The Effects of Diet Quality on Developmental Plasticity of Size and Flight Energetics in the Hawk Moth *Manduca sexta*

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ABSTRACT

Energy metabolism is one of the most widely measured structural backbones of physiological and life history traits, where variation in body size and morphology significantly impact flying insects' flight energetics. Both size and energy metabolism are a central feature of a species and individuals' ecology and evolution. In holometabolous insects, the larval stage of development is crucial in determining the final size of adults but also various body proportions. Generally, larger individuals will beat its wings during flight at a lower frequency. However, variations in wing proportions can affect wingbeat frequency, therefore affecting the energy that is required for flight. The flight muscles associated with the metabolic properties of flight vary with body mass and wing proportions, connecting morphological variation with muscle metabolic properties. To assess this, we experimentally induced developmental plasticity by subjecting *Manduca sexta* fifth instar larvae with varying intermittent starvation treatments: (1) controls being fed *ad libitum*; (2) our 'mild' group subject to intermittent feeding with 24 hour starvation periods between rations where animals are subject to intervals of one full day of feeding following one full day of starvation; (3) our 'severe' group subject to 48-hour starvation treatment where animals are subject to intervals of two full days of starvation followed by one full day of feeding. Our results show a significantly longer developmental period in mild and severe groups, along with significantly smaller sized larvae at wandering, pupae, and adults. Sexual dimorphism was observed in treated males showing morphological variation of body to wing proportions, along with reduced thorax size while females' morphological variation remained unchanged throughout nutrient restriction. Although males showed higher flight wingbeat frequency than females, wingbeat frequency did not change among treatments, along with showing no relationship between wingbeat frequency and body mass. Further, flight metabolic rate was not significantly different between treatment and sex. These findings suggest that individuals under nutrient-restricted developmental conditions can have a lasting impact on morphology, which may contribute to reduced flight capability, where males are more affected. Further research is needed to understand the metabolic and biomechanical thresholds that are responsible for successful flight under nutrient restricted individuals. These future investigations can provide insight into how developmental plasticity impacts flying insects in terms of energy balance and flight performance.

RESUME

Le métabolisme énergétique est l'un des piliers structurels les plus largement mesurés des traits physiologiques et de l'histoire de vie, où la variation de la taille et de la morphologie du corps a un impact significatif sur l'énergétique du vol des insectes volants . La taille et le métabolisme énergétique sont des caractéristiques centrales de l'écologie et de l'évolution d'une espèce et d'un individu. Chez les insectes holométaboles, le stade de développement larvaire est crucial pour déterminer la taille finale des adultes ainsi que les différentes proportions corporelles. Généralement, les plus gros individus battent des ailes pendant le vol à une fréquence plus basse. Cependant, les variations dans les proportions des ailes peuvent affecter la fréquence des battements d'ailes, affectant ainsi l'énergie nécessaire au vol. Les muscles du vol associés aux propriétés métaboliques du vol varient en fonction de la masse corporelle et des proportions des ailes, reliant la variation morphologique aux propriétés métaboliques musculaires. Pour évaluer cela, nous avons induit expérimentalement une plasticité développementale en soumettant des larves de cinquième stade de *Manduca sexta* à différents traitements de famine intermittents : (1) des contrôles nourris à volonté ; (2) notre groupe « doux » soumis à une alimentation intermittente avec des périodes de famine de 24 heures entre les rations, où les animaux sont soumis à des intervalles d'une journée complète d'alimentation après une journée complète de famine ; (3) notre groupe « sévère » soumis à un traitement de famine de 48 heures où les animaux sont soumis à des intervalles de deux jours complets de famine suivis d'une journée complète d'alimentation. Nos résultats montrent une période de développement significativement plus longue dans les groupes légers et sévères, ainsi que des larves de taille significativement plus petite au moment de l'errance, des pupes et des adultes. Un dimorphisme sexuel a été observé chez les mâles traités, montrant une variation morphologique des proportions du corps par rapport aux ailes, ainsi qu'une taille réduite du thorax, tandis que la variation morphologique des femelles est restée inchangée tout au long de la privation de nutriments. Bien que les mâles aient montré une fréquence de battements d'ailes en vol plus élevée que les femelles, la fréquence des battements d'ailes n'a pas changé entre les traitements, et n'a montré aucune relation entre la fréquence des battements d ailes et masse corporelle. De plus, le taux métabolique de fuite n'était pas significativement différent entre le traitement et le sexe. Ces résultats suggèrent que les individus dans des conditions de développement dépourvues de nutriments peuvent avoir un impact durable sur la morphologie, ce qui peut contribuer à réduire la capacité de vol, là où les mâles sont plus affectés. Des recherches supplémentaires sont nécessaires pour comprendre les seuils métaboliques et biomécaniques responsables du succès du vol chez des individus privés de nutriments. Ces recherches futures pourraient donner un aperçu de l’impact de la plasticité développementale sur les insectes volants en termes de bilan énergétique et de performances de vol.

1. INTRODUCTION

1.1 Overview

Energy metabolism is one of the most widely measured structural backbones of physiological and life-history traits, where animal size directly impacts metabolic rate. Both size and energy metabolism are known to be central features of a species and individuals' ecology and evolution, affecting survival, growth, immunity, predation, and reproduction. Not only does size vary across species, but environmental conditions experienced during development can induce substantial variation in adult size (Atkinson, 1994; Davidowitz et al., 2003; Kingsolver & Huey, 2008). These tremendous variations in animal size have profound impacts on most of their function (Biewener, 1991; Alexander, 2005). These changes also impact the systems and tissues responsible for energy supply and demand where variation in animal size is responsible for most of the variation in metabolic activity (West et al., 1997; Hulbert & Else, 2000; Gilooly et al., 2001). The physiological and molecular bases of developmental plasticity have gained much interest given the pivotal role they can play in organismal adaptation (Burggren, 2018). The impact of developmental plasticity on size variation can therefore be substantial and affect animal function such as locomotion that is highly dependent on animal size. To further explore the role of the impact of size on animal function, by using the hawk moth, we can experimentally induce developmental plasticity to investigate the influence of size variation on flight function.

1.2 Developmental Plasticity – Body Size and Growth

Developmental phenotypic plasticity is a major underlying cause of intraspecific size variation. It can be defined as the property of individual genotypes to produce different phenotypes due to the environmental conditions and surroundings that the organism is exposed to (Pigluicci et al., 2006). Such environmental conditions may be attributed to variables such as temperature or nutrient quality in the organism's diet. In all ectotherms ranging from vertebrates to invertebrates, temperature affects body size during development (Atkinson, 1994). Higher temperatures will show a significant reduction in body size and lower temperatures causing a larger size (Atkinson, 1994). Apart from temperature, reduced diet quality can also have its effects on body size, where nutritional stress can cause decreased growth rate and decreased final body mass (Saastamoinen et al., 2010; Nijhout and Grunert, 2010). Intermittent starvation has also been shown to significantly affect body size as well as affecting morphology, where allocation of resources changes between body parts (Nijhout & Emlen, 1998; Mangel & Munch, 2005; Nijhout et al., 2006).

Body size is ultimately determined by genetic and environmental factors. In a theoretically optimal and constant environment, insects will grow to be a characteristic species-specific size (Nijhout and Grunert, 2010). However, a common pattern in many insects shows that typical consequences of poor developmental conditions include increased development time (Esperk et al., 2007; Gibbs et al., 2012) and small body size (Blanckenhorn, 1999; Boggs and Freeman, 2005: Gibbs et al., 2012; Nylin and Gotthard, 1998), which ultimately leads to a negative correlation between development time and body size at maturity. In other words, it is ultimately the rate and duration of growth during ectothermic larval development that determines adult body size of insects (Mirth and Shingleton, 2012). Further, these poor

developmental conditions can also influence the distribution of growth between body parts causing morphological differences at the adult stages of development (Mangel & Munch, 2005; Nijhout et al., 2006; Hector & Nakagawa, 2012). For example, a study conducted on the tropical butterfly, *Bicyclus anynana,* under intermittent-starvation conditions experience a prolonged average developmental time from 30 to 33 days, with the final larval instar extending from an average of 6 to 9 days and with the food-stressed larvae's final mass being significantly smaller than the control (Saastamoinen et al., 2010). This decrease in body mass was also not evenly distributed between body parts, where proportionally more weight was lost from the abdomen than from the thorax (Saastamoinen et al., 2010). Similarly, a semi-starvation study done on *Speyeria mormonia* resulted in semi-starved individuals having significantly smaller body mass and forewing length than individuals fed ad libitum, where females body mass decreased more rapidly with wing length under semi-starved conditions (Boggs and Freeman, 2005). These effects of developmental plasticity not only reduce overall body size, but also disrupt the morphology and allometric relationships between wing area and body mass, showing how environmental conditions during development completely alter the individual's morphology.

1.3 Regulation of growth in fifth instar *Manduca sexta*

In holometabolous insects, the larval stage of development is crucial in determining the final size of adults but also various body proportions. In the fifth and final larval instar of *M. sexta*, the species grows from an average mass of approximately 1.2g to about 11g, meaning ~90% of the final mass of the larva is gained during the final instar (Nijhout et al., 2006). In insects, a key mechanism behind important developmental events involves the release of the hormone ecdysone, which initiates the cessation of feeding in the fifth instar and triggers the

metamorphic molt (Nijhout et al., 2006). During the fourth day of the fifth larval instar, *M. sexta* larvae developing under 'normal' conditions reach a specific mass, called the critical weight, which triggers the first secretion of ecdysone called the 'commitment peak', causing the cessation of feeding (Fig 1. Zitnan and Adams, 2012). This cessation of feeding causes the body to stop growing; therefore, the timing at which the release of the first ecdysone peak occurs allows ecdysone to regulate growth and size of *M. sexta* (Grunert et al., 2015). The second larger ecdysone peak, or 'prepupal peak' following the wandering stage occurs on the seventh day of the fifth larval instar and triggers the first metamorphic molt (Walter et al., 1980).

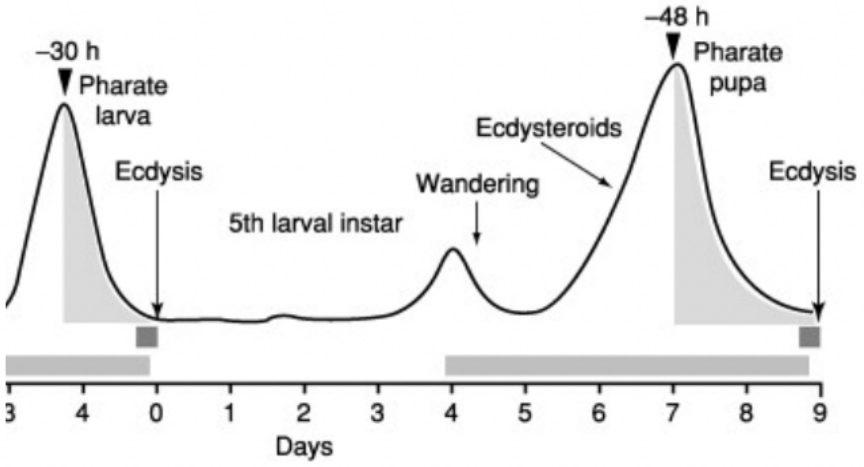

Figure 1. Visual timeline of the fifth larval instar depicting ecdysone hormone peaks of *M. sexta* from Zitnan and Adams (2012). This diagram outlines the two ecdysone peaks showing the first peak (commitment peak) on the fourth day and the second peak (prepupal peak) on the seventh day of the fifth larval instar.

The quantity and quality of nutritional resources during larval development in insects alters the timing of ecdysone hormone peaks during the last larval instar, causing developmental plasticity (Nijhout, 2003). In *M. sexta*, the critical weight that is needed for release of ecdysone decreases with decreasing diet quality (Davidowitz et al., 2003). This decrease in critical weight

accounts for a large proportion of phenotypic variation in body size of *M. sexta* (Davidowitz et al., 2003), where decreased diet quantity and quality results in smaller individuals (Nijhout and Grunert, 2010). Recent publications have outlined the allometric relationship between wing area and body size changes in relation to variation in nutrition (Nijhout and Callier, 2015). As we know that nutrition plays a role in the stimulation of ecdysone release (Grunert et al., 2015), when larvae are starved between their minimal viable weight and critical weight, there is an intense increase in secretions of ecdysone resulting in early cessation of larval growth, causing metamorphosis to occur earlier compared to non-starved controls (Nijhout and Grunert, 2010). However, the growth of wings occurs after the larva have stopped feeding when the body has stopped growing (Nijhout and Callier, 2015). Thus, when larvae are starved after the critical weight has been reached, there is an arrest in larval body growth and wing disks will continue to grow (Tobler and Nijhout, 2010). Therefore, the food-deprived animals would be smaller in body size, but their wings would be disproportionally large (Tobler and Nijhout, 2010).

1.4 Allometry of Body and Wing Proportions

Specifically, allometry refers to the change of one part of the body in relation to changes in the overall body mass of the individual or species (Teissier and Huxley, 1936). The law of relative growth or law of simple allometry is explained by the equation $y = ax^b$ (Teissier and Huxley, 1936). If we were measuring the allometric relationship between one specific part of the body, such as wing area, and body mass, '*y*' represents the body part, in this case wing area, and '*x*' represents the whole body, being body mass (Teissier and Huxley, 1936). This relationship can be made linear using a log transformation, changing the equation to *log(y) = log(b) + alog(x)*, where *'b'* represents the type of allometric relationship (Shingleton et al.,

2007). Therefore, the value of '*b*' represents the growth ratio between two body parts (Teissier and Huxley, 1936). In our case, '*b*' shows the growth ratio between wing area in relation to body mass.

When individuals within a species vary in body masses, but the proportions between wing area and body mass remain unchanged, we see an isometric relationship where wing area increases proportionally to body mass where $b = 2/3$ (Thompson, 1917). Broad comparisons over 160 species across insect orders follow this general formula (Byrne et al., 1988; Darveau, 2024). Individuals may have disproportionate growth rates between wing area and body mass, where *b* could be > or < 2/3 showing hyper allometry or hypo allometry, respectively. For example, we see different species of bees showing both hyper and hypo allometry, where stingless bees show the wing area scaling hypo allometrically where $b = 0.57$ (Duell et al, 2022), whereas we see hyper allometry in orchid bee species with an exponent of 0.87 (Darveau et al., 2005b; Rodriguez et al., 2015). These relationships are due to the function of the individual, where having a hyper allometric relationship is common in species that need to generate extra lift during flight or if carrying heavy loads, such as pollen for bumblebees, while hypo allometry occurs in individuals that perform long-distance flight where efficiency is prioritized over maneuverability (Dudley, 2000).

Moreover, such changes in proportion of wing size affect the calculated ratio of wing loading. Wing loading refers to the pressure that is exerted by the wings on surrounding air and is calculated by using the formula: p_w = *wet weight/wing area* (Gilchrist and Huey, 2004). Changes in wing area directly impact wing loading, having a large influence on the individual's capacity for flight success along with the energetic demands needed for hovering flight (Dudley,

2000; Gilchrist and Huey, 2004). For instance, smaller wings cause an increase in wing loading, leading to a higher energetic demand for flight creating difficulty in maintaining efficient flight during hovering (Dudley, 2000). A poor nutrient diet has been previously shown to result in hyper allometry, where having limited nutritional resources causes growth to be diverted toward essential structures like wings, ultimately resulting in disproportionally larger wing area relative to body mass (Nijhout & Callier, 2015; Tobler & Nijhout, 2010). These changes will further have an impact on wing loading, ultimately affecting flight energetics, showing how the effects of body size and growth play major roles in the function of the individual due to developmental plasticity.

If *M. sexta* larvae are fed diet that is poor in nutrient quality, the larval growth rate is slow, which is also accompanied by a slow growth rate of the imaginal disks in the wings (Nijhout et al., 2007). However, once the larvae enter the wandering stage and stop feeding, the control of the imaginal disk growth rate changes from being nutrient-dependent to nutrient-independent (Nijhout et al., 2007). This means that wing growth is completely independent of the initiation of the commitment peak that is accompanied by the cessation of feeding, while imaginal disks will continue an uninterrupted growth period (Nijhout et al., 2007). Further, intermittent starvation has been shown to extend the growth period in *M. sexta*, allowing for more time for imaginal disks to continue growing after the critical weight has been reached (Nijhout et al., 2007; Saastamoinen et al., 2010). These larvae will follow the same trend, showing $b > 2/3$ as these larvae will take longer to reach maturity compared to those on a nutrient-rich diet (Mangel & Munch, 2005), following the same general trend where organisms under intermittent starvation often have an exponent b value of > 2/3, due to extended

developmental periods allowing for prolonged wing growth (Boggs & Freeman, 2005). These developmental effects, causing prolonged wing growth under nutrient-stressed conditions, can significantly impact the way the individual functions, particularly in terms of flight.

1.5 Outcome of Developmental Plasticity's Effects on Flight Energetics

The energetic cost associated with hovering flight is represented by the flight metabolic rate, where species and individual variation in flight metabolic rate is associated to variation in body mass and wing proportions. Casey (1976) was able to explore this relationship by measuring the energetic cost of hovering flight in five species of sphinx moths, showing that metabolic rate during hovering flight increases as the species body mass increases (Casey, 1976). Moreover, two key functions in hovering flight can also explain variations in metabolic rate: wing loading and wingbeat frequency (Casey, 1976). As discussed previously, wing loading is determined by the ratio of body mass to wing area (Gilchrist and Huey, 2004) whereas wingbeat frequency is a central factor affecting the production of force and generating lift (Deora et al., 2017), where for a given size, larger wings will beat at a lower frequency (Casey, 1976). The frequency at which an insect beats its wings directly impacts its metabolic rate during flight (Darveau, 2024).

The effect of developmental plasticity on adult *M. sexta* metabolic rate is not well understood due to most of the published literature being dated or focuses on their larval form. However, work conducted on a lineage of orchid bee species shows that metabolic rate during hovering flight increases allometrically as body mass increases (Darveau et al., 2005). When expressed on a mass-specific basis, this study showed that flight metabolic rate decreses with increasing species size, which paralleled the decline in wingbeat frequency with increasing size.

Further, an increase in wing loading is accompanied by an increase in wingbeat frequency, resulting in an increase in metabolic rate within the orchid bee species (Darveau et al., 2005). This ultimately depicts that wings that are proportionally smaller to body size require a higher wingbeat frequency, thus increasing metabolic rate. Although this is also observed among individuals within a bumblebee species (Skandalis and Darveau, 2012; Darveau et al., 2014; Billardon & Darveau, 2019), it provides a link to inter and intraspecific variation.

1.6 Metabolism in *Manduca sexta*

Regarding *M. sexta*, metabolism can be analyzed on a cellular level by identifying key metabolic enzymes and metabolites. To identify which metabolic enzymes and metabolites to analyze, we must first understand which metabolic pathways predominantly fuel flight in *M. sexta*. One of the main fuel sources involved with flight metabolism in *M. sexta* is lipids (Ziegler and Schulz, 1986). During flight, *M. sexta's* adipokinetic hormones mobilize lipids and influence the uptake of lipids by the flight muscles via transportation (Ziegler and Schulz, 1986). After 30 minutes of flight, adult *M. sexta* haemolymph lipid levels rapidly decreased by a concentration of 20 mg x ml^{-1}, and then maintained a steady state for the next 60 minutes (Ziegler and Shulz, 1986). Therefore, with longer flight duration, increasing amounts of lipids are mobilized from fat and after 30 minutes of flight, a new steady state equilibrium is reached between use in the flight muscles and mobilization from the fat body (Ziegler and Schulz, 1986). The large reduction followed by a steady state of haemolymph lipid levels indicate that fat bodies are an important fuel source used by flight muscles (Ziegler and Schulz, 1986).

M. sexta also use carbohydrates to fuel flight, also controlled by adipokinetic hormone (Van Marrewijk et al., 1983). During the first 5 minutes of flight, haemolymph sugar levels decreased

rapidly by about 8 mg x ml^{-1}, followed by little change for another 85 minutes of flight (Ziegler and Schulz, 1986). Further, glycogen phosphorylase is an enzyme involved in carbohydrate metabolism that degrades and mobilizes carbohydrates as a fuel source for flight (Ziegler and Schulz, 1986). About 7% of glycogen phosphorylase is in its active form before flight of *M. sexta*, however after 60 minutes of flight, 35% was in its active form (Ziegler and Schulz, 1986). Particularly, analysis of energy budgets shows that in *M. sexta*, glycogen oxidation supplies approximately 39% of the energy that is needed for the warm-up phase of flight (Joos, 1987). From this, we can estimate that about 40% of the metabolic energy requirements needed for flight in *M.* sexta comes from carbohydrates, while the other 60% is covered by lipids.

To assess the effects of developmental plasticity on metabolism in *M. sexta*, we can study metabolism on a cellular level through enzyme assays. In regards to this study, the mass specific metabolic rate of individuals will increase with decreasing body mass (Darveau et al., 2005), meaning that the metabolic capacity in small individuals is going to be more active than that of a larger individual. The common enzymes that are involved in the metabolism behind fuel utilization in invertebrates are trehalase (TR), hexokinase (HK), citrate synthase (CS) and β-hydroxyacyl CoA-dehydrogenase (Darveau et al., 2014, Rondot & Darveau 2024) as these enzymes are rate controlling steps in beta-oxidation and mitochondrial Krebs cycle that control the flow of the pathway.

2. OBJECTIVES AND STUDY QUESTIONS

In the present study, our primary interest is to assess the effects of size variation on animal function through inducing developmental plasticity by manipulating nutrient intake during development in *M. sexta*. To address our main objective, this study aims to evaluate how nutrient restriction during development impacts size variation, and how the resulting variation in size affects morphological and physiological traits that are essential to flight in *M. sexta*. Through this study, our main questions we are looking to address are: (1) how does developmental plasticity through nutrient restriction affect size and morphology in *M. sexta*? (2) What are the effects of size and morphological variation on the physiological mechanisms responsible for flight, such as wingbeat frequency, flight metabolic rate, and flight success? And (3) does variation in body size and morphological traits affect flight muscle metabolic phenotype? Given that smaller sized species or individuals have higher wingbeat frequency, and mass-specific flight metabolic rate due to smaller wings, I hypothesize that nutrient restriction during development will cause smaller sizes accompanied by increased wing frequency and mass-independent flight metabolic rate. Furthermore, additional changes in wing proportions will increase or decrease this effect with disproportionately smaller and larger wings, respectively. I also hypothesize that flight metabolic phenotype will change as a function of flight metabolic rate as a plastic compensatory response.

3. METHODS

3.1 Animal Rearing

The hawk moth *M. sexta* were received as eggs in several cohorts of 250-500 eggs from Carolina Biological Supply company and were developed under common conditions outlined by the Carolina Biological Supply care guide until the fifth instar. Animals were maintained under common larval rearing conditions as described by Kingsolver, 2007, in a room maintained at 25^{o}C with a 16h light:8h dark photocycle. Diet consisting of commercial hornworm chow was obtained through Super Cricket Farms (Saskatoon, Saskatchewan) and was rehydrated following the company's procedure. The selected treatments were used to prolong the larval developmental period to induce developmental plasticity by altering body mass and wing morphology (Bauerfeind and Fischer, 2009; Nijhout et al., 2007; Miner et al., 2000). Following the shedding of the head capsule in the fifth instar, larvae were randomly assigned to one of three treatment groups and transferred to 4 oz specimen containers with punctured lids: (1) no dietary stress feeding ad libitum referred to as the control group; (2) intermittent feeding with 24 hour starvation periods between rations where animals are subject to intervals of one full day of feeding following one full day of starvation (referred to as 'mild'); (3) 48-hour starvation treatment where animals are subject to intervals of two full days of starvation followed by one full day of feeding (referred to as 'severe') (Bauerfeind and Fischer, 2009). Each individual larvae entering the fifth instar started their treatment with a full day of feeding. Larvae were monitored daily and when reaching the wandering stage indicative of the initiation of metamorphosis, they were transferred from food vials to conical vials filled with hamster bedding and wrapped in tin foil, sealed with a foam stopper to eliminate any light entering the

vial to simulate pupation conditions. The resulting pupae were observed under a dissection microscope to identify their sex organ, then separated into cages according to gender. The cages contained a 3D printed model flower with a glass nectar feeding tube attached.

3.2 Morphological Measurements

Individual larvae growth and survival were tracked and monitored until the wandering stage. Larvae were measured for total mass once entering the wandering stage that ends the larval cycle. After flight experiments, adults were anesthetized using nitrogen and placed in a –80^{o}C freezer. Body mass was measured followed by dissection of body parts (head, thorax, abdomen, legs, wings) then weighed to the nearest 0.1 mg using an analytical balance (Metler-toledo). Wing area measurements (cm^2) were made using Axios Vision software by taking images with a dissection microscope (Discovery V8, Zeiss, Oberkochen, Germany). Individuals with extensive wing wear, underdeveloped, or uninflated wings were not included.

3.3 Respirometry and wingbeat frequency measurements

Rate of CO_2 production was measured using a FoxBox flow-through field respirometry system (Sable Systems International, Las Vegas, NV, USA). The system was connected to a laptop computer where data was analyzed using Expedata (Sable Systems International). The CO_2 detector was calibrated daily. The baseline CO_2 level was measured before and after each measurement. Air was drawn into a flight chamber through PharmMed BPT tubing (Fisher Scientific, Pittsburgh, PA, USA) at a rate of approximately 1700 ml/min. The quality of flight in the chamber was ranked according to the following criteria on a scale of 0-2: (0) the individual failed to beat its wings upon stimulation; (1) the individual failed to generate any lift upon

stimulation of flight; (2) the individual successfully performed hovering flight or was able to reach a plateau in CO2 production. Only flight values of 1 and 2 were included in analyses. Stimulation of flight involved visual stimulation through UV light, olfactory stimulation through attaching tobacco plant leaves to the top of the flight chamber, or physical stimulation through applying slight pressure to the abdomen by forceps (Claassen and Kammer, 1985). Hovering flight quality was assessed based on our qualitative ranking as pervious work using similar flight assay shows a connection between hovering flight energetics and morphological parameters among individuals (Skandalis & Darveau, 2012).

Wingbeat frequency measurements are performed through a high-speed camera (PROMON U1000 Mono, AOS Technologies, Switzerland) capturing the flight video of each individual during hovering flight at 112 frames per second. The camera is linked to a computer where we use video analysis (AOS Imaging Studio v4.6.0.5, AOS Technologies, Switzerland) to analyze the flight in slow motion and track the wingbeat frequency in Hz using the software Tracker (Open Source Physics).

3.4 Enzyme and protein assays

The activity of selected metabolic enzymes was measured on the thorax stored at –80^{o}C. Thoracic homogenates were prepared as described in Darveau et al. (2014) and minced with scissors using nine volumes of ice-cold homogenization buffer containing 25 mmol l^{-1} tris-potassium phosphate, pH 7.8 at 4^{o}C, 2 mmol l^{-1} ethylene diamine tetraacetic acid, 5 mmol l^{-1} dithiothreitol and 0.5% (v/v) Triton X-100. The minced thoraces were homogenized three times in 10 second intervals at 7,000 rpm with 30 second cooling periods using the Polytron PT 1300

D (Kinematica) homogenizer. The homogenates were then sonicated for 5 seconds three times with 30 second cooling periods using the Vibra-Cell™ Ultrasonic Processor at 20% amplitude. The homogenates were centrifuged at 2400 *g* at 4°C using a Sorvall Legend Micro 21R Centrifuge. The homogenates were further diluted prior to measuring the activities of four enzymes involved in carbohydrate oxidation, hexokinase (HK), trehalase (TRE), lipid metabolism, 3-hydroxyacl CoA dehydrogenase (HOAD) and the mitochondrial enzyme citrate synthase (CS).

Enzyme activities were measured using a Synergy 2 Multi-Detection Microplate Reader (Biotek Instruments, Winooski, VT, USA) adjusted to maintain a temperature of 37°C. HK and TRE reactions were monitored using the rate of appearance of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm using a millimolar extinction coefficient (ϵ) of 6.22. The CS reaction was monitored using the rate of appearance of 5,5' dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm using ϵ = 13.6. The HOAD reaction was monitored using the rate of disappearance of nicotinamide adenine dinucleotide (NADH) at 340 nm using ϵ = 6.22. Enzyme activities are expressed in U g-1 thorax, where U = µmol min-1.

Enzyme assay conditions are as follows: HK: 100 mmol l^{-1} Tris-imidazole, pH 8.1 at 25°C, 100 mmol l^{-1} KCl, 10 mmol l^{-1} MgCl2, 5 mmol l^{-1} D-glucose (omitted for control), 1 mmol l^{-1} $NADP^+$, 5 mmol l^{-1} ATP, and 1.25 U ml^{-1} glucose-6-phosphate dehydrogenase; TR: 100 mmol l^{-1} potassium phosphate, pH 6.6 at 25°C, 1.1 mmol l^{-1} MgCl2, 0.75 mmol l^{-1} NADP, 1.1 mmol l^{-1} ATP, 10 mmol l^{-1} trehalose, 1.25 U hexokinase and 1.25 U glucose-6-phosphate dehydrogenase; CS: 50 mmol l^{-1} Tris-HCl, pH 7.4 at 37°C, 0.5 mmol l^{-1} oxaloacetate (omitted for control), 0.3 mmol l^{-1} acetyl-CoA, 0.1 mmol l^{-1} dithiobis-2-nitrobenzoic acid; HOAD: 50 mmol l^{-1} imidazole-HCl, pH 7.4 at

25°C, 0.1 mmol l^{-1} acetoacetyl CoA, 0.16 mmol l^{-1} nicotinamide adenine dinucleotide, 1 mmol l^{-1} ethylenediaminetetraacetic acid, 5 mmol l^{-1} dithiothrietol.

Protein assays were performed using the bicinchoninic acid method (Sigma Chemicals). Individual assays were performed using the BSA protein standard provided within the kit. Enzyme activities were analyzed when expressed both per gram of tissue and per milligram of protein.

3.5 Statistical analysis

Statistical analyses were conducted using the software SYSTAT 13.0 (Chicago, IL, USA) and Graphpad Prism 10.2.3 (Boston, MA, USA). Larval mass and larval duration of growth were analyzed using an ordinary one-way ANOVA to find differences among treatments as larvae are unable to be sexed. Pupal and adult mass were analyzed using ordinary two-way ANOVA's followed by a Tukey's multiple comparisons test when analyzing differences in sex and treatment. Since interindividual variation is strongly determined by variation in body mass, we therefore examined all relationships with body mass using log-transformed data. Dependent variables (wing area, thorax mass, flight metabolic rate, wingbeat frequency, HK activity, TRE activity, HOAD activity, CS activity, and protein content) were analyzed with analyses of covariance (ANCOVA) with sex and treatment as factors and body mass as covariate. The final model presented in the results was simplified by first removing the non-significant interaction term. Cohorts were also in the model to account for differences in batches but not reported. Following ANCOVA analyses where a sex or treatment interaction was detected, males and females along with treatment groups were analyzed separately to identify the nature of the

difference. To assess the differences in relationships of main effects with body mass, we performed regressions on body mass, wing area, thorax mass, flight metabolic rate, wingbeat frequency, and enzyme activity (HK, TRE, HOAD, and CS) in order to obtain the scaling exponent *b* from the equation $Y=ax^b$. We performed a pearson chi-square analysis to examine the relationship between flight success and treatment. Average flight grade was calculated between treatment groups along with flight success proportions (%).

4.0 RESULTS

4.1 Diet effects on larval and pupal development

Larval mass at wandering decreased significantly under intermittent starvation conditions from 6.763 g (± 0.129) to 4.664 g (± 0.129) in the mild group and 3.877 g (± 0.142) in the severe group (Fig. 2A). Similarly, the duration of growth is significantly longer in nutrient restricted conditions, where controls reach wandering at 4.661 (± 0.070) days and mild individuals at 6.971 (± 0.192) days and severe individuals at 7.327 (± 0.217) days (Fig. 2B, Table 3). Pupae shows similar results where size decreased from 3.922 g (± 0.058) to 2.734 g (± 0.071) in the mild group and 2.283 g (± 0.075) in the severe group. Pupae mass ranged from 5.100 g to 0.9570 g where a two-way ANOVA shows pupal mass being strongly dependent on sex and treatment (Fig 3. treatment: $F_{2,216} = 161.9$, $P < 0.001$; sex: $F_{1,216} = 11.59$, $P < 0.001$; treatment x sex: $F_{2,216} = 0.859$, $P = 0.425$). Female pupae are larger than males under nutrient-rich diet ($P = 0.016$), however the effect of sexual dimorphism is lost under nutrient restricted conditions (Fig. 3, Table 3).

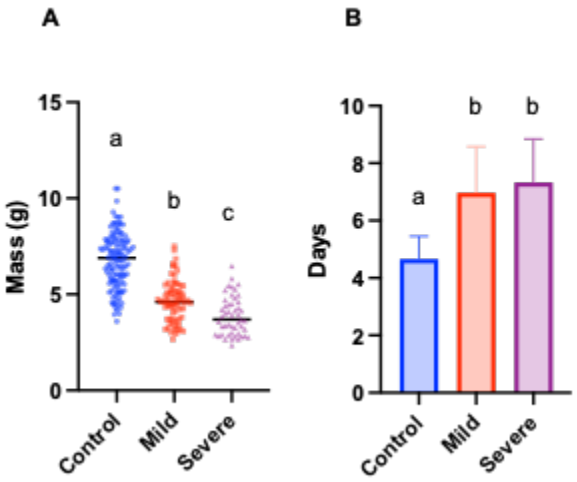

Figure 2: Poor nutritional conditions cause smaller size and a longer development period. Comparison of means (±SEM) of (A) body mass (g) at wandering and (B) duration of growth (days) among treatment groups (control: n = 124, mild: n = 70, severe: n = 50). Differences in means were determined using one-way ANOVAs followed by Tukey's multiple comparisons test finding differences between groups. Groups that do not share letters are statistically different ($P < 0.05$).

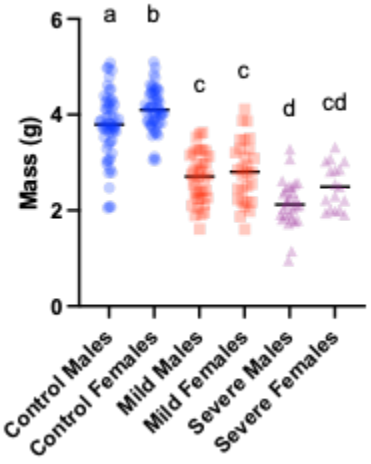

Figure 3: Poor nutritional conditions cause smaller pupae where males and females do not differ in size. Comparison of means (±SEM) of pupal mass between treatment groups and sexes (See Results and Table 3 for statistics). Differences in means were determined using a two-way ANOVA followed by Tukey's multiple comparisons test finding differences between groups. Groups that do not share letters are statistically different ($P < 0.05$).

4.2 Interindividual variation, sexual dimorphism, and morphological differences in adults

Adult *M. sexta* varied in body mass from 0.455 g to 2.18 g where a two-way ANOVA shows body mass being strongly dependent on treatment and sex where females are larger than males in the control group, but are not different in nutrient restricted conditions (Fig. 4; treatment: $F_{2,129}$: 62.80, $P < 0.001$; sex: $F_{1,129}$: 13.11, $P < 0.001$; treatment x sex: $F_{2,129}$: 0.968, $P = 0.382$). Wing area measurements varied from 6.828 cm^2 to 2.553 cm^2 where an ANCOVA shows wing area being strongly dependent on body mass and differs between sexes where females have larger wings for a given size (Fig. 5, Table 1; mass: $F_{1,121} = 88.553$, $P < 0.001$; treatment: $F_{2,121} = 4.372$, $P = 0.015$; sex: $F_{1,121} = 17.515$, $P < 0.001$; treatment x mass: $F_{2,121} = 6.530$, $P = 0.002$). The effect of body mass on wing area differs in males, where wing area in mild males increase with body mass at a steeper slope than male controls (Fig. 5A, Table 2, scaling exponent *b* values of 0.586 and 0.239, respectively; $P = 0.003$), while the slopes do not differ in females (Fig 5B, Table 2). To further asses the significant interaction, regression analyses were performed on each treatment group within both sexes, showing the relationship between body mass and wing area in severe males is not significant ($P = 0.631$, $r^2 = 0.027$) where sample size was also the lowest among our groups ($n = 11$).

Wing loading ranged from 0.055 g cm^{-2} to 0.206 g cm^{-2} per animal where an ANCOVA shows wing loading being strongly dependent on treatment and sex (Fig. 5C and D; mass: $F_{1,104} = 0.005$, $P = 0.944$; treatment: $F_{2,104} = 4.181$, $P = 0.018$; sex: $F_{1,104} = 16.891$, $P < 0.001$). The effect of body mass on wing loading was significant for all treatments and both sexes (Fig. 5C and D, Table 1), however the slopes do not differ between groups in males and females. Males and females under mild and severe conditions had higher intercept than controls (males: Fig. 5C and Table 1, scaling coefficient *a* values of -1.012, -0.9330, and –0.898 for the control, mild and

severe groups, $P < 0.001$; females: Fig 5D and Table 1, scaling coefficient a values of –1.033, -1.011, and –0.974 for the control, mild and severe groups, $P = 0.035$).

Thorax size ranged from 0.534 g to 0.154 g per animal where an ANCOVA shows a thorax mass being strongly dependent on body mass and treatment with a significant interaction term where males under nutrient restricted conditions have a smaller thorax for a given size (Fig. 5E and F; mass: $F_{1,126} = 155.436$, $P < 0.001$; treatment: $F_{2,126} = 5.375$, $P = 0.006$; sex: $F_{1,126} = 0.565$, $P = 0.453$; sex x treatment: $F_{2,126} = 4.508$, $P = 0.013$). The effect of body mass on thorax mass was significant for all treatments and both sexes (Fig 5C and D, Table 1) while males in the mild and severe groups had lower intercepts than male controls (Fig 5C, Table 1, scaling coefficient a values of -0.511, -0.493, and –0.431, respectively; $P < 0.001$).

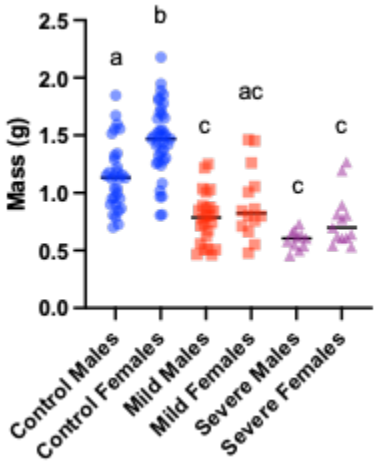

Figure 4: Adult body size is different among treatments where males are typically smaller than females. Comparison of means (±SEM) of adult mass (g) between treatment groups sexes (see Results and Table 2 for statistics) Differences in means were determined using two-way ANOVAs followed by Tukey's multiple comparisons test finding significance between groups. Groups that do not share letters are statistically different ($P < 0.05$).

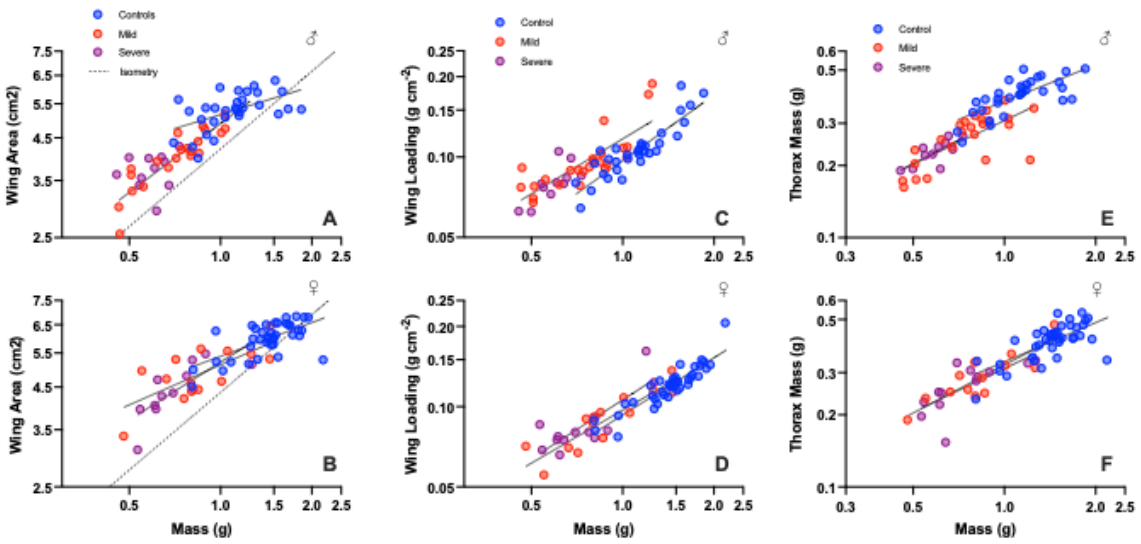

Figure 5: Allometry of morphological variation in males and females and across treatments. Relationship between body mass and wing area (A and B), wing loading (C and D) and thorax mass (E and F) in males and females. Wing area is strongly dependent on body mass apart from the severe male group while males and females have higher wing loading under nutrient restricted conditions than controls. Males under nutrient restricted conditions have smaller thoraces for a given size (see Results and Table 1 for details). Dashed line in A and B represents isometry, where b = 2/3.

4.3 Flight energetics and metabolic enzyme activity

Wingbeat frequency measurements ranged from 25 to 34 Hz per animal. Wingbeat frequency was different between sexes but was not dependent on body mass and did not differ between nutritional treatment (mass: $F_{1,80}$ = 0.036, P = 0.851; treatment: $F_{2,80}$ = 0.389, P =

0.679; sex: $F_{1,80}$ = 7.839, P = 0.006). Males calculated mean WBF is 29.75(±0.35) Hz while females calculated mean WBF is 28.56(±0.27) Hz.

Flight metabolic rate measurements ranged from 2.910 to 44.60 ml CO2 h^{-1} per animal. Flight metabolic rate was significantly affected by body mass but was not dependent on sex or differ between nutritional treatment. Flight metabolic rate was also not dependent on flight quality (mass: $F_{1,104}$ = 16.874, P < 0.001; treatment: $F_{2,104}$ = 0.678, P = 0.510; sex: $F_{1,104}$ = 0.622, P = 0.432; flight quality: $F_{2,104}$ = 0.347, P = 0.707). A separate ANCOVA analysis including wingbeat frequency as a covariate shows flight metabolic rate being dependent on both mass and wingbeat frequency (not shown: WBF: $F_{1, 79}$ = 11.612, P = 0.001; mass: $F_{1, 79}$: 18.294, P < 0.001; treatment: $F_{2, 79}$ = 0.251, P = 0.778; sex: $F_{1, 79}$ = 0.632, P = 0.429).

Treatment was strongly associated with flight success ($x^2_{2, 122}$ = 12.113, P = 0.002; Fig. 7). Looking at proportions of rankings, we see the proportions changed with treatment where mild and severe groups had a greater proportion of poor quality flight. Calculated averages for flight grades were as follows: control males: 1.379(0.191), n = 32; control females: 1.837(0.075), n = 39; mild males: 1.068(0.068), n = 24; mild females: 1.371(0.361), n = 14, severe males: 1.375(0.239), n = 11; severe females: 1.625(0.191), n = 13. Calculated flight success (%) were as follows: control males: hovering flight = 59.38%, short flight = 31.25%, no flight = 9.37%; control females: hovering flight = 79.49%, short flight = 20.51%, no flight = N/A; mild males: hovering flight = 29.17%, short flight = 54.17%, no flight = 16.67%; mild females: hovering flight = 42.86%, short flight = 42.86%, no flight = 14.28%; severe males: hovering flight = 36.36%, short flight = 45.45%, no flight = 18.18%; severe females: hovering flight = 46.15%, short flight = 53.85%, no flight = N/A.

HK activity ranged from 3.177 to 36.15 U g^{-1} thorax per animal. HK activity was different between sexes but was body mass and treatment invariant (mass: $F_{1,101}$ = 0.038, P = 0.846; treatment: $F_{2,101}$ = 1.293, P = 0.279; sex: $F_{1,101}$ = 7.147, P = 0.009). Males had higher activity with a calculated mean HK activity of 18.42(0.82) U g^{-1} thorax while females calculated mean HK activity was 15.36(1.215) U g^{-1} thorax. For the TRE, HOAD, and CS assays, there was no significance between enzyme activity and treatment, mass, or sex, apart from a significant relationship between body mass and enzyme activity in mild females for TRE and mild males for HOAD (Fig 8D and E: scaling exponent b values of 0.589 and –1.638, respectively. P = 0.025 for both, see Table 1 for details). The muscle protein content did not differ with nutritional quality and the enzyme activity expressed per unit protein yielded the same results, showing no effect of treatment.

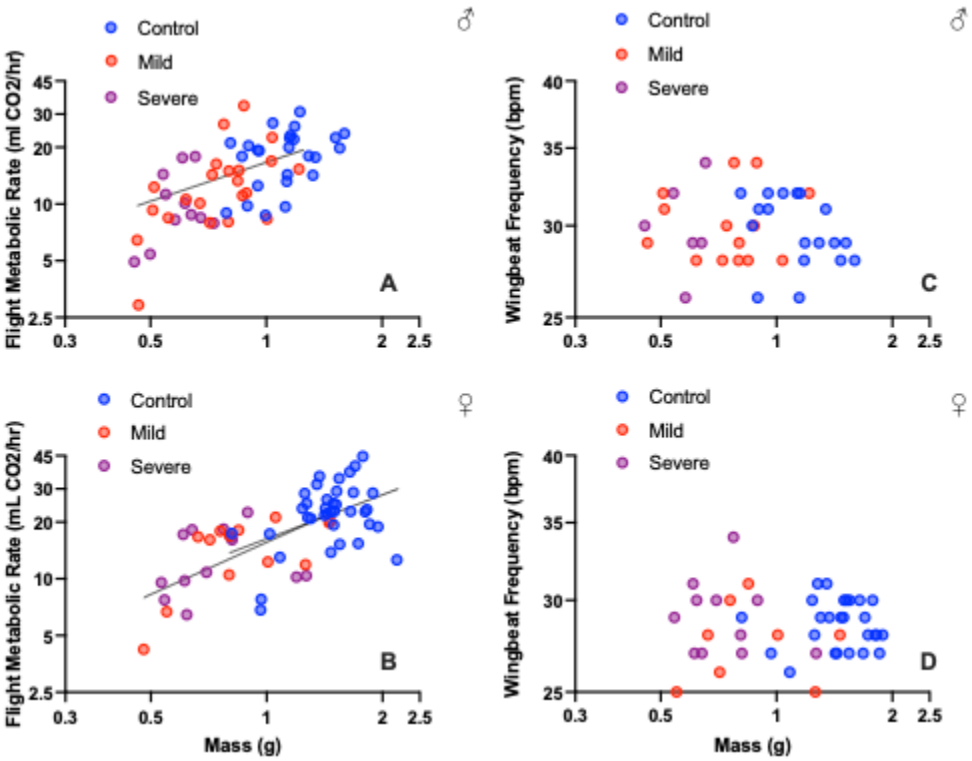

Figure 6. Allometry of flight energetics variation in both males and females among each treatment group. Relationship between body mass and (A and B) flight metabolic rate and (C and D) wingbeat frequency during hovering flight in both males (A and C) and females (B and D) among each treatment group in *M. sexta*. Significant effects were found in the control group for females and 24h group for flight metabolic rate in both sexes (see Results and Table 2 for details).

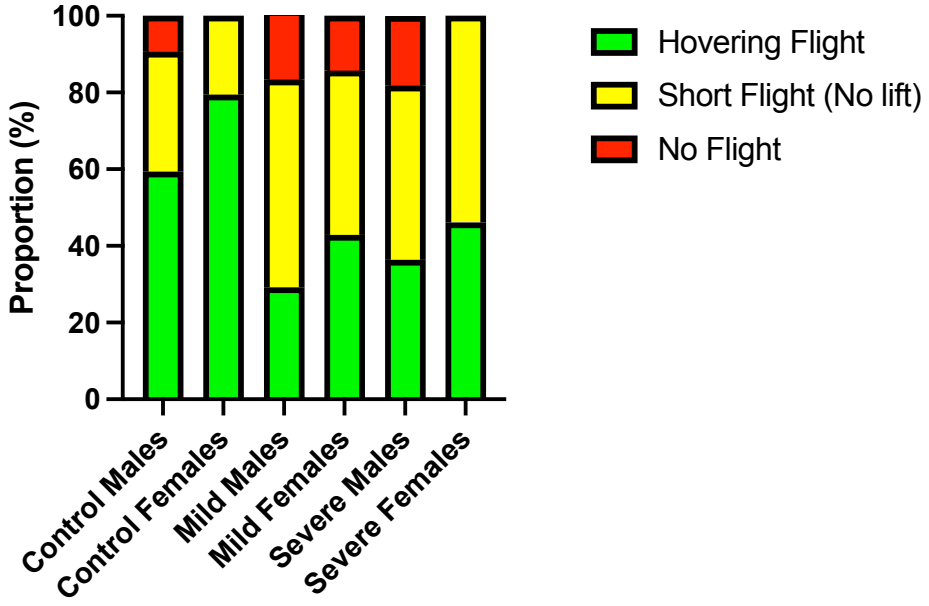

Figure 7. Variation in flight success between treatment groups and sexes. Values are shown as flight proportion based on flight grade of each individual between sexes among treatment groups. Hovering Flight = flight grade of 2; Short Flight = flight grade of 1; No Flight = flight grade of 0. Treatment is shown to be strongly associated with flight success (see Results).

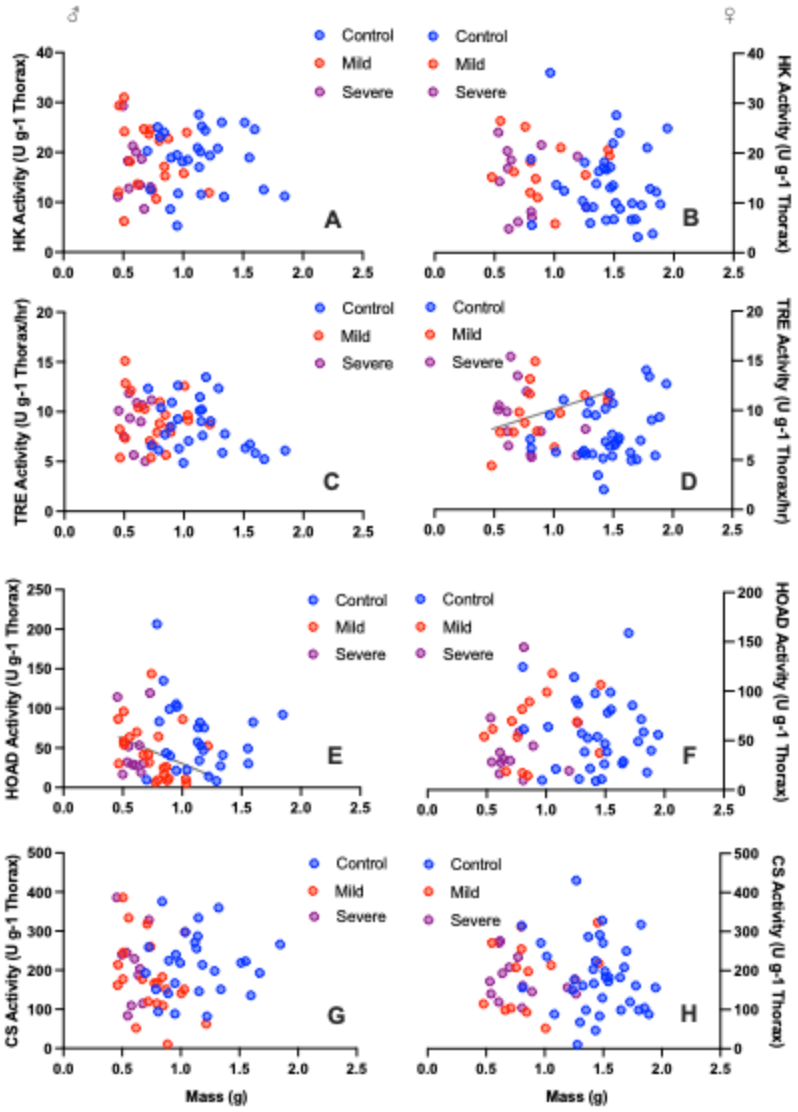

Figure 8. Flight metabolic enzyme activity variation in males and females among each treatment group. Relationship between body mass and (A and B) hexokinase, (C and D) trehalase, (E and F) 3-hydroxyacl CoA dehydrogenase, and (G and H) citrate synthase in both males (A, C, E, G) and females (B, D, F, H) among each treatment group in *M. sexta.* Significant effects were found for TRE in the mild female group along with HOAD mild males (See Results and Table 1 for details).

Table 1. Relationship between body mass, morphological parameters, flight energetics measurements, and enzyme activity for both sexes and each treatment group in the hawk moth (*M. sexta*).

Variable	Sex	Treatment	*a*	*b*	r^2	*P*
Wing Area (cm2)	Male	Control	0.713(0.008)	0.239(0.070)	0.290	0.002
		Mild	0.690(0.012)	0.586(0.067)	0.803	<0.001
		Severe	0.597(0.060)	0.126(0.253)	0.027	0.631
	Female	Control	0.732(0.010)	0.287(0.055)	0.423	<0.001
		24h	0.710(0.015)	0.340(0.097)	0.507	0.004
		48h	0.717(0.023)	0.492(0.129)	0.593	0.003
Thorax Mass (g)	Male	Control	-0.431(0.010)	0.504(0.085)	0.537	<0.001
		Mild	-0.511(0.022)	0.605(0.125)	0.517	<0.001
		Severe	-0.493(0.045)	0.658(0.190)	0.572	0.007
	Female	Control	-0.478(0.017)	0.543(0.092)	0.485	<0.001
		Mild	-0.490(0.015)	0.667(0.098)	0.793	<0.001
		Severe	-0.506(0.031)	0.604(0.178)	0.511	0.006
Wing Loading (g cm^{-2})	Male	Control	-1.012(0.009)	0.816(0.078)	0.789	<0.001
		Mild	-0.933(0.019)	0.685(0.106)	0.654	<0.001
		Severe	-0.898(0.060)	0.874(0.254)	0.569	0.007
	Female	Control	-1.033(0.010)	0.713(0.055)	0.819	<0.001
		Mild	-1.011(0.968)	0.660(0.097)	0.794	<0.001
		Severe	-0.974(0.027)	0.735(0.153)	0.676	<0.001
Flight Metabolic Rate (mL CO2 h-1)	Male	Control	1.211(0.033)	0.691(0.349)	0.146	0.059
		Mild	1.221(0.059)	1.135(0.341)	0.356	0.003
		Severe	1.268(0.221)	1.238(0.935)	0.163	0.222
	Female	Control	1.212(0.053)	0.776(0.288)	0.171	0.011
		Mild	1.194(0.049)	0.920(0.311)	0.443	0.013
		Severe	1.136(0.072)	0.297(0.413)	0.045	0.488
Wingbeat Frequency (Hz)	Male	Control	1.478(0.009)	-0.099(0.105)	0.064	0.364
		Mild	1.479(0.013)	0.014(0.075)	0.003	0.851
		Severe	1.490(0.084)	0.061(0.343)	0.008	0.868
	Female	Control	1.457(0.008)	-0.004(0.042)	0.001	0.920
		Mild	1.446(0.013)	0.033(0.094)	0.017	0.738
		Severe	1.455(0.017)	-0.056(0.102)	0.033	0.592
HK (U g-1 Thorax)	Male	Control	0.055(0.305)	1.241(0.036)	0.001	0.859
		Mild	0.389(0.478)	1.378(0.087)	0.032	0.426
		Severe	-0.656(0.842)	1.045(0.201)	0.071	0.607
	Female	Control	-0.473(0.471)	1.166(0.084)	0.028	0.322
		Mild	-0.216(0.408)	1.223(0.062)	0.023	0.601

		Severe	-0.147(0.775)	1.136(0.134)	0.003	0.853
TRE (U g-1 Thorax)	Male	Control	-0.334(0.248)	-4.159(0.029)	0.068	0.190
		Mild	0.171(0.239)	-4.077(0.040)	0.028	0.484
		Severe	-1.444(2.700)	-4.296(0.645)	0.034	0.607
	Female	Control	0.376(0.481)	-4.309(0.079)	0.023	0.441
		Mild	0.589(0.227)	-4.036(0.036)	0.379	0.025
		Severe	-0.837(0.580)	-4.298(0.101)	0.188	0.183
HOAD (U g-1 Thorax)	Male	Control	-0.316(0.665)	1.700(0.074)	0.009	0.639
		Mild	-1.638(0.678)	1.277(0.124)	0.226	0.025
		Severe	0.074(1.635)	1.618(0.391)	<0.001	0.965
	Female	Control	0.028(0.618)	1.648(0.110)	<0.001	0.964
		Mild	0.525(0.583)	1.758(0.090)	0.063	0.385
		Severe	0.213(0.776)	1.581(0.135)	0.007	0.789
CS (U g-1 Thorax)	Male	Control	0.148(0.312)	2.293(0.037)	0.009	0.639
		Mild	-0.973(0.577)	2.040(0.105)	0.124	0.107
		Severe	-0.609(1.179)	2.143(0.282)	0.032	0.619
	Female	Control	-0.259(0.529)	2.229(0.095)	0.007	0.628
		Mild	0.356(0.456)	2.248(0.070)	0.048	0.451
		Severe	-0.313(0.315)	2.194(0.055)	0.083	0.341

Least-squares regression analyses were performed using log-transformed data such that log$Y$=log$a$+$b$log$X$ where X is body mass in g. Standard errors are presented in parentheses

5.0 Discussion

5.1 Larval and pupal development

We examined the developmental effects of reduced diet quality on size and body proportions, ultimately to test if these changes affect flight energetics by treating larvae with three separate diets: controls being fed *ad libitum,* mild larvae being fed in 24-hour increments and severe larvae being fed in 48-hour increments. Larvae under dietary stress have been known to increase developmental time while achieving smaller body size at maturity (Esperk et al., 2007; Gibbs et al., 2012; Blanckenhorn, 1999; Boggs and Freeman, 2005: Gibbs et al., 2012; Nylin and Gotthard, 1998). Our results stay consistent with previous literature as larvae fed under dietary restraints were significantly smaller at wandering and at the pupal stage than those being fed freely along with treated larvae having a significantly longer developmental period. Our findings suggest that these nutrient restricted larvae may face some constraints in resource allocation, where they are prioritizing survival over growth. This adaptive trade-off is critical in insects, where smaller sized individuals can suffer from long term fitness consequences such as flight ability and reproductive success (Boggs, 2009). Further, smaller body sizes often influence population dynamics and gene flow, where reduced body size correlates with decreased mating opportunities and dispersal ability (Harrison & Roberts, 2000), as decreased size impairs performance in activities that require high energy expenditure, such as flight (Sibly & Atkinson, 1994). These results highlight the importance of diet quality in molding crucial life history traits. As nutrient restriction during development imposes changes to certain traits linked to flight energetics, resource allocation can also cause variation in morphology.

5.2 Developmental plasticity's effect on interindividual variation and sexual dimorphism of body size and morphology

Many morphological traits of adult insects show a plastic response to a change in larval developmental conditions. As predicted, nutrition restriction during the late stage of development induces differences in adults' size, up to five-fold in our study. These results are consistent with previous insect food-stress studies showing decreased nutrient intake/intermittent starvation causing significantly decreased body size (Merry et al., 2011; Boggs and Freeman, 2005; Boggs and Niitepold, 2016; Bauerfeind, Fischer, & Larsson, 2005; Saastamoinen et al., 2013).

These large decreases in body mass can occur due to nutrition deficiency, but it can also affect the proportion of central flight phenotypes. The wing area or wing length of wings are also affected in nutrient restricted conditions during development. For instance, Boggs and Freeman (2005) found both body mass and wing length to be significantly smaller while conducting a semi-starvation treatment on *Bicyclus anyanana.* In addtion to *Bicyclus anyanana*, other species such as *Pararge* aegeria, *Drosophila melanogaster*, and *Hermetia illucens,* have shown this same effect under nutrient restricted treatments, where treated with varying nutritional content during development results in smaller body and wing sizes (Gibbs et al., 2011; Pocas et al., 2022; Gobbi et al., 2013). This suggests that developmental plasticity is widespread across species showing how nutritional deficiencies result in smaller body and wing proportions, which in turn can cause compromised flight ability, affecting traits such as wing loading and wingbeat frequency. Changes in wing size directly impact wing loading, influencing the individual's capacity to generate lift for

flight success, affecting the energetic demands needed for hovering flight (Dudley, 2000; Gilchrist and Huey, 2004) However, wing area or wing length does not always decrease in size to the same extent as the body, causing variation in morphology, further affecting fight ability.

Changes in allometry between male body mass and wing area is consistent with previous studies from Nijhout and Emlen (1998), with their experimental results on removal of imaginal discs from the hind wings in *Precis coenia.* Nijhout and Emlen demonstrated how there can be tradeoffs between resource allocation to different parts of the body due to changes in demands on developmental resources. Here, Nijhout and Emlen removed imaginal discs of the hindwing at the beginning of the last larval instar, resulting in a compensatory response in the relative size of the adult fore wings. These experimental results show that allometric relationships between different body parts may change through developmental constraints when competing for resource allocation. Boggs and Freeman (2005) further support this case on their semi-starvation study on *Bicyclus anyanana*, where nutrient restricted diet during development caused adult females to allocate resources toward their abdomen, resulting in disproportionately larger abdomens. This suggests that female *Bicyclus anyanana* are potentially selecting toward increased fecundity, as opposed to allocating resources to their thorax where they would select toward flight success and maneuverability.

From manipulating nutrient content in the larval diet, developmental plasticity also affects the size of the thorax and abdomen in flying insects, altering flight ability. As the thorax houses the muscles necessary for flight, variation in thorax size will cause variation in muscle mass, ultimately causing changes in energy production affecting flight (Srygley & Chai, 1990; Harrison et al., 2017; Berwaerts et al., 2002). Conversely, as we see in Boggs and Freeman's

(2005) results where female tropical butterfly's exhibit a larger abdomen under semi-starvation conditions, this may lead to inefficiencies in flight energetics by altering body mass distribution creating more drag for the individual (Marden, 2000; Marden & Chai, 1991; Srygley & Kingsolver, 2008). These changes in thorax and abdomen proportions through nutritional stress show to be different among sexes across species. This suggests there may be an adaptive response to resource limitations allowing for reproductive or survival strategies between the sexes.

Through our manipulation of nutrient content during development, we see greater competition of resource allocation among treated males, where male wing area grows disproportionately larger in relation to body mass along with males having smaller thoraces for a given size - a trend that is absent among treated females. The different responses to developmental conditions between sex appear to vary between species. We see in previous studies conducted on *Speyeria mormonia* that females wing area increases at a shallower slope to body mass in semi-starved developmental conditions than well-fed individuals (Boggs and Freeman, 2005, Boggs and Niitepold, 2016). Further, Angelo and Slansky (1984) found that in four different species of noctuid moths (*Anticarsia gemmatalis, Spodoptera frugiperda, Helicoverpa zea,* and *Spodoptera latifascia*) subjected to different larval starvation treatments, each species showed a different allometric relationship between wing area and body mass with varying slopes between species. Each of the four species also showed no differences in relationship between males and females. Boggs and Niitepold (2016) also found the proportion of resources invested in the thorax was greater for males than for females on nutrient-stressed larvae in *Speyeria mormonia*, paralleling the findings from Saastamoinen et al. (2010) in *Bicyclus*

anynana. The sexual dimorphism we observe under nutritional stress in our male *M. sexta* may be species-specific, as other Lepidopterans have shown to respond to nutrient stressed developmental conditions between sexes very differently.

Regardless, this steeper slope in allometry under nutrient restriction may impose constraints on smaller individuals where wing loading is greater for a given size in both males and females in the mild and severe groups. This could result in changes in flight capability and metabolism, where previous work done on *P. occidentalis* has shown that experimental trimming of wing size substantially increases wing loading, thus increasing wingbeat frequency during hovering flight (Kingsolver, 1999). These reductions in body and wing size ultimately suggest a higher wing loading and higher wingbeat frequency, all at the cost of greater energetic output.

5.3 Effects of developmental plasticity on flight energetics, flight ability and muscle metabolic phenotype

As we see variations in morphological characteristics due to restricted nutrients during development, we notice these individuals' capacity for flight is greatly affected, where morphological changes influence flight ability. Nutrient restriction imposes changes in insect adult size and may further impact important body proportions associated with flight, such as wing area that may become disproportionate in some species (Boggs and Freeman, 2005, Boggs and Niitepold, 2016). These changes affect crucial properties such as wing loading that is known to directly explain differences in flight metabolic rate (Dudley, 2000; Gilchrist and Huey, 2004). We predicted that a nutrient restricted diet will cause smaller body sizes with a hyper

allometric relationship between body mass and wing area (Nijhout & Callier, 2015, Tobler & Nijhout, 2010), ultimately causing lower wing loading resulting in lower frequency and flight metabolic rate (Darveau et al., 2005). As stated previously, we see a significantly smaller size with our treated individuals along with males showing a hyper allometric relationship between wing area and body mass while females relationship remains the same.

Further, we see higher wing loading relative to size in both mild and severe groups for both males and females, along with differences in wingbeat frequency between males and females, but no differences in wingbeat frequency among treatment groups with no significant relationships between wingbeat frequency and body mass. Studies conducted on orchid bee species by Darveau et al. (2005), however, find that wingbeat frequency during hovering flight significantly decreases with body mass. A similar study conducted by Yu et al. (2022) shows the same results with a strong negative relationship between body mass and wingbeat frequency among 77 species in 3 orders (Lepidoptera, Neuroptera, and Odonata) and 14 families of migratory insects, showing a broad pattern across species. Many studies have shown this trend, where larger wings for a given size will beat at a lower frequency, which also applies to birds and bats (Darveau, 2024). Since we see no relationship between body mass and wingbeat frequency, this may impose flight challenges to our smaller individuals as they are not following general trends in body mass to wingbeat frequency relationships, where smaller individuals would need to have a higher wingbeat frequency to generate lift. This leads to suggestions that plasticity of morphology is not accompanied by co-plasticity in energetics, thus compromised flight.

When looking at our results, we see that as expected, body mass has a significant effect on flight metabolic rate along with our model showing that wingbeat frequency is related to flight metabolic rate independent of mass. Further, our smaller-sized individuals have lower flight metabolic rate than larger individuals, following the same trend shown on other studies of bees and sphinx moths (Darveau et al., 2005; Darveau et al., 2014; Casey, 1976). This positive correlation of flight metabolic rate with body mass has been well documented among flying insects, birds, and mammals, where metabolic rate increases with size (Niven & Scharlemann, 2005; Darveau et al., 2024). Due to the lack of frequency differences among treatments, treatment showed no effect on flight metabolic rate. Surprisingly, sex also showed no differences in metabolic rate, although males had a higher wingbeat frequency than females by approximately 1 hz. We see across species that Eusocial bumblebees' male drones show larger wings and lower wingbeat frequency for a given size (Darveau et al., 2014). Further, similar sex dimorphism of lower wing loading for males has been observed across several species of honey bees, showing that differences in flight-related morphologies may be associated with differences in flight performance and flight energetics (Darveau, 2024). In our case, our treated males show hyper allometry between wing area and body mass, along with smaller thoraces for a given size, ultimately imposing challenges in flight ability and energetic consequences regarding flight.

Flight success was significantly affected by treatment and sex, where treated individuals have significantly lower flight success along with males showing more difficulty generating hovering flight. Our smaller-sized individuals had much difficulty sustaining longer periods of flight, where both males and females in our mild and severe groups had difficulty generating

any lift upon stimulation of flight. Under nutrient restricted conditions, we see a 30.21% decrease in hovering flight in our male control group to our mild group, along with a 36.63% decrease in our female control group to mild group. Likewise, a study conducted on *Spodoptera litura* testing different larval diets with varying lipid content reared significantly smaller individuals that were also unable to generate any flight (Sakamoto et al., 2004). We see that our treated individuals' wingbeat frequency remains unchanged for both males and females, while as stated previously, it is well known that smaller individuals need a higher wingbeat frequency to sustain flight (Byrne et al., 1998). These results bring about a question as to whether there is a threshold size that is needed to meet the energetic demands that are necessary to generate flight, where individuals that are below a certain threshold size may not have sufficient muscle mass, wing area, or energy stores needed to reach the metabolic demands of hovering flight. From our results, we can see an imbalance between smaller body sizes, thorax size, and wing size in nutrient restricted individuals, where this imbalance may fail to compensate for the high energetic power and lift demands of hovering flight.

The interplay between nutrient restriction's effect on body size and enzyme activity further highlight challenges that smaller individuals face when discussing the metabolic demands of flight, particularly when observing enzyme activities of flight muscle. Among each of the tested enzymes in our study, we only found HK to show sexual dimorphism in activity along with a significant relationship between body mass and enzyme activity in TRE for mild females and HOAD for mild males. While we see very little trends in our results, prior research indicates a correlation to muscle metabolic activity and flight metabolic rate, where small size or high wing loading results in higher wingbeat frequency, thus higher metabolic rate per gram

of muscle tissue and higher enzyme activity to support it (Darveau et al., 2014; Marden, 2000). Across species of *Drosophila*, we see that individuals that possess a high metabolic rate would have an overall increase in in enzyme content (Clark and Wang, 1994), however this is not the case for us. As flight metabolic rate increases with size, this further warrants our previous results showing decreased flight ability among nutrient restricted individuals, where no difference in enzyme content gives smaller individuals an energetic disadvantage regarding flight. This may suggest a lack of plasticity in flight muscle, where we also see from Skandalis and Darveau (2012) that experimentally changing wing loading does not result in changes in flight muscle properties. For us, change in flight properties through developmental plasticity does not yield compensatory changes at the level of flight muscle which may be in part why they are compromised.

6.0 PERSPECTIVE AND FUTURE DIRECTIONS

Nutrient restriction during the last instar of *M. sexta* larvae results in significantly smaller adults, with changes on morphology resulting in varying wing area and thorax allometry between males and females. Although we see a reduced size, our results indicate no significant differences in functional key factors such as flight metabolic rate, wingbeat frequency or the activities of metabolic enzymes involved in flight muscle metabolism. Our findings suggest that despite the physiological systems related to flight energetics remaining stable, the smaller-sized individuals resulting from nutrient restriction may have a negative impact on flight ability due to the prior mentioned physical constraints, such as reduced lift generation. This outlines the importance of body size in determining flight performance, even with physiological and metabolic factors remaining unchanged, further supporting previous studies on flight energetics in *M. sexta* and *Locusta migratoria* (Snelling et al., 2012; Marden, 1995).

Investigating the role of body size and morphological variation on muscle function in nutrient restriction individuals could provide crucial information into how developmental plasticity affects flight biomechanics. Future studies should focus on evaluating whether there is a threshold size below which these metabolic and wingbeat systems can no longer compensate for smaller sizes, leading to flight failure. Additionally, exploring the genetic basis of how a nutrient restricted diet can impact allometric scaling could open concepts on potential evolutionary adaptations to changes in environmental conditions.

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