalose use constrains flight metabolism, we simulated low trehalose levels by injecting a subset of individuals with a trehalase inhibitor and predicted that these individuals should show reduced flight metabolic rate.

MATERIALS AND METHODS

Rearing and handling of butterflies

We used the common garden-reared offspring of wild-caught butterflies originating from the Åland Islands in SW Finland (Hanski, 2011). Larvae were reared in sibling groups and fed *ad libitum* with fresh leaves of the host plant *Plantago lanceolata*. Upon eclosion, adults were numbered with a felt tip pen, transferred to large cylindrical cages (50×40 cm), and split between the two resource limitation experiments, involving either oxygen or food.

Effect of food limitation on metabolism

To determine the effect of energy limitation on metabolite levels, we randomly assigned adults to one of two treatment groups upon eclosion. Adults were either fed *ad libitum* the control diet of 20% honey-water solution or were placed under a starvation regime, where they were provided with water only. Butterflies were maintained under controlled conditions at 28°C for 12 h in the light and 18°C for 12 h in the dark. Individuals were sampled for metabolites at 3, 5 or 10 days of age. This starvation experiment also served as a control for the metabolite measurements.

Post-flight sampling of metabolites

Haemolymph was sampled from adult butterflies following a post-flight rest period of 10 min. Adults were immobilized on ice and fixed to a microscope slide with cellophane tape with wings spread to expose the dorsal thorax. Jeweller's forceps were used to pierce the thorax at the triangular junction between hard plates of the mesothorax and metathorax. The tip of a 2 µl micropipette was inserted into the opening and 1–2 µl haemolymph was withdrawn. Samples were diluted 1:10 in 1× phosphate buffered saline (PBS), pH 6.6 and immediately frozen in liquid nitrogen.

Quantification of metabolites

Haemolymph glucose, trehalose and triglycerides assays were modified from previously described methods (Havula et al., 2013; Bartok et al., 2015; Mattila et al., 2015). Haemolymph samples were thawed on ice, heated for 5 min at 70°C, and centrifuged for 1 min at 12,000 ***g*** to denature and pellet proteins. A 2 µl aliquot was reserved for protein determination prior to heat treatment. Heat-treated haemolymph was further diluted 1:10 in 1× PBS, pH 6.6 and 2 µl was used to determine circulating glucose concentration using a glucose oxidase/peroxidase assay kit (Sigma). Reactions were scaled to 50 µl assay volume in 384-well plate format. The absorbance of the reporter dye *o*-dianisidine at 540 nm was measured using a Perkin Elmer Enspire 2300 spectrophotometer (PerkinElmer, Hamburg, Germany) and compared with that of pure glucose standards for each sample. Samples were measured in three technical replicates with controls lacking glucose oxidase and sample. Circulating glucose concentration in µg ml^{-1} was calculated as the mean glucose concentration of the three measurement wells minus that of the controls.

Circulating levels of trehalose, a disaccharide composed of two linked glucose molecules, were determined by digesting haemolymph samples with trehalase and then determining the glucose concentration as described above. The 12 µl trehalase digests contained 2 µl of 1:50-diluted haemolymph, 9 µl of Tris-buffered saline, pH 6.6 and 1 µl of 2.3 units ml^{-1} trehalase. Samples were digested for 16 h at 37°C. Trehalose content was calculated by subtracting the glucose content of undigested samples from that of the trehalase-digested samples and is reported as mg ml^{-1} glucose.

Circulating triglycerides were measured from adult haemolymph and total triglyceride content was measured for whole pupae using a serum triglyceride determination kit (Sigma). In the assay, fatty acids were cleaved from the glycerol backbone with lipoprotein lipase and the quantity of total glycerol (TG) was measured colorimetrically. Free glycerol (FG) present in haemolymph or homogenate samples that was not due to lipase cleavage of triglycerides was also quantified. The true quantity of triglycerides (TRI) was calculated as TRI=TG−FG and is interpreted as mg ml^{-1} triolein (a triglyceride composed of glycerol and three oleic acids) equivalents standardized to body mass. For each sample, the absorbance of the reporter quinoneimine dye was measured at 540 nm using a Perkin Elmer Enspire 2300 spectrophotometer (PerkinElmer, Hamburg, Germany) and compared with that of pure glycerol standards for each sample (9.61 mg ml^{-1} triolein equivalents/1 mg ml^{-1} glycerol). Assay volume was scaled to 100 µl and samples were measured in three technical replicates with controls lacking lipase and lacking sample. The protein content of haemolymph was determined by Bradford protein assay (Bradford, 1976) using Bradford reagent (Sigma).

Effects of oxygen limitation on flight metabolism

Flight metabolic rate (FMR) was measured using flow-through respirometry [see Mattila and Hanski (2014) for the description of the respirometry set-up]. Two ready-made gas mixtures were used to simulate hypoxic or normoxic conditions. Based on a pilot experiment measuring the ability of butterflies to fly in a range of oxygen environments (7, 14, 21 and 28 kPa oxygen), a 10 kPa O_2/90 kPa N_2 mix was selected for the hypoxia treatment (Linde AG, Munich, Germany) with a 21 kPa O_2/79 kPa N_2 mix (Linde AG, Munich, Germany) used as the normoxia control (all atmospheres were normobaric). Gas was pumped through a 1 litre transparent jar at the rate of 1.0 l min^{-1}. As the purpose of the measurement was not to detect short-term temporal changes in CO_2 production rate, the large chamber volume to flow rate ratio helps to smooth out unwanted artificial peaks in the data. This flow rate might lead to a relatively slow response time and a systematic underestimation of peak metabolic rates. However, a flying butterfly in the chamber acts like a fan, mixing the CO_2 in the air and providing a faster response than when measuring stationary subjects.

Adults were 3–5 days old at the time of the measurement and were randomly assigned to the two treatments. Prior to the experiment, individuals were maintained at 23°C and provided with a 20% honey-water solution. Individuals were flown for 10 min by gently tapping or shaking the respirometry chamber whenever the butterfly landed. The measurement chamber was kept at a constant temperature using an electric heater with the jar illuminated under a UV source to promote active flight. Before and after the experiment the chamber was covered with a black cloth to obtain a stable baseline of resting metabolic rate (RMR). The order of measurements was randomized amongst treatment groups.

The average measurement temperature was 30.0°C (range, 28.3–31.3°C; s.d., 0.6). We calculated from the original measurements peak FMR (FMR_{peak}) and integrated FMR (FMR_{int}). The former is the highest level of CO_2 production during the experiment, whereas the latter gives the total volume of CO_2 produced over the 10 min

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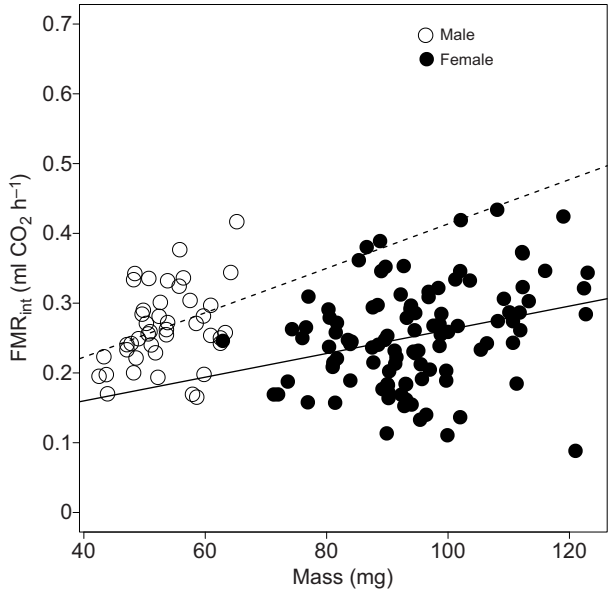

Fig. 1. Multiple regression of integrated FMR against adult body mass for each sex in the Glanville fritillary butterfly (*Melitaea cinxia*). Both regressions are significant (female, R^2=0.07, N=104, P=0.003; male, R^2=0.09, N=43, P=0.03). Residuals from these regressions were used for subsequent analyses.

experiment. To characterize flight endurance, we calculated the same measures for the last 5 min of the experiment. All measures of FMR were corrected for variation in body mass by regressing FMR against adult mass (Fig. 1) and using the residuals from this regression in subsequent analyses.

Effect of trehalase inhibition on flight metabolism

To experimentally test whether trehalose levels limit flight metabolic rate, we injected 20 adult butterflies from a single family with either a control solution or a trehalase inhibitor and measured FMR and RMR. The trehalase inhibitor prevents trehalose being metabolized into glucose, which is used in flight muscles. Each butterfly was spanned with a net on a soft sponge with ventral side up to ensure that it was immobilized. Butterflies were either injected with 2 µl PBS or 2 µl of 1.66 µg $µl^{-1}$ validamycin A (Sigma, 32347) in PBS with a Hamilton syringe into the thorax. The needle was introduced at a very low angle and just as deep as necessary to avoid extra damage to tissue. The validamycin A concentration was determined through a pilot experiment selecting the highest non-lethal concentration where butterflies were still able to take flight. After injection, butterflies were maintained under controlled conditions as above, with only water for 24 h before metabolic rate was measured using atmospheric air.

Statistical analyses

The results were analysed using the statistical platform R v.3.1.2 (http://www.R-project.org) and the package *lme4* v.1.1-7 (http://cran.r-project.org/web/packages/lme4/index.html). Models were constructed to test the change in metabolite levels over time in the starvation experiment, using the age of the butterfly, treatment (starvation or control) and sex as fixed effects, and family as a random effect. The models were compared using likelihood ratio tests in a stepwise backward fashion starting with the full model with all biologically meaningful interactions included. Covariates were excluded if they did not significantly improve the model fit (details of model selection are given in Table S1). To test sources of variation in flight metabolism, we used linear mixed-effects (LME) models with treatment (hypoxia or normoxia) and sex as fixed effects, temperature during the flight measurement as a covariate and their interaction terms. Family was included as a random effect.

To investigate the influence of hypoxia on the relationship between flight metabolism and metabolite levels we fitted separate models for each treatment, with metabolite quantity, sex and FMR as fixed effects; temperature during FMR measurements as a covariate; and their interaction terms. Family was included as a random effect. Metabolite levels were log transformed. There was a weak positive relationship between glucose level and the time of day of the experiment (Fig. S1), but including time-corrected glucose values did not change the result. We therefore retained the uncorrected glucose levels in the analysis. To compare the different measures of FMR as explanatory factors for log trehalose level we used the function *model.sel* in the package *MuMIn* v.1.13.4 (http://cran.r-project.org/web/packages/MuMIn/index.html) to rank the models based on their Akaike information criterion for finite sample sizes (AICc) (Johnson and Omland, 2004). While measuring the effect of trehalase inhibition on metabolic rate, linear models (LMs) were used as all individuals had originated from the same family. Treatment and sex were fitted as fixed effects, with temperature during measurement fitted as a covariate, and the best-fitting model was selected using *model.sel* (Table S1).

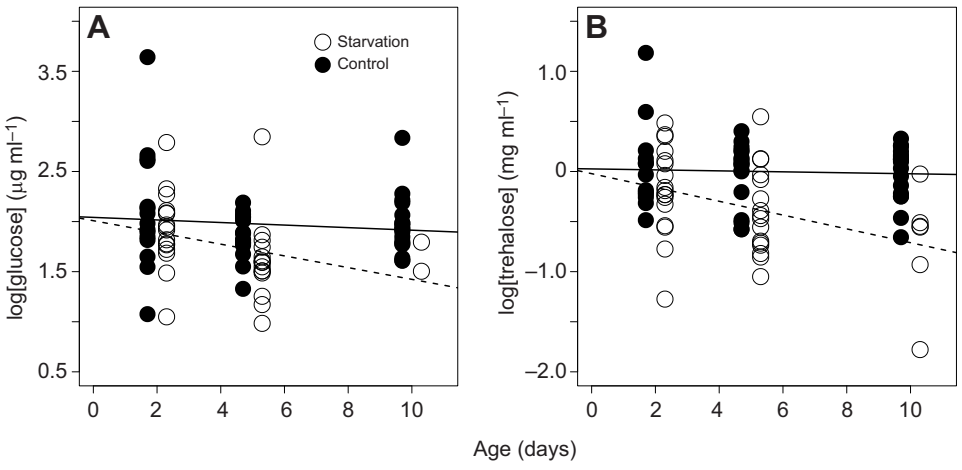

Fig. 2. Glucose and trehalose levels during 10 days of starvation in *M. cinxia*. (A) Glucose (N=85) and (B) trehalose (N=89) levels during 10 days of starvation versus control treatment. The two treatment groups are offset for clarity. Lines represent regressions. Under starvation, butterflies had a significant drop in both glucose (χ^2=6.62, P=0.01) and trehalose levels (χ^2=13.79, P=0.0002).

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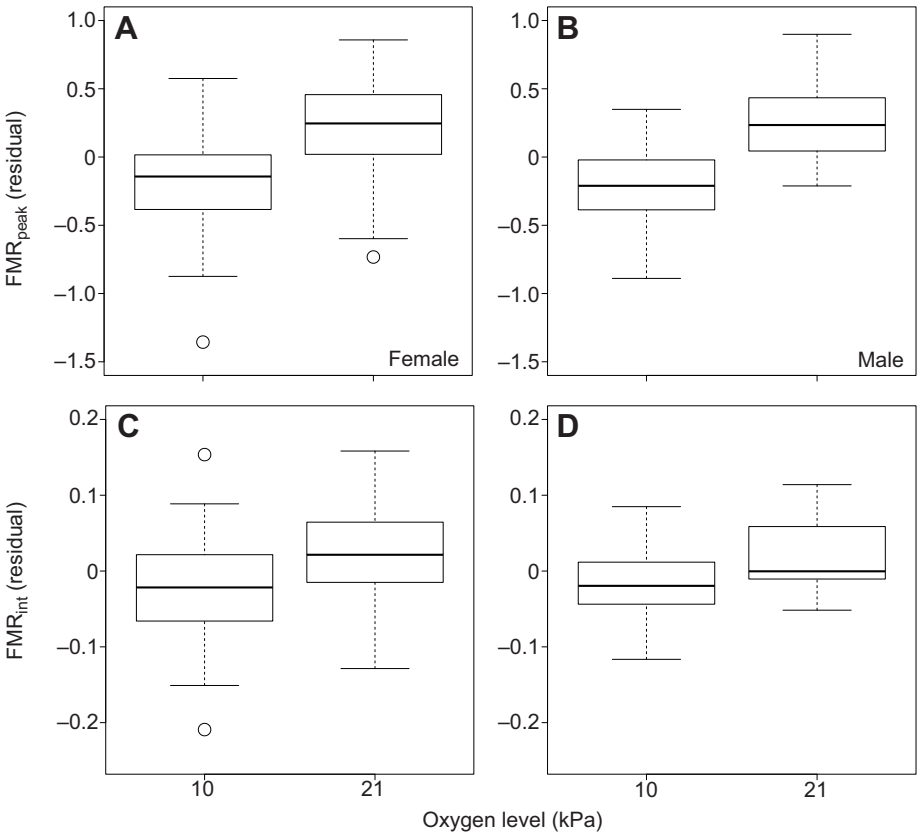

Fig. 3. Peak and integrated FMR in *M. cinxia* under hypoxia. FMR$_{peak}$ (A,B) and FMR$_{int}$ (C,D) under hypoxia (10 kPa O_2) and normoxia (21 kPa O_2) in females (A,C; hypoxia *N*=55, normoxia *N*=49) and males (B,D; hypoxia *N*=24, normoxia *N*=19). FMR was corrected for adult weight using sex-specific regressions (see Fig. 1). Under hypoxia, there was a significant reduction in both peak (χ^2=51.38, *P*=7.61e^{-13}) and integrated FMR (χ^2=20.91, *P*=4.81e^{-06}). Whiskers indicate the maximum and minimum values within 1.5 times the interquartile range, bar is median value and box is the upper and lower quartiles.

RESULTS

To assess the influence of nutrient availability on metabolite levels, we performed an acute starvation experiment. Under starvation butterflies had a significant drop in both glucose (Fig. 2A; *P*=0.01) and trehalose levels (Fig. 2B; *P*=0.0002) over a period of 10 days. By contrast, triglycerides did not show a significant difference between the starvation and control treatments (Fig. S2), but there was a highly significant difference between the two sexes (χ^2=41.97, *P*=9.28e^{-11}), males having 13 times higher levels of triglycerides than females. There was a significant effect of sex in trehalose level (χ^2=4.77, *P*=0.03), but not in glucose concentration (Fig. S3).

FMR was significantly reduced under hypoxia (10 kPa O_2) in comparison with normoxia (21 kPa O_2). The peak flight metabolic rate and the integrated rate over the 10 min experiment were reduced by 20% and 17%, respectively, in hypoxia compared with normoxia (Fig. 3; FMR$_{peak}$, *P*=7.61e^{-13}; FMR$_{int}$, *P*=4.81e^{-06}). The two sexes responded similarly to oxygen limitation (FMR$_{peak}$, χ^2=0.002, *P*=0.96; FMR$_{int}$, χ^2=0.004, *P*=0.95). There was no difference in the concentrations of glucose, trehalose and triglyceride in the haemolymph between the oxygen treatments (Fig. S4).

Haemolymph was collected from adult butterflies following a post-flight rest period of 10 min. We examined whether individual variation in post-flight metabolites was related to the corresponding variation in FMR$_{int}$ (results were similar when peak flight metabolic rate was analysed instead). There was no significant relationship between FMR and the post-flight glucose (Fig. 4A) or triglyceride concentration (Fig. S5), either under normoxia or hypoxia. By contrast, the post-flight trehalose concentration was highly significantly related to flight metabolism among the butterflies under normoxia (Fig. 4B; χ^2=7.71, *P*=0.005). Conversely, no correlation between FMR and

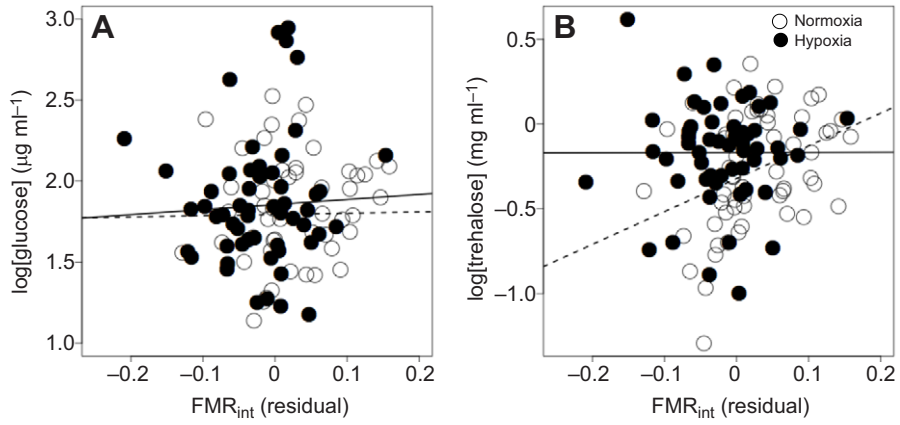

Fig. 4. Correlation between post-flight glucose and trehalose levels and the integrated FMR in *M. cinxia* butterflies under hypoxia. FMR (corrected for adult mass using sex-specific regressions, see Fig. 1) plotted as a function of (A) glucose (*N*=109) and (B) trehalose (*N*=113) concentrations. While there was no significant relationship between FMR and glucose, trehalose concentration was strongly and highly significantly related to integrated FMR under normoxia (χ^2=7.71, *P*=0.005).

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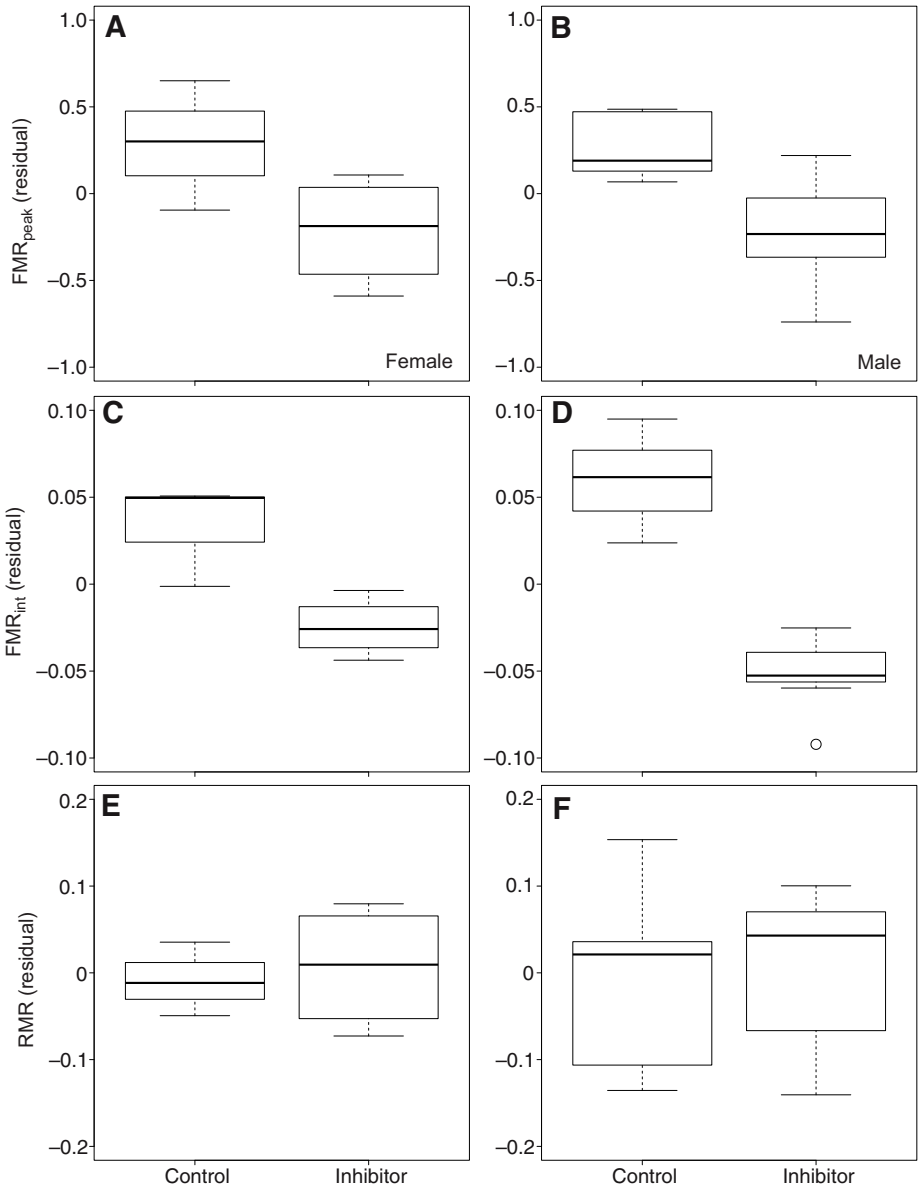

Fig. 5. Peak and integrated FMR and resting metabolic rate in *M. cinxia* treated with trehalase inhibitor. FMR_{peak} (A,B), FMR_{int} (C,D) and RMR (E,F) in control (n=9) and trehalase inhibitor-treated (n=11) females (A,C,E) and males (B,D,F). Metabolic rates were corrected for adult mass using sex-specific regressions. Butterflies injected with trehalase inhibitor had significantly reduced peak (F=15.51, d.f.=1, P=0.001) and integrated FMR (F=63.93, d.f.=1, P=2.47e^{-07}). There was no difference in RMR between the treatments. Whiskers indicate the maximum and minimum values within 1.5 times the interquartile range, bar is median value and box is the upper and lower quartiles.

post-flight trehalose levels was observed under hypoxia (χ^2=0.0001, P=0.99). Including sex in the model as a categorical factor showed no significant gender effect (normoxia: χ^2=1.68, P=0.20; hypoxia: χ^2=0.27, P=0.60).

We repeated the analysis for two other measures of flight metabolism that reflect flight endurance, namely FMR_{int} and FMR_{peak} during the last 5 min of the experiment (5–10 min from the beginning of the experiment). The result was qualitatively the same as in Fig. 4, but the correlation between flight metabolism and post-flight trehalose concentration was even stronger (Fig. S6; end FMR_{int}, P=0.002; end FMR_{peak}, P=0.0003). A comparison between all models (ranked by AICc) showed that integrated end-flight metabolism was the best explanatory variable for post-flight trehalose concentration.

Butterflies that were injected with trehalase inhibitor had significantly reduced FMR (Fig. 5A–D; FMR_{peak}, F=15.51, d.f.=1, P=0.001; FMR_{int}, F=63.93, d.f.=1, P=2.47e^{-07}). Including sex as a fixed factor did not improve the model fit (effect of sex on FMR_{peak}, χ^2=0.11, P=0.73; FMR_{int}, χ^2=0.03, P=0.85). Importantly, there was no difference in resting metabolic rate between the treatments (Fig. 5E,F; F=0.04, d.f.=1, P=0.85).

DISCUSSION

Both oxygen and energy availability are essential to insect flight, one of the most resource-demanding activities in the natural world, but their relative roles have remained less well studied. Here, we found that flight metabolic rate is sensitive to oxygen availability, with individuals flown under hypoxic conditions (10 kPa O_2) having 20% lower flight metabolic rate in 10 min experiments than individuals flown under normoxia (21 kPa O_2). Naturally, this result is contingent on the degree of hypoxia used in the experiment. The second main result was a highly significant relationship between FMR and the post-flight level of haemolymph trehalose among individuals flown under normoxia, but not under hypoxia, suggesting the hypothesis that under normoxia low circulating trehalose levels can constrain flight activity. This hypothesis was supported by the experiment showing reduced FMR in trehalase-inhibited individuals. Since trehalose levels were reduced upon

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starvation, nutrient availability in the wild is likely to limit flight performance under normoxia.

The primary fuel source for flight can be estimated from the respiratory quotient (RQ), i.e. the ratio between CO_2 produced and oxygen consumed. The amount of CO_2 produced is dependent on the oxidized substrate, with an RQ of 0.7 indicating lipids as the fuel and an RQ of 1.0 indicting carbohydrates, with an intermediate ratio suggesting that both are used (O'Brien, 1999). The Glanville fritillary butterfly has an estimated RQ close to 1.0 (Haag et al., 2005), indicating that carbohydrates are used as the fuel source for flight, which has also been observed in many other insects [e.g. *Decapotoma lunata* (Auerswald and Gade, 1995) and *Locusta migratoria* (van der Horst et al., 1978)]. Whilst oxygen availability is thought to be the primary limiting factor for peak metabolism, energy limitation is thought to be crucial in endurance metabolism (Turner et al., 2006). Our results are consistent with this hypothesis, because the relationship between flight metabolism and trehalose concentration after the flight was strongest when flight metabolism was measured as CO_2 production during the last 5 min of the experiment. Similarly, in the experiment involving trehalose inhibition, the reduction in flight metabolic rate was greater in total CO_2 emission during the experiment than in peak flight metabolism.

Based on previous studies it is not surprising that glucose levels remained stable under hypoxia and were not associated with flight performance. Blood glucose levels were shown to be stable in response to flight endurance in blow flies, whereas trehalose levels declined dramatically (Clegg and Evans, 1961), indicating that trehalose availability to the flight muscles was the limiting factor. A study of honeybees demonstrated that flight speed was positively correlated with food concentration, and that after an exhaustive flight glucose obtained from food was incorporated to trehalose within 2 min of feeding (Gmeinbauer and Crailsheim, 1993).

In the present study, trehalose was not limiting when butterflies were flown under hypoxic conditions, based on the observation that there was no relationship between trehalose levels and FMR. A likely explanation is that oxygen availability constrained metabolic rate, which in turn reduced energy use. Nonetheless, trehalose levels were not significantly higher in the hypoxia treatment than in normoxia, suggesting that sugar was utilized in some capacity.

As the Glanville fritillary uses primarily (or only) carbohydrates as energy for flight, unlike long-distance migrants such as the Monarch butterfly (Cenedella, 1971; Brown and Chippendale, 1974), it is not surprising that we found no significant association between flight metabolic rate and triglyceride content. However, we found a highly significant sex difference in lipid content, with males having a higher amount of triglycerides than females, consistent with previous observations on Lepidoptera (Gilbert and Schneiderman, 1961). A large percentage of lipids is thought to be broken down during the metamorphosis from pupa to adult and females are expected to convert a large proportion of lipids into eggs (Gilbert and Schneiderman, 1961).

This study highlights a crucial interaction between oxygen availability and energy metabolism in enhancing flight capacity, and indicates that oxygen supply is the more limiting resource. If this finding is a general trend across species, it has important implications for the ecology of butterflies, whereby oxygen availability acts as a potential limit to range expansion across altitudinal gradients. Climate change has resulted in many insect species shifting their distributions to higher altitudes (Hickling et al., 2006; Hill et al., 2011). Our results suggest that even where suitable habitat and energy resources are available, expansion may be constrained by reduced oxygen availability that compromises flight ability. To test this hypothesis, experiments should be conducted with a fine-grained series of O_2 levels using populations originating from different altitudes.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
T.F., R.G.M., V.H. and I.H. designed the study; T.F., R.G.M., S.I., A.R. and L.W. performed the experiments; T.F. and R.G.M. analysed the data; T.F., R.G.M., V.H. and I.H. wrote the paper.

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