PHYSIOLOGICAL AND BIOCHEMICAL MECHANISMS UNDERLYING PHENOTYPICALLY PLASTIC RESPONSES TO ENVIRONMENTAL CONDITIONS IN THE TRUE ARMYWORM, *Mythimna unipuncta.*

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ABSTRACT

We hypothesized that the migratory true armyworm moths, *Mythimna unipuncta*, would have phenotypically plastic responses to unfavourable, short day breeding environments. These phenotypic responses may include higher wing aspect ratios, lower wing loading and a larger thorax to allow for more energy-efficient migration (Vincze *et al.*, 2019). Additionally, migratory moths were predicted to have other factors that may aid in migration such as increased levels of long, unsaturated fatty acids, higher metabolic rates and an increased mitochondrial volume density (Hill *et al.*, 2012). Morphometry of the moths' wings, body compositions and eggs were measured, gas chromatography was used to analyze fatty acids of the fat bodies and lipid assays were used to calculate haemolymph lipid concentrations. Additionally, a respirometer was used to determine resting and active metabolic rates and a spectrophotometer was used to measure citrate synthase activity. It was found that moths in unfavourable, short day conditions were phenotypically different from the reproductive moths—the short day moths had lower wing loading for more energy-efficient flight and female short day moths had no eggs, unlike the long day female moths, in order to preserve energy for migration. However, the migratory and reproductive moths did not differ in aspect ratio, thorax mass, haemolymph lipid concentrations and mitochondria and myofibril densities.

INTRODUCTION

Phenotypic plasticity is the ability of a single organism with a fixed genotype to express multiple, genetically controlled phenotypes (Hill *et al.*, 2012). The phenotype is a product of interactions between genes and the environment; individuals can rapidly respond to changes in the environment via adaptive phenotypic plasticity (Liu *et al.*, 2022). One highly plastic activity is migration—phenotypic plasticity influences migration timing and energy budgets which can consequently also affect an individual's breeding performance (Liu *et al.*, 2022). Migration is beneficial for animals as it allows individuals a choice of when and where to reproduce by utilizing variable habitats (Hill and Gatehouse, 1993). In such cases, diapause—a period of suspended development—is triggered, delaying reproduction so that the organism can migrate to allow reproduction to occur in a different, more favourable environment (Hill and Gatehouse, 1993).

In *Propylea japonica*—a species of lady beetle—reproductive diapause is determined by the concentration of juvenile hormone (JH) in the haemolymph (HuangFu *et al.*, 2021). High concentrations of JH in the haemolymph signals to adult female insects' reproductive tract to begin oogenesis and produce eggs to lay and fertilize; meanwhile if JH concentrations remain low, reproduction is delayed and the individual experiences diapause and egg production does not occur (HuangFu *et al.*, 2021). When in environments unfavorable to reproduction (such as short photoperiod, cold temperatures), diapause occurs to allow the insect to migrate (Hill and Gatehouse, 1993).

Long-distance flight migration is accompanied by decreased fertility and reproductive diapause as energy must be shared between flight and migration (Xiao *et al.,* 2016). Fatty acids (FA) are a component of triglycerols (TAG), which acts as the one of the main fuels for flight (Sakamoto *et al.,* 2014). FAs are long chains of 12-24 carbons with a carboxylic acid group and can be saturated (no double bonds) or unsaturated (one or more double bonds) (Hill *et al.,* 2012). The degree of unsaturation and the length of FAs in storage deposits can have substantial effects on an organism's performance. For example, longer polyunsaturated fatty acids (PUFA), such as ARA 20:4 n6, are strong PPAR agonists which promotes the breakdown of FAs to produce energy (Hill *et al.,* 2012). Additionally, unsaturation also increases relative mobilization from lipids and may be beneficial to migratory performance (Price *et al.,* 2008). For example, decreased chain lengths and increased double bonds leads to higher relative mobilization in ruffs (*Philomachus pugnax*) and white-crowned sparrows (*Zonotrichia leucophrys*) during migration (Price *et al.,* 2008). Flight activity in insects triggers the release of adipokinetic hormone (AKH) which stimulates the release of lipids from the fat bodies, in the form of a lipoprotein, into the haemolymph to be used as fuel for flight (Orchard *et al.,* 1991).

Meanwhile, high wing loading is associated with flapping (faster, more powerful flight), which is more energetically costly than the soaring that occurs with low wing loading (Vincze *et al.*, 2019). In migratory birds, high wing aspect ratio and low wing loading is preferred for energy-efficient migratory flight (Vincze *et al.*, 2019).

Factors such as intense physical activity, the ambient temperature, ingestion of food and reproductive condition can impact an animal's metabolic rate (the rate at which an animal converts chemical energy to heat and external work) (Hill *et al.,* 2012). In insects, metabolism

increases with increased levels of physical activity (such as flight during migration), temperature and after ingestion of a meal (Hill *et al.,* 2012).

The maintenance of high exercise performing machinery such as insect flight muscles (located in the thorax) also contributes to an increased metabolic rate (Hill *et al.,* 2012). It is also more expensive to maintain due to its large size (up to 65% of an insect's body mass in strong fliers) and increased relative proportion of mitochondria in order to support the high aerobic metabolic rates required for flight (Niven and Scharlemann, 2005). Muscle aerobic capacity and mitochondrial volume can be measured using the activity of citrate synthase (CS) which is essential to the first step of the Krebs cycle (Hill *et al.,* 2012). In addition to CS activity, mitochondrial volume density and myofibril volume density can also indicate mitochondrial volume and muscle aerobic capacity.

The true armyworm moth, or *Mythimna unipuncta,* is known for its high mobility and as a North American agricultural pest to grasses such as wheat, oats, rice and barley (Hobson *et al.,* 2012). The true armyworm spends 25 days and 6 instars in its caterpillar larval stage and afterwards pupates for two weeks (Batallas *et al.,* 2020). Adult moths feed on nectar and females produce up to 2000 eggs when conditions are favourable—the true armyworm can be migratory or non-migratory depending on the environmental conditions during emergence (Hobson *et al.*, 2012). Increased JH levels indicate to adult moths to breed when under favourable breeding conditions such as long days (LD) and warm temperatures (Hobson *et al.,* 2012). However, under short days (SD) and cold temperatures the moths migrate and overwinter in the southern United States to avoid the cold and later return northward in spring to Canada (Hobson *et al.,* 2012). In order to migrate, the moths undergo reproductive diapause due to lower JH levels and instead allocate energy towards long distance migration (Hobson *et al.,* 2012). Migratory insects, such as the true armyworm moth, make tradeoffs to optimize the cost of migratory flight with future reproduction (Anparasan *et al.,* 2020).

The objective of this experiment is to investigate the environmental conditions that lead to the underlying phenotypically plastic responses in regards to the true armyworm moths. If high wing aspect ratio, low wing loading and a larger thorax is preferred for energy-efficient migratory flight (Vincze *et al.*, 2019), then the migrant moths (SD male and female adult moths) will have more of the energy-efficient phenotypic traits associated with efficient flight compared to reproductive moths (LD females and males). Additionally, migrant moths are predicted to

have increased levels of adipocytes in the form of unsaturated FAs and long chain PUFAs as both help to increase the relative mobilization from adipocytes, which is beneficial for highly intense aerobic activity such as migration (Price *et al.*, 2008). If flight activity triggers the release of AKH which stimulates the release of lipids from the fat bodies into the haemolymph to be used for flight (Orchard *et al.,* 1991), then the migrant SD moths will have increased levels of circulating lipids as flight time increases compared to the LD moths due to increased release of AKH and increased relative mobilization of adipocytes. The circulating lipids in LD moths are also predicted to increase over flight duration, but at a slower rate than the SD moths which are phenotypically adapted to maximize flight efficiency. Finally, as physical activity and the maintenance of high exercise performing machinery—such as insect flight muscles—increases metabolic rate (Hill et al., 2012), the migrant SD moths are predicted to have increased metabolic rates and CS activity as well as a greater number of mitochondria and myofibrils to support the migratory flight machinery compared to the LD moths. This is because the reproductive LD moths will have traded-off migratory efficiency and machinery for reproductive investment.

MATERIALS AND METHODS

All materials and methods used in this study were from G. Guglielmo and M. Bernard's (2022) Techniques in Physiology and Biochemistry.

STUDY CONDITIONS AND TREATMENTS

M. unipuncta from Ontario were brought into the laboratory and allowed to reproduce for a minimum of two generations. Larvae were raised on a formulated pinto bean diet under stable light and temperature conditions (14/10 hr photoperiod at 25°C) until they reached the pupal stage.

Emerged adults were collected at the start of each day and assigned to either a long day (LD) treatment with a longer photoperiod and warm temperatures (16/8 hr photoperiod at 25°C) or a short day (SD) treatment with a shorter photoperiod and cooler temperatures (12/12 hr photoperiod at 10°C). Adult moths were fed an 8% sucrose solution.

Day 0 moths were frozen immediately after pupal emergence and day 3 and 6 moths were frozen after 3 or 6 days of different temperature treatments, respectively.

ANALYSIS OF MORPHOMETRY, BODY COMPOSITION AND EGGS

The weight and lengths of moths were measured and the area of the moth wings were analyzed using ImageJ (Madison, Wisconsin).

A wet mass and a dry mass (recorded after drying for 2 days in a 70°C oven) was obtained from male moths. The dried moths were then extracted for 8 h in petroleum ether in a Soxhlet extractor, then allowed to evaporate overnight before being re-dried at 70°C.

Insect saline (0.7% NaCl) was used to keep the ovarioles of the female moths hydrated during extraction. The ovaries were then stained with Grenacher borax carmine and after 15 minutes, they were rinsed twice with 70% ethanol. The oocytes were then counted.

GAS CHROMATOGRAPHY

Fat extracted from the abdomen and the thorax of a moth was placed in 1:1 chloroform-methanol. Then, 17:0 internal standard (15 nmol/μL heptadecanoic acid) was added. Samples were then vortexed and allowed to sit for 5 minutes before 0.25% KCl in water was added to each sample. Samples were then placed in a 50°C water bath for 5 minutes. 1 mL of the organic phase for each sample was separated and centrifuged at high speed for 3 minutes. Clean extracts were then separated into culture tubes for transesterification using a nitrogen evaporator set to 70°C and 0.5 mL of BF_3 for each sample before being placed in a 70°C oven. After drying in the oven overnight, double distilled water and ethyl ether was added to the samples and the ether extract was transferred to dry under nitrogen at room temperature. The FAMEs were then dissolved with Ch_2Cl_2 before being analyzed on an Agilent 6890N GC (Column type: DB-23, 30 m x 0.025 mm internal diameter; Stationary phase: 0.25 mm thick; Mobile phase: He flowing at 1.9 mL/min; Injector: splitless mode at 250°C; FID: 280°C; Injection volume: 1 μL; Oven condition: 80°C for 2 min, 80–180°C ramp at 5°C per min hold at 180°C for 3 min, 180–200°C ramp at 1.5°C per min, 200–240°C ramp at 10°C per mind hold at 240°C for 3 min).

STANDARD METABOLIC RATE

QUBIT SYSTEMS™' (Kingston, ON) flow-through open system respirometer at a range of 2000 ppm and LabPro Interface (Beaverton, Oregon) was used to measure the standard metabolic rate of the moths. The flow rate of the respirator was set to 10 mL/min for 10 minutes

for background data collection. 60 minutes of data at a flow rate of 10 mL/min was recorded with the moth in the respirometer in a settled state.

FLIGHT METABOLIC RATE

QUBIT SYSTEMS™' (Kingston, ON) flow-through open system respirometer was modified by attaching a 300 mL Erlenmeyer flask. The flow rate of the respirator was set to 70 mL/min for 10 minutes for background data collection. 60 minutes of data was recorded at a flow rate of 70 mL/min with the moth suspended inside the Erlenmeyer flask, continuously in flight.

HAEMOLYMPH LIPID CONCENTRATION: LIPID ASSAY STANDARD CURVE

A lipid assay standard curve was created using 0 µL, 8 µL, 16 µL, 24 µL and 32 µL of triglyceride standard (5 µg/µL canola oil in Chloroform ($CHCl_3$)-Methanol 1:1 (v/v)) in a culture tube. Concentrated sulphuric acid (H_2SO_4) was added to each tube before being placed in a 95°C water bath for 10 min. Once cooled, phosphovanillin reagent (1 part 0.6 (w/v) vanillin in H_2O, 4 parts concentrated phosphoric acid (H_3PO_4)) was added to each tube, vortexed and allowed to sit at room temperature for 30 min. A spectrophotometer (Biochrom Novaspec™ II Spectrophotometer) was warmed up and set to 530 nm and a zero reference was set using phosphovanillin reagent. The solution in each tube was measured for absorbance at 530 nm.

HAEMOLYMPH LIPID CONCENTRATION: FLIGHT EXPERIMENT ASSAY

Moth haemolymph was sampled using a 10 cm, 50 µL microcapillary tube for moths at rest, moths after flying on a flight mill for 15 min and moths after flying on a flight mill for 30 min. The haemolymph collected was placed in a culture tube and concentrated sulphuric acid (H_2SO_4) was added to each tube before being placed in a 95°C water bath for 10 min. Once cooled, phosphovanillin reagent was added to each tube, vortexed and allowed to sit at room temperature for 30 min. The solution from each tube was measured for absorbance at 530 nm using a spectrometer.

CITRATE SYNTHASE ACTIVITY

The thorax of moths were frozen in liquid nitrogen (-196°C) and stored in a -80°C freezer. Each thorax was put into a culture tube and 19 volumes of ice cold homogenization buffer (25 mM HEPES, 2 mM EDTA, 0.1% Triton X-100 (v/v), pH 7.5). The thoraxes were then homogenized on ice with a handheld homogenizer for 3 x 10 s with a 20 s rest in between. The samples were then cryogenically frozen for 5 min. The homogenates were then thawed and centrifuged at 2000 g for 5 min. 20 µL of supernatant from the centrifuged homogenate was added to homogenization buffer and vortexed to further dilute the homogenate. The diluted homogenate was added to 5 mM oxaloacetate, 2 mM Acetyl CoA and 1 mM DTNB, then immediately placed into the Novaspec spectrophotometer (set to 412 nm with a zero reference using assay buffer (50 mM tris-HCl)) and absorbance was recorded for 3 minutes, at 15 second intervals. This process was repeated for each of the diluted homogenates twice.

STEREOLOGY

The thorax of female moths was opened dorsally and flight muscle was placed in fixative (2% glutaraldehyde in 0.1 M sodium phosphate buffer pH 6.9) for 3 hours before being rinsed overnight in phosphate buffer and rinsed again for 30 min in the morning. Membranes and lipids were stained using 2% osmium tetraoxide in water for 30 min and then rinsed 2 x 10 min in water. Afterwards, the tissue was dehydrated for 10 min each in a series of 20%, 50%, 70% and 90% acetone/water before 2 x 30 min in 100% acetone. The tissue was prepared for embedding in epoxy resin by being treated with 1:3 and 1:1 resin/acetone for 1 hour each, followed by 3:1 resin/acetone overnight and 100% resin for 4 hours. The tissue embedded in 100% resin was polymerized for 36 h at 60°C. Blocks were trimmed and sectioned (60–80 nm), post-stained with uranyl acetate followed by lead citrate in preparation for transmission electron microscopy (TEM) using an ultramicrotome. Sections were enlarged to reach a final magnification of 19 000 x on micrographs. The micrographs were examined using a coherent test system of points on a transparency and the number of mitochondria and myofibrils were recorded.

DATA ANALYSIS

Two-way ANOVA was used to compare the effects of treatment, age and sex and Tukey's HSD test and least-squares means was used to identify where differences were among treatments, ages and sexes when there was a significant interaction.

All data analysis was performed with the software R (v. 4.1.2; R Development Core Team 2022) and statistics were considered significant if $P<0.05$.

RESULTS

BODY MASS, WING LOADING AND ASPECT RATIO

The body mass of day 0 moths did not significantly differ between both sex (Figure 1; ANOVA; $F_{1,28}=0.003$, $P=0.96$) or treatment type (ANOVA; $F_{1,28}=3.80$, $P=0.062$). On day 6, body mass did not significantly differ between sexes (Figure 2; ANOVA; $F_{1,44}=0.41$, $P=0.53$) however, the day 6 moths did significantly differ between treatments (ANOVA; $F_{1,44}=11.22$, $P=0.0017$). During the long day (LD) treatment, day 6 female moths had significantly higher body masses than the LD males (Least-squares mean; $P=0.0028$) and the short day (SD) treatment, day 6 female moths had a significantly lower body masses than the SD males (Least-squares mean; $P=0.028$). There was no significant difference between LD and SD day 6 females (Least-squares mean; $P=0.17$) and LD and SD day 6 males (Least-squares mean; $P=0.19$).

The wing loading of day 0 moths did not significantly differ between both sex (Figure 3; ANOVA; $F_{1,26}=0.11$, $P=0.74$) or treatment type (ANOVA; $F_{1,26}=1.62$, $P=0.22$). On day 6, wing loading significantly differed between sex and treatment groups (Figure 4; ANOVA; $F_{1,39}=11.58$, $P=0.0016$). There was a significant increase in wing loading for day 6 LD females (TukeyHSD; $P=0.0051$) compared to the day 6 SD females, however, there was no significant difference in wing loading between day 6 LD and SD males (TukeyHSD; $P=0.074$). Day 6 female LD moths also had significant higher wing loading than the day 6 male LD moths (TukeyHSD; $P=0.026$), and day 6 male SD moths had significantly higher wing loading than the day 6 female SD moths (TukeyHSD; $P=0.017$).

The aspect ratio of day 0 moths did not significantly differ between both sex (Figure 5; ANOVA; $F_{1,28}=0.003$, $P=0.96$) or treatment type (ANOVA; $F_{1,28}=3.79$, $P=0.062$). On day 6, there was also no significant difference in aspect ratio between sex (Figure 6; ANOVA; $F_{1,35}=0.31$, $P=0.58$) and treatment type (ANOVA; $F_{1,35}=0.045$, $P=0.834$).

EGG COUNT

The number of eggs in the thorax did not significantly differ between ages (Figure 7; ANOVA; $F_{1,60}$=2.18, P=0.15) however, it did significantly differ between treatments (ANOVA; $F_{1,60}$=7.20, P=0.0094). The day 6 LD moths had a significantly greater number of eggs than the day 6 SD moths (Least-squares mean; P=0.017) as well as the day 0 LD moths (Least-squares mean; P=0.031).

THORAX MASS

There was no significant difference in thorax mass between sexes (Figure 8; ANOVA; $F_{1,26}$=3.80, P=0.062) and treatments (ANOVA; $F_{1,26}$=2.93, P=0.099). Additionally, thorax mass did not significantly differ between ages (Figure 9; ANOVA; $F_{1,36}$=0.20, P=0.66) nor between treatments (ANOVA; $F_{1,36}$=2.01, P=0.16) of female day 6 moths

FATTY ACID CONCENTRATIONS

There was no significant difference between treatment and levels of 16:00 (Figure 10; ANOVA: $F_{1,24}$=0.97, P=0.33) , 18:3 (Figure 11; ANOVA: $F_{1,24}$=0.235, P=0.63), and 20:4 (Figure 12; ANOVA: $F_{1,24}$=0.759, P=00.39) FA stores of the fat body.

HAEMOLYMPH LIPID CONCENTRATION

There was no significant difference in haemolymph lipid concentration between sexes and treatments at 0 minutes (Figure 13; ANOVA: $F_{1,16}$=0.75, P=0.40), 15 minutes (ANOVA: $F_{1,16}$=0.173, P=0.68) and 30 minutes (ANOVA: $F_{1,16}$=1.752, P=0.21). The haemolymph lipid concentration was also not significantly different between the LD males and the LD females (TukeyHSD; P=0.21).

METABOLIC RATE

There was no significant difference in resting metabolic rate between different sexes (Figure 14; ANOVA: $F_{1,8}$=0.20, P=0.67) and treatments (ANOVA; $F_{1,8}$=0.052, P=0.83). There was no significant difference in the active metabolic rate between different sexes (ANOVA; $F_{1,8}$=0.80, P=0.38) but there existed a significant difference between treatments (ANOVA;

$F_{1,8}$=4.19, P=0.047). The LD male moths had a significantly higher active metabolic rate than the SD moth (TukeyHSD; P=0.011).

CITRATE SYNTHASE ACTIVITY

There was a significant difference in CS activity between sexes (Figure 15; ANOVA; $F_{1,26}$=14.32, P=0.0008) but not between treatments (ANOVA; $F_{1,26}$=0.27, P=0.61). The CS activity was significantly higher in male SD moths than female SD moths (Tukey; P=0.0063) and significantly higher in male SD moths than female LD moths (Tukey; P=0.03).

MITOCHONDRIA AND MYOFIBRIL VOLUME DENSITY

There was no significant difference in the percent mitochondrial volume density in the thorax between the SD and LD treatment female moths (Figure 16; ANOVA; $F_{1,15}$=3.31, P=0.090)

There was no significant difference in the percent myofibril volume density in the thorax between the SD and LD treatment female moths (Figure 17; ANOVA; $F_{1,15}$=0.29, P=0.60).

DISCUSSION

OVERVIEW

It was predicted that the migrant true armyworm (*Mythimna unipuncta*) moths (SD male and female adult moths) would have lower wing loading, a higher aspect ratio and a larger thorax than female LD moths as it is preferred for energy-efficient migratory flight (Vincze *et al.*, 2019). Additionally, as migration is a highly intense aerobic activity, the migrants were predicted to have increased levels of adipocytes in the form of unsaturated FAs and long chain PUFAs (both of which increase the relative mobilization from adipocytes to support their energy-intensive flight) (Price *et al.*, 2008). This increase in relative mobilization in migratory moths leads to predicted increases in levels of circulating lipids as flight time increases compared to the reproductive LD moths. Finally, the migrant moths were also predicted to have increased metabolic rates, CS activity and mitochondria and myofibril density to support their intense physical activity and flight muscles required for migratory flight (Hill et al., 2012).

Like in our predictions, the day 6 migrant moths were observed to have significantly lower wing loading than the reproductive LD females, however, there was no significant

difference in aspect ratio or thorax mass between the treatments and sexes. Additionally, there was also no significant difference in the unsaturated 18:3 FAs and the 20:4 long chain PUFAs between the treatments as well as no significant difference in haemolymph lipid concentration between sexes or treatments. At rest, there was no significant difference in metabolic rates between migrants and reproductive moths, but after flight, migrant SD male moths had significantly lower active metabolic rates than the LD male moths which was not what we predicted. However, like in our predictions, the SD moth had a significantly increased CS activity compared to the LD and SD females. There was no significant difference between mitochondria and myofibril density between the treatments.

WING MORPHOLOGY AND THORAX MASS

There was no significant difference between mass, wing loading and aspect ratio between sexes and treatments on day 0. This may be because *M. unipuncta* were kept under identical treatments during larval and pupal stages and day 0 specimens were frozen immediately after pupal emergence. As these moths never experienced the different treatments, they did not express different phenotypes and therefore had statistically insignificant differences between mass, wing loading and aspect ratio.

Day 6 moths did have significant differences in mass, wing loading and aspect ratio based on the treatments, showing phenotypic plasticity. For example, day 6 LD female moths were significantly heavier than day 6 LD male moths. Day 6 LD females were also found to have a significantly higher number of eggs than all other treatments and ages, which may explain why day 6 LD female moths were significantly heavier that day 6 LD male moths. However, day 6 SD female moths were significantly lighter than day 6 SD male moths—despite the fact that in many species of moths (such as spruce budworm, *Choristoneura fumiferana*), males are typically lighter than females. This may be due to the fact that the day 6 SD females moths had—on average—almost no eggs due to reproductive diapause. Future studies may investigate if the day 6 SD females develop eggs later in their 7 to 21 day lifetime (Hobson *et al.,* 2018) and have a heavier body mass similar to the day 6 SD females during reproductive maturation.

As predicted, the day 6 SD migrant moths had significantly lower wing loading than the reproductive day 6 LD females. This shows evidence of phenotypic plasticity as a lower wing loading is preferred for energy-efficient migratory flight (Vincze *et al.*, 2019). However, despite

the fact that a higher aspect ratio wing is better suited for endurance and long-distance flight, day 6 moths showed no significant difference in aspect ratio between sexes and treatments. This could be due to their age—perhaps as they get older, the more significant differences in wing aspect ratio may become. An alternate explanation is that *M. unipuncta* may not require aspect ratio optimization like the Arctic terns, *Sterna paradisaea* (which have high aspect ratio wings)—*M. unipuncta* only migrate within North America meanwhile, *S. paradisaea* travel annually from the Arctic to the Antarctic and back (Vincze *et al.*, 2019).

Finally, there was no significant difference in thorax mass between sexes and treatments which opposes our original prediction. Instead, it supports the notion that migratory SD moths may not develop increased high exercise performing machinery such as insect flight muscles compared to the reproductive LD counterparts. Future areas of interest may look at wing aspect ratios and thorax mass, and determine if there are correlations between phenotypically plastic emergences of energy-efficient flight anatomy and migratory distance.

FATTY ACID CONCENTRATIONS AND CIRCULATING LIPIDS

16:0 FAs were chosen to represent short, saturated FAs; meanwhile 18:3 FAs represented long, unsaturated FAs; and 20:4 represented long-chain PUFA. Unsaturated FAs and long chain PUFAs may help increase the relative mobilization of adipocytes, which provides fuel for migration (Price *et al*., 2008), however, there are no significant differences in 16:0, 18:3 and 20:4 in the fat bodies of moths between sexes and treatments. Sakamoto *et al.* (2004) discusses that the absence of 20:4 FA in mosquitos, *Culex pipiens*, causes flightlessness and that the absence of unsaturated FA (such as 18:3 FAs) led to low flight ability in the common cutworm, *Spodoptera litura*. Perhaps the true armyworm moths only require enough unsaturated FAs and long chain PUFAs to remain flighted, unlike other animals that may modify FA composition leading up to a more intense migration (Price *et al.,* 2008). Finally, it is possible that true armyworm moths may modify levels of different FAs to better sustain migration and future studies could look into levels of FAs other than 16:0, 18:3 and 20:4, over flight duration.

The lack of significant differences in FAs between the sexes and treatments also supports the lack of a significant difference in haemolymph lipid concentrations between sexes and treatments during flight. However, this finding differs from McNeil and Toby (2001)'s findings where SD males and females had a marked increase in lipid mobilization compared to the LD

males and females in *M. unipuncta*. This difference could be attributed to different methods (such as 30 min of flight compared to hours of flight) as well as age of the subjects (for example, moths older than 6 days may mobilize lipids more efficiently than day 6 moths).

METABOLIC RATE, CS ACTIVITY AND MITO. AND MYO. DENSITY

Unlike our prediction that increased metabolic rates would be required to support the intense physical activity and flight muscles of the SD moths, LD males showed a significant increase in active metabolic rate compared to the SD males. As previously mentioned, SD moths had lower wing loading compared to LD moths. High wing loading is associated with flapping which is more energetically costly than the soaring that occurs with low wing loading, which is preferred by migratory birds (Vincze *et al.*, 2019). Additionally, Drosophilid flies have low active metabolic rates due to their slow wingbeat frequencies which saves energy during flight (Niven and Scharlemann, 2005). The lower metabolic rate may be due to the SD males' low wing loading, which preserves energy during migratory flight. Additionally, the moths were not in a fasted state during respirometry which may have impacted the metabolism data as metabolic rate is heavily impacted by the ingestion of a meal (Hill *et al.,* 2012). In future studies, moths should be in fasted states before respirometry tests to ensure more accurate resting and active metabolic rates.

As predicted, the SD male moths had a significantly higher CS activity than the LD females. This supports the notion that SD moths may have increased muscle aerobic capacity and mitochondrial volume compared to the reproductive LD females (Hill *et al.*, 2012).

In addition to CS activity, mitochondrial volume density and myofibril volume density can also indicate mitochondrial volume and muscle aerobic capacity. However, there was no significant difference in mitochondrial volume density and myofibril volume density between female LD and SD moths. Future studies may include LD and SD males as the males showed a significant difference in CS activity, which impacts mitochondrial volume density (Hill *et al.,* 2012).

CONCLUSIONS

It was supported that day 6 migrant moths had phenotypically plastic responses to the short day treatments due to their significantly lower wing loading compared to the LD

reproductive females. Additionally, the significant difference in eggs between the day 6 LD and SD females showed that the SD females underwent reproductive diapause in response to the unfavourable environment in order to preserve energy for migration. However, aspect ratio, thorax mass, haemolymph lipid concentrations and mitochondria and myofibril densities did not significantly differ between the sexes or treatments—perhaps due to the less strenuous nature of the *M. unipuncta'*s migration compared to other migratory animals.

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FIGURES

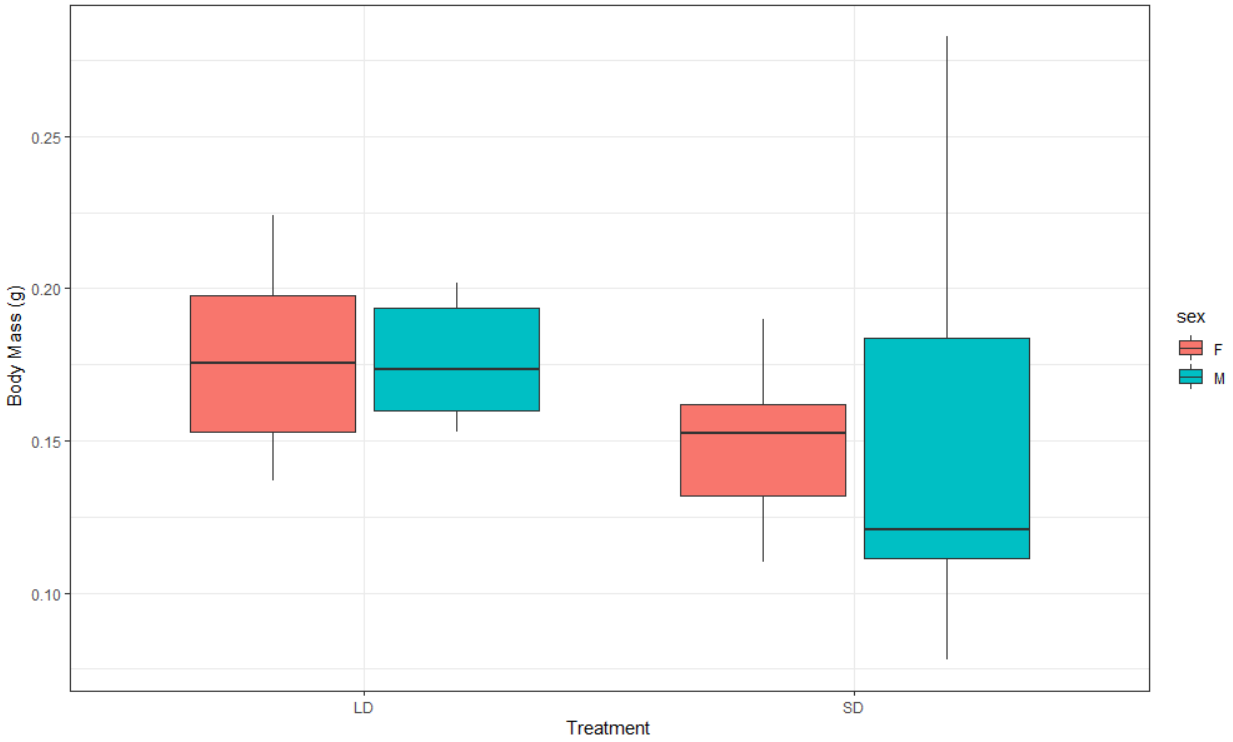

Figure 1. There were no significant differences in body mass between the long day (LD) and short day (SD) treatments as well as female (F) and male (M) day 0 true armyworm moths, *Mythimna unipuncta*. Data presented are median, first and third quartiles and the minimum and maximum, N=8 for each treatment and sex combination.

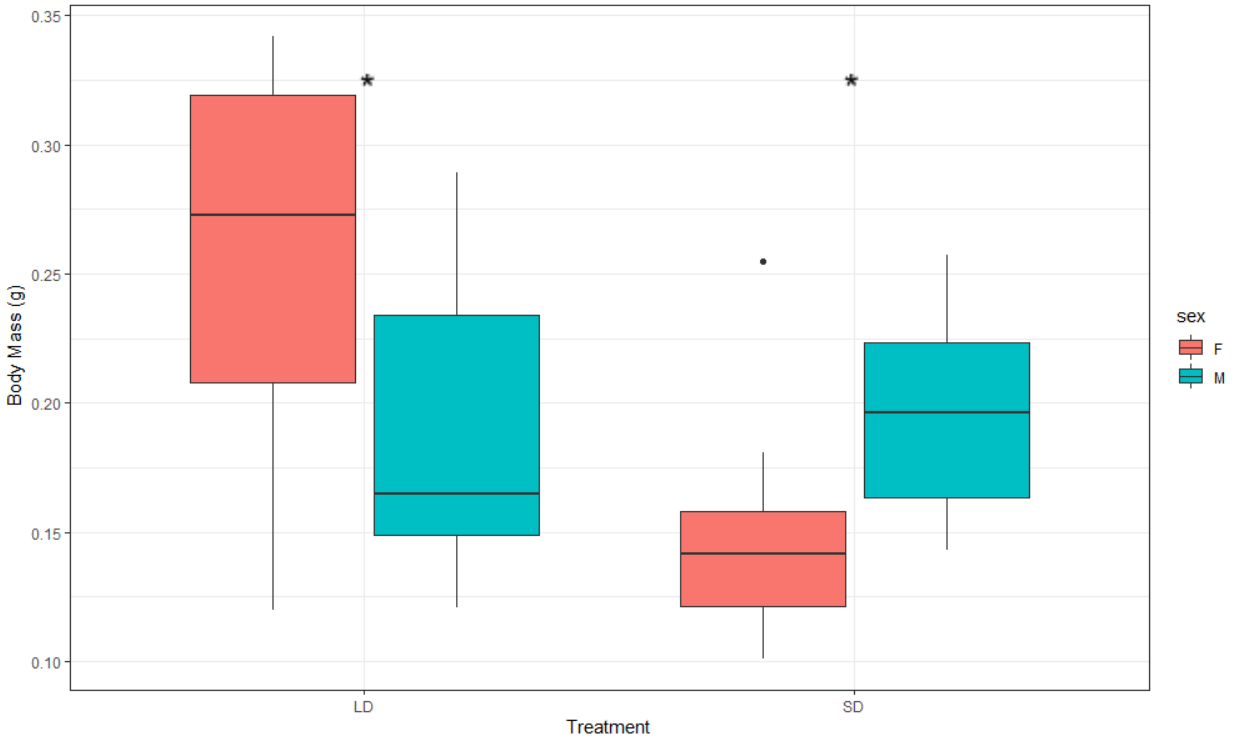

Figure 2. Day 6 female (F) true armyworm moths, *Mythimna unipuncta,* under the long day (LD) treatments were significantly heavier than LD day 6 male (M) moths. Meanwhile, day 6 F SD moths were significantly lighter than the day 6 M SD moths. Data presented are median, first and third quartiles and the minimum and maximum, N=8 for each treatment and sex combination. * indicates significant differences between sexes within treatments and ° indicates significant differences between treatments.

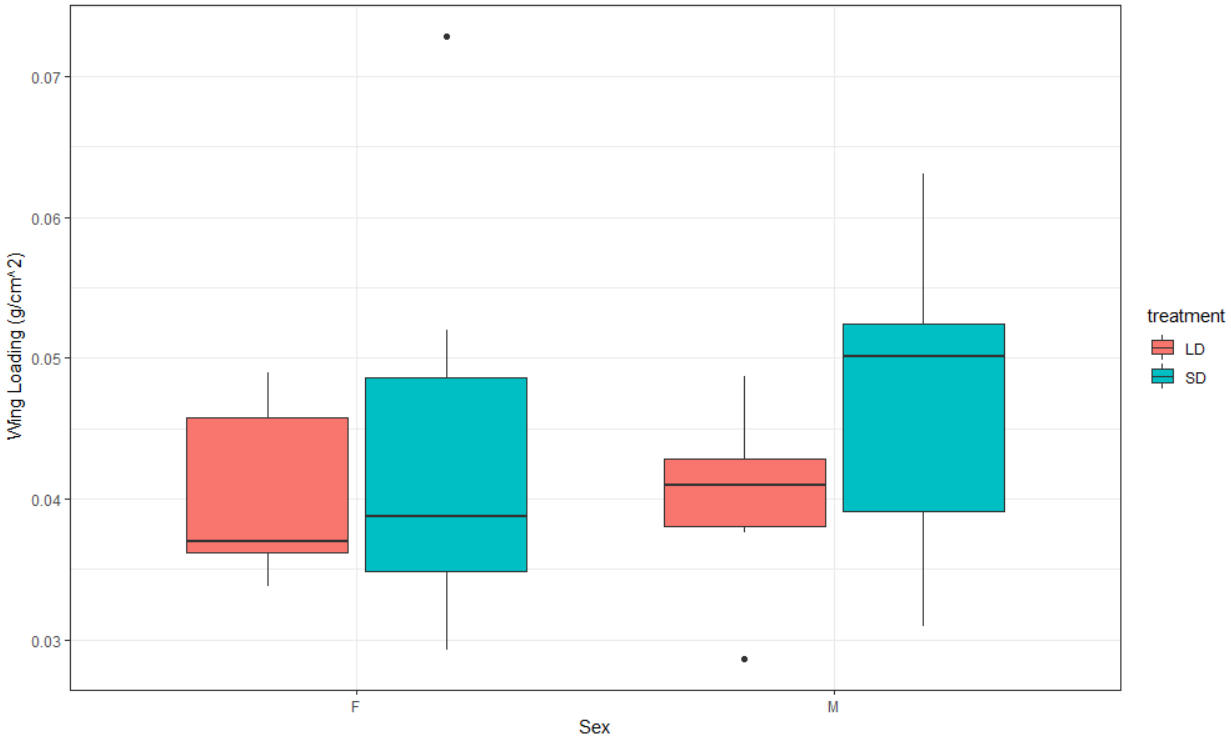

Figure 3. There was no significant difference in wing loading between the long day (LD) and short day (SD) treatments as well as female (F) and male (M) day 0 true armyworm moths, *Mythimna unipuncta*. Data presented are median, first and third quartiles and the minimum and maximum, N=8 for each treatment and sex combination.

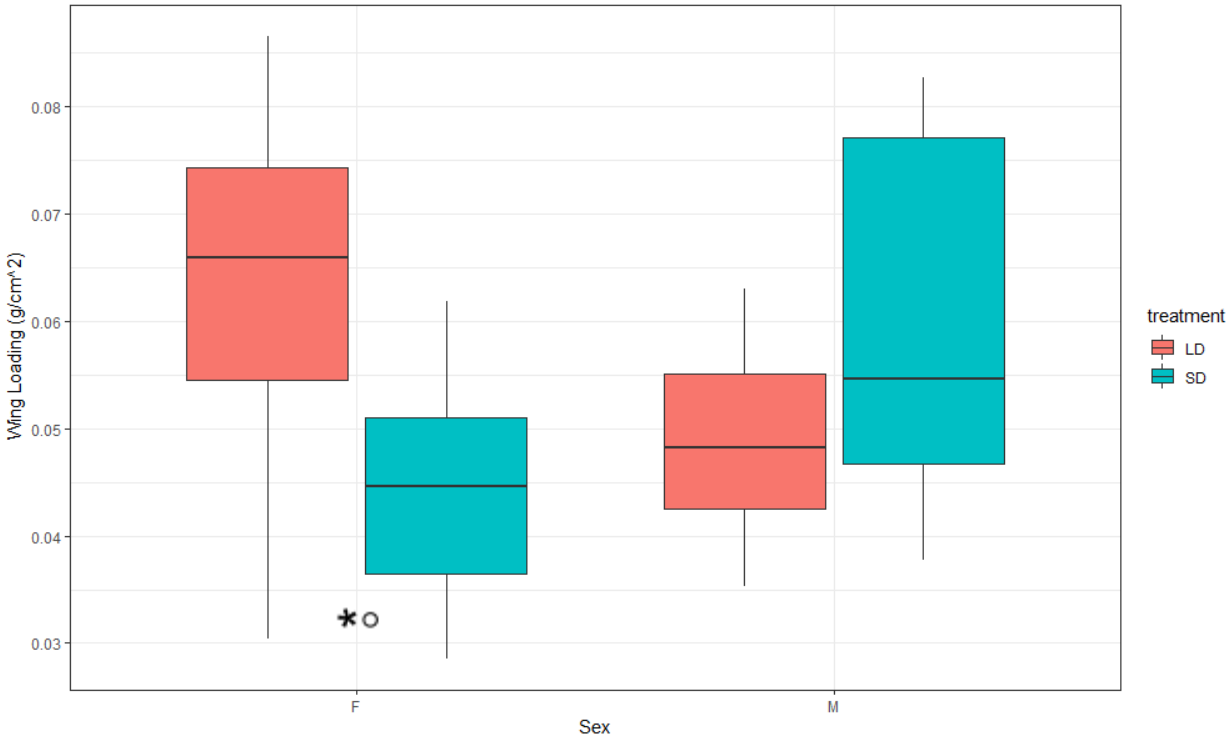

Figure 4. On day 6, female (F) true armyworm moths, *Mythimna unipuncta*, under the long day (LD) treatments had significantly higher wing loading than the short day (SD) females. Additionally, the day 6 female LD moths had significantly higher wing loading than the day 6 male (M) LD moths and the day 6 male SD moths had significantly higher wing loading than the day 6 female SD moths. Data presented are median, first and third quartiles and the minimum and maximum, N=8 for each treatment and sex combination. * indicates a significant difference within sexes and ° indicates a significant difference between treatments.

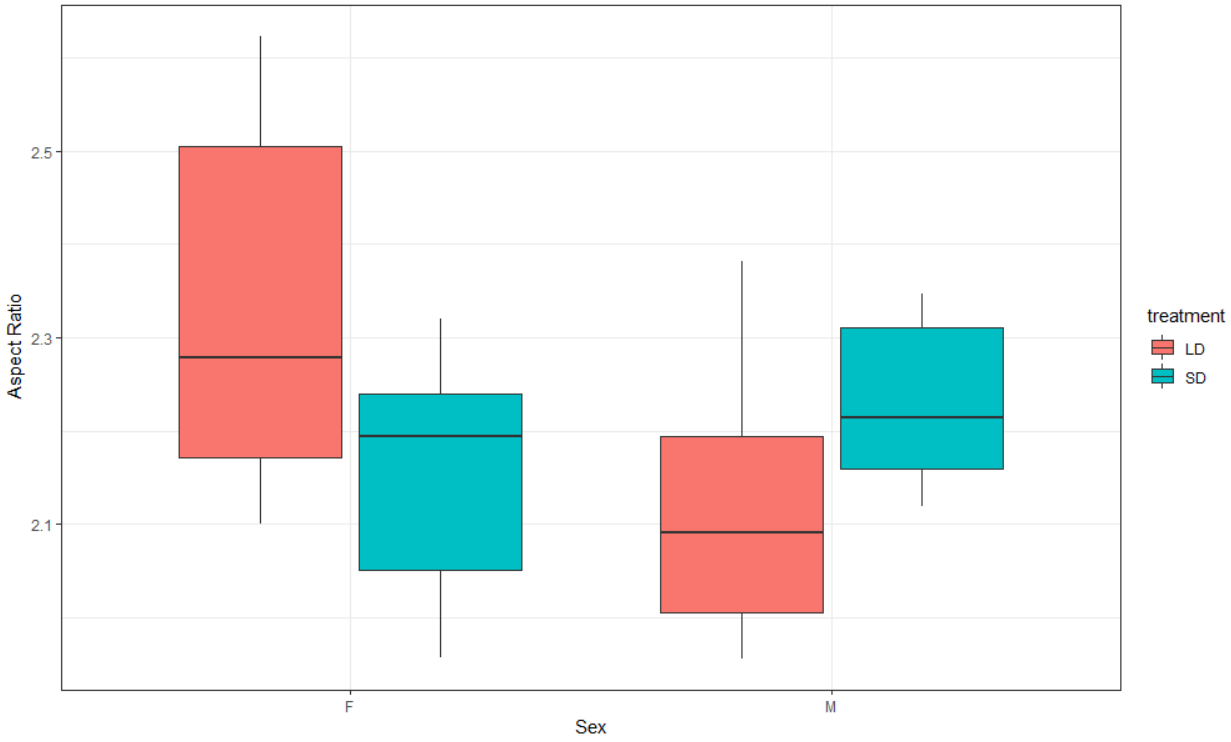

Figure 5. There was no significant difference in aspect ratio between the long day (LD) and short day (SD) treatments as well as female (F) and male (M) day 0 true armyworm moths, *Mythimna unipuncta*. Data presented are median, first and third quartiles and the minimum and maximum, N=8 for each treatment and sex combination.

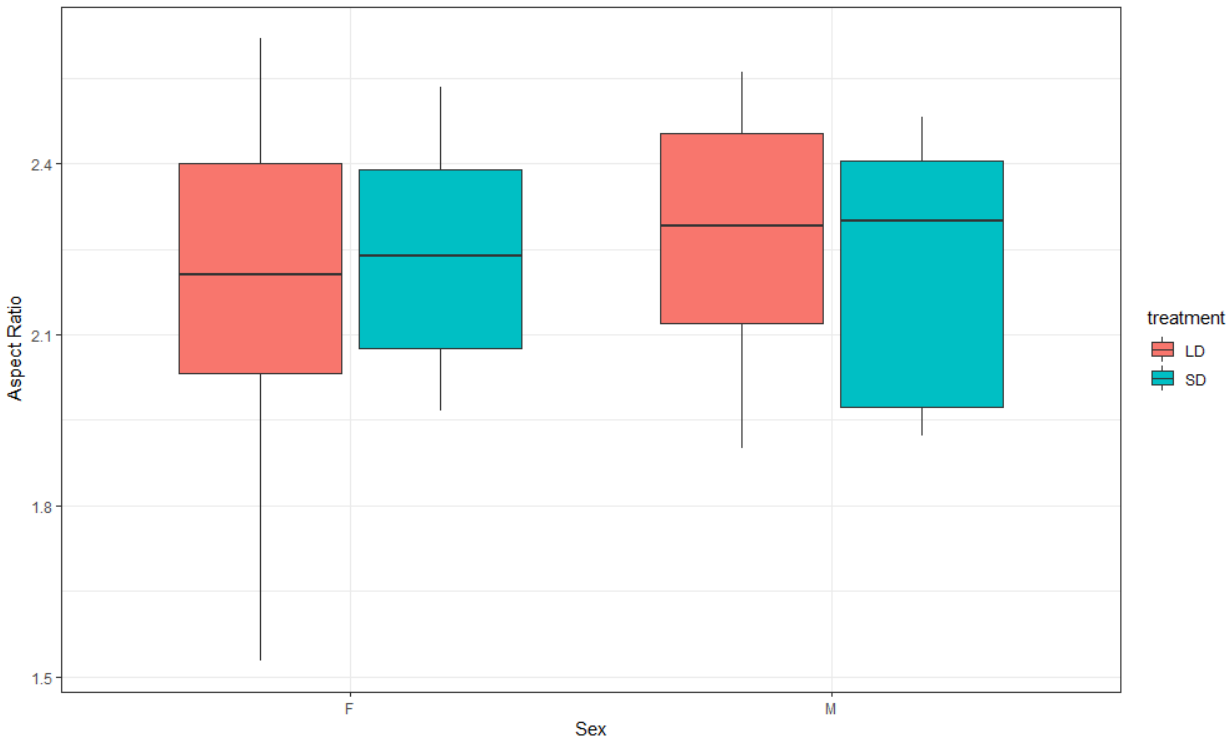

Figure 6. There was no significant difference in aspect ratio between the long day (LD) and short day (SD) treatments as well as female (F) and male (M) day 6 true armyworm moths, *Mythimna unipuncta*. Data presented are median, first and third quartiles and the minimum and maximum, N=8 for each treatment and sex combination.

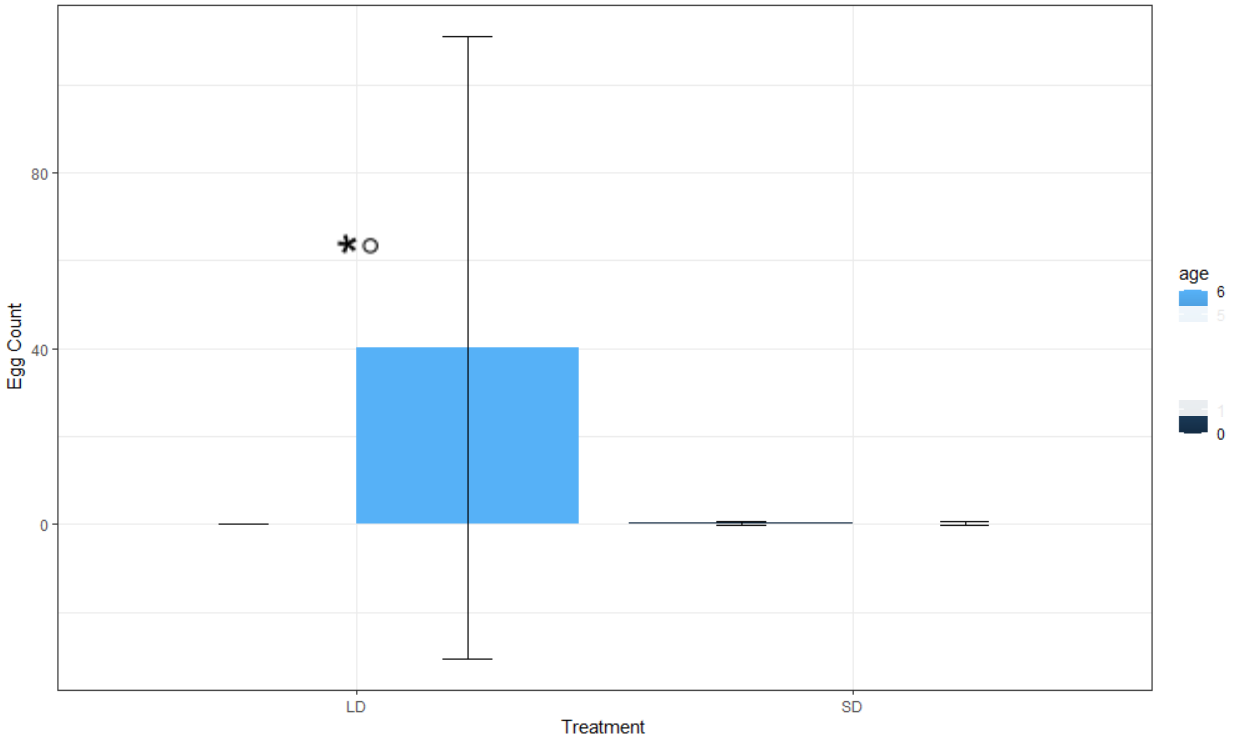

Figure 7. Day 6 female true armyworm moths, *Mythimna unipuncta*, under the long day (LD) treatments had a significantly larger number of eggs than the day 6 short day (SD) females and the day 0 LD females. Data presented are mean ± SD, N=10 for each treatment and age combination. * indicates a significant difference within treatments and ° indicates a significant difference between ages.

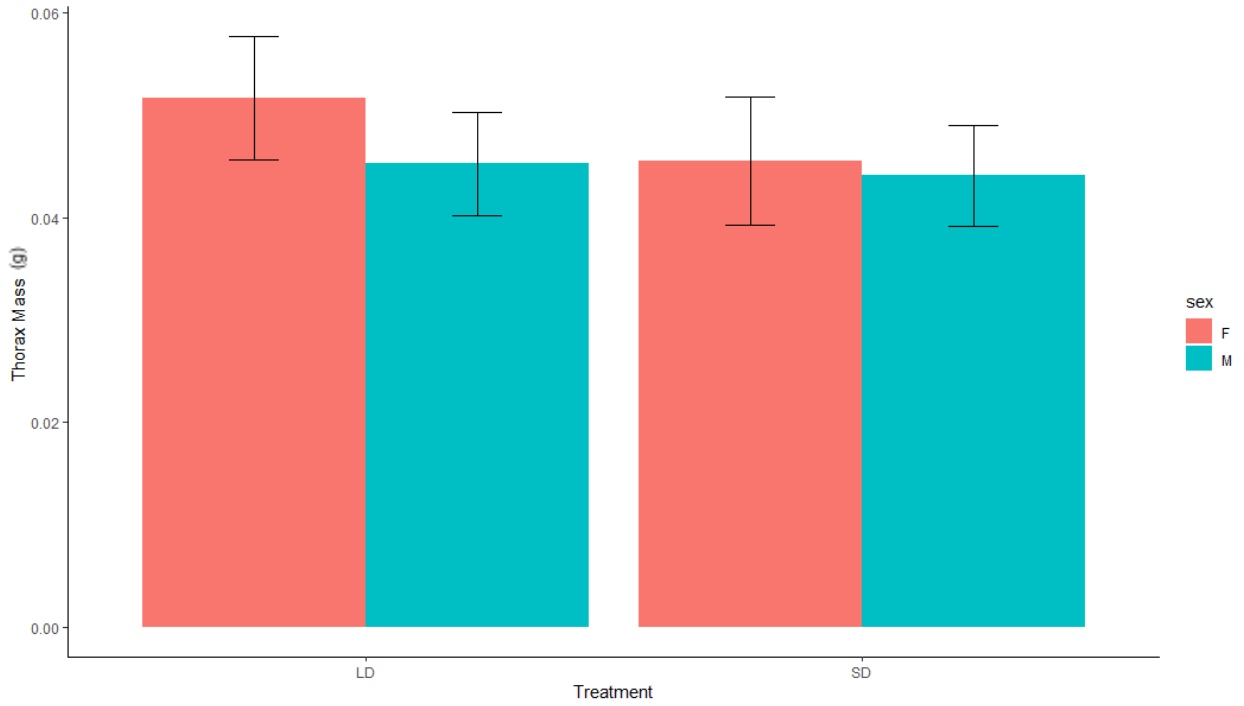

Figure 8. True armyworm moths, *Mythimna unipuncta*, did not have significantly different thorax masses between treatments (LD: Long day, SD: Short day) or sexes (M: Male, F: Female). Data presented are mean ± SD, N=15 for each treatment and sex combination.

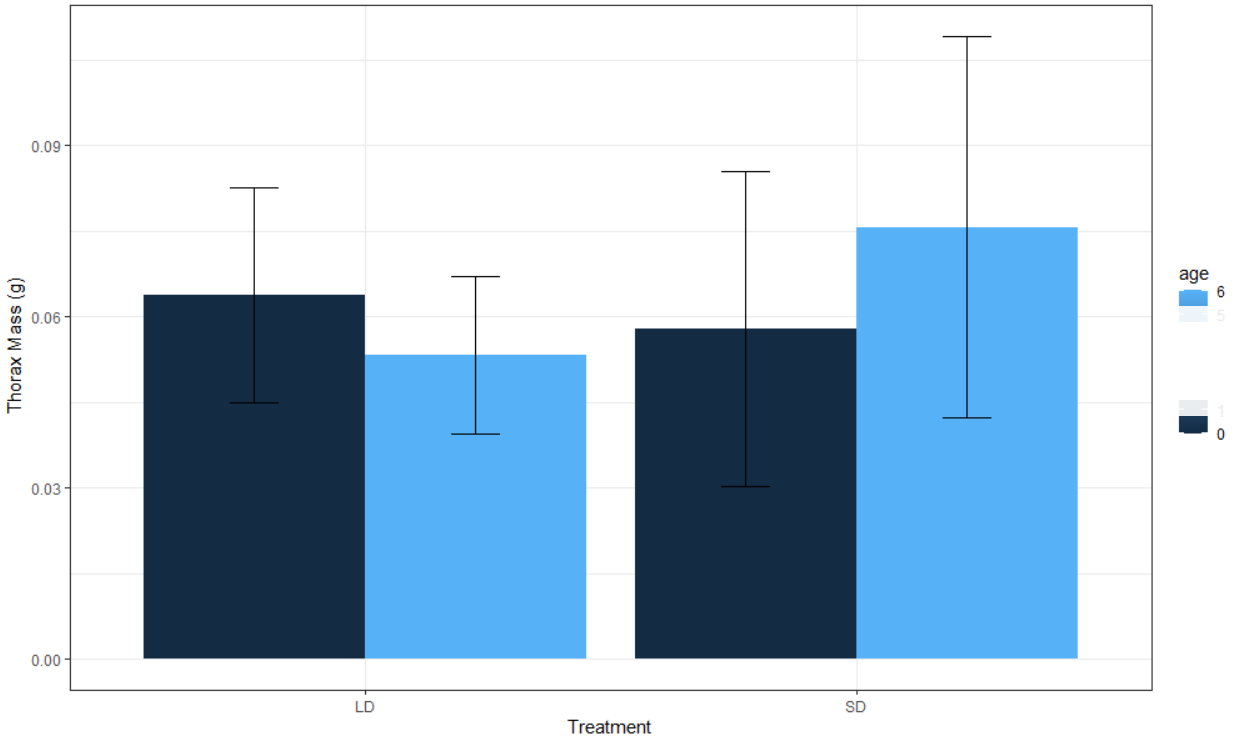

Figure 9. The long day (LD) and short day (SD) treatments and age made no significant difference in the thorax mass of the female true armyworm moths, *Mythimna unipuncta*. Data presented are mean ± SD, N=10 for each treatment and age combination.

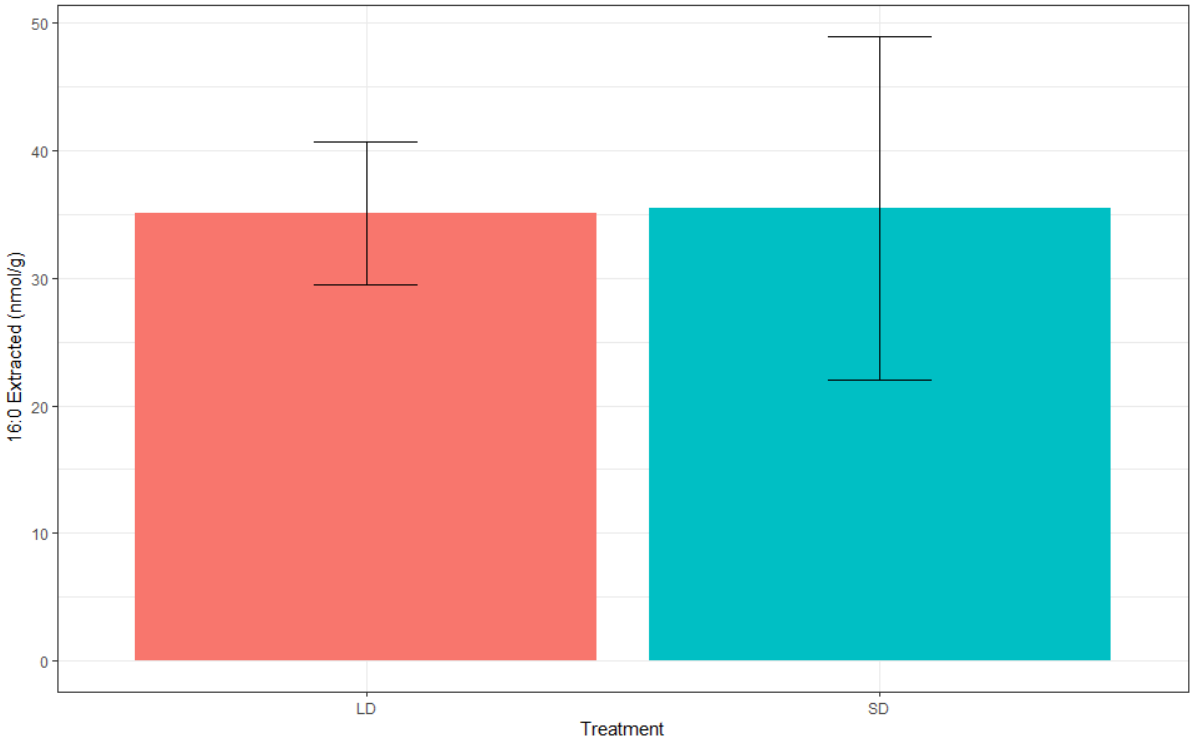

Figure 10. There was no significant difference in 16:0 fatty acid stores of the fat body between the long day (LD) and short day (SD) treatments for the true armyworm moths, *Mythimna unipuncta*. Data presented are mean ± SD, N=6 for each treatment.

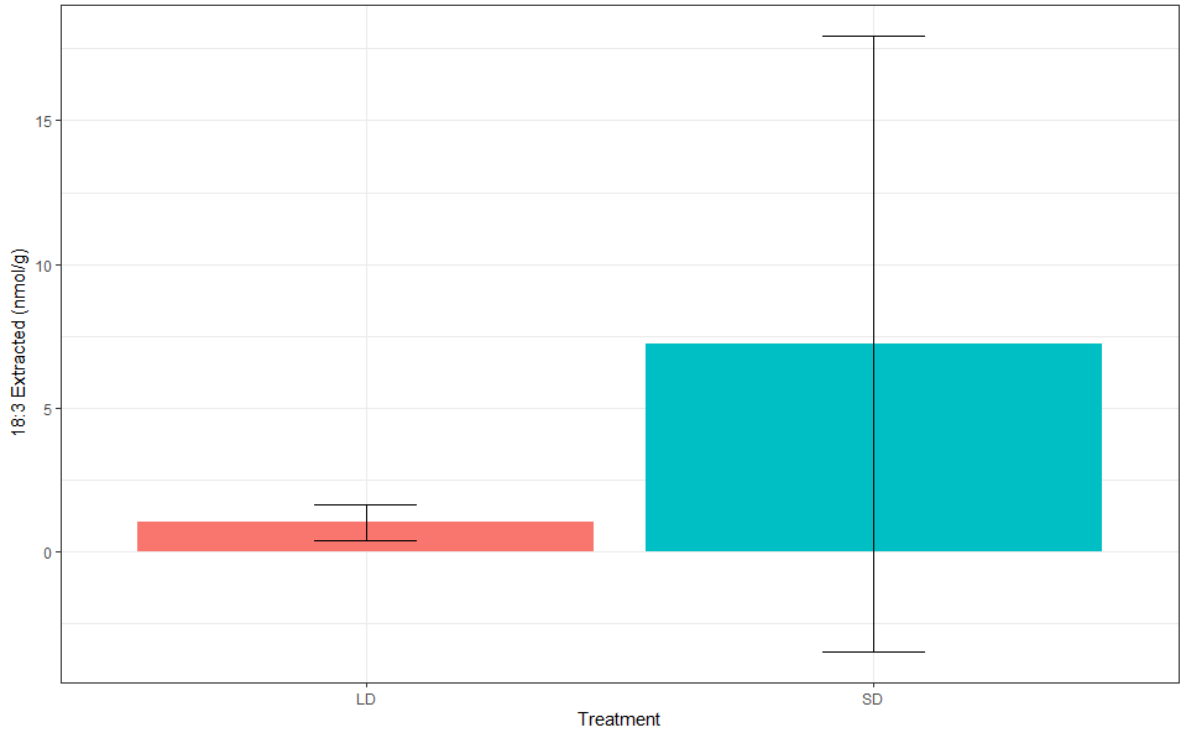

Figure 11. There was no significant difference in 18:3 fatty acid stores of the fat body between the long day (LD) and short day (SD) treatments for the true armyworm moths, *Mythimna unipuncta*. Data presented are mean ± SD, N=6 for each treatment.

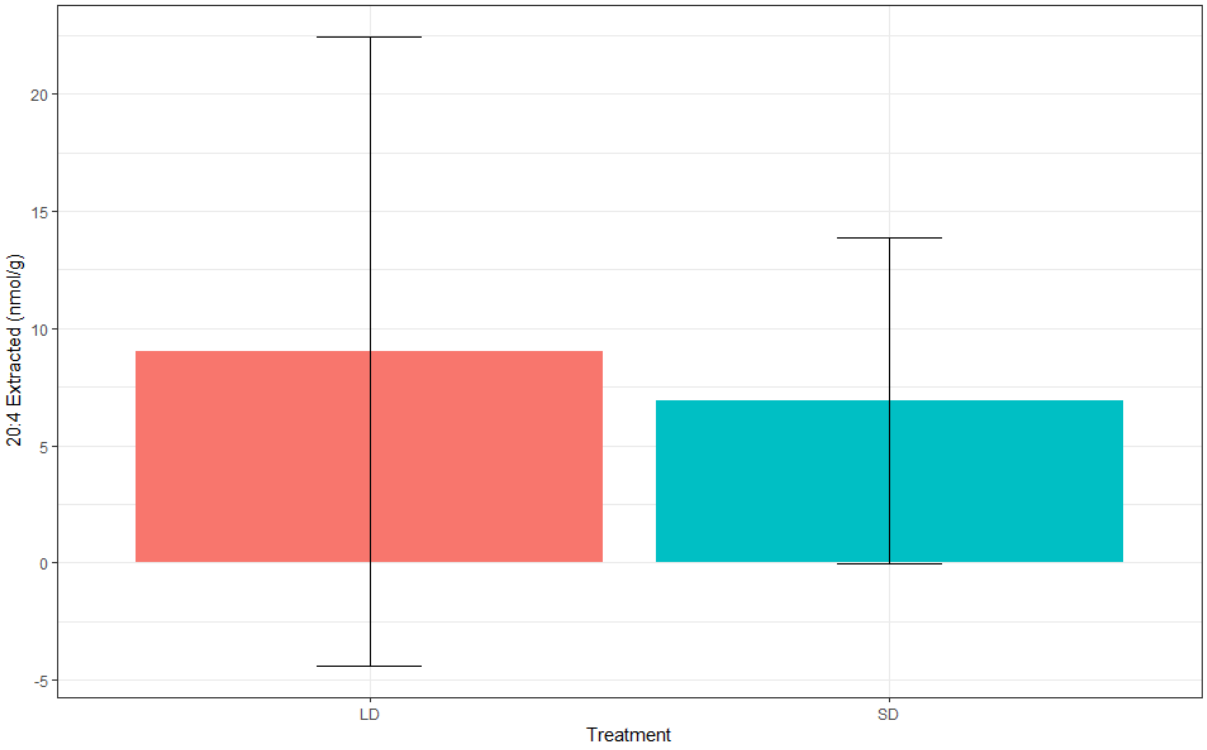

Figure 12. There was no significant difference in 20:4 fatty acid stores of the fat body between the long day (LD) and short day (SD) treatments for the true armyworm moths, *Mythimna unipuncta*. Data presented are mean ± SD, N=6 for each treatment.

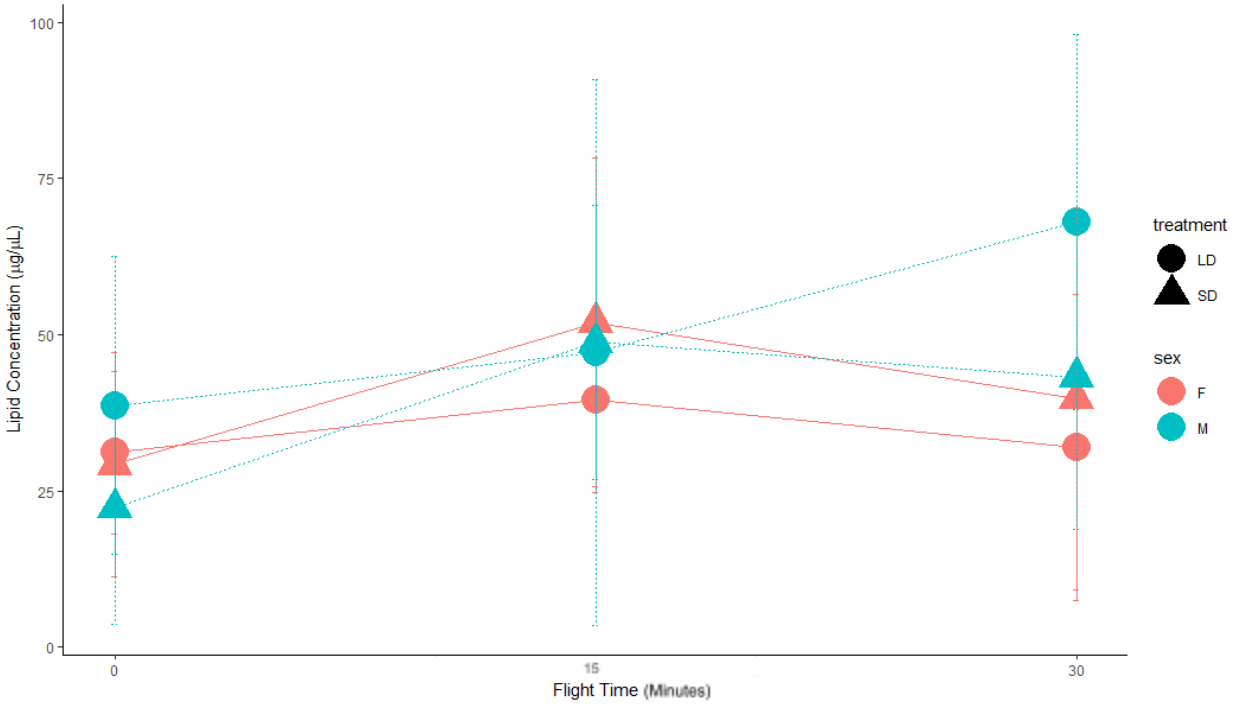

Figure 13. There was no significant difference in haemolymph lipid concentration between the long day (LD) and short day (SD) treatments as well as female (F) and male (M) in the true armyworm moths, *Mythimna unipuncta*. Data presented are mean ± SD, N=15 for each sex and treatment combination.

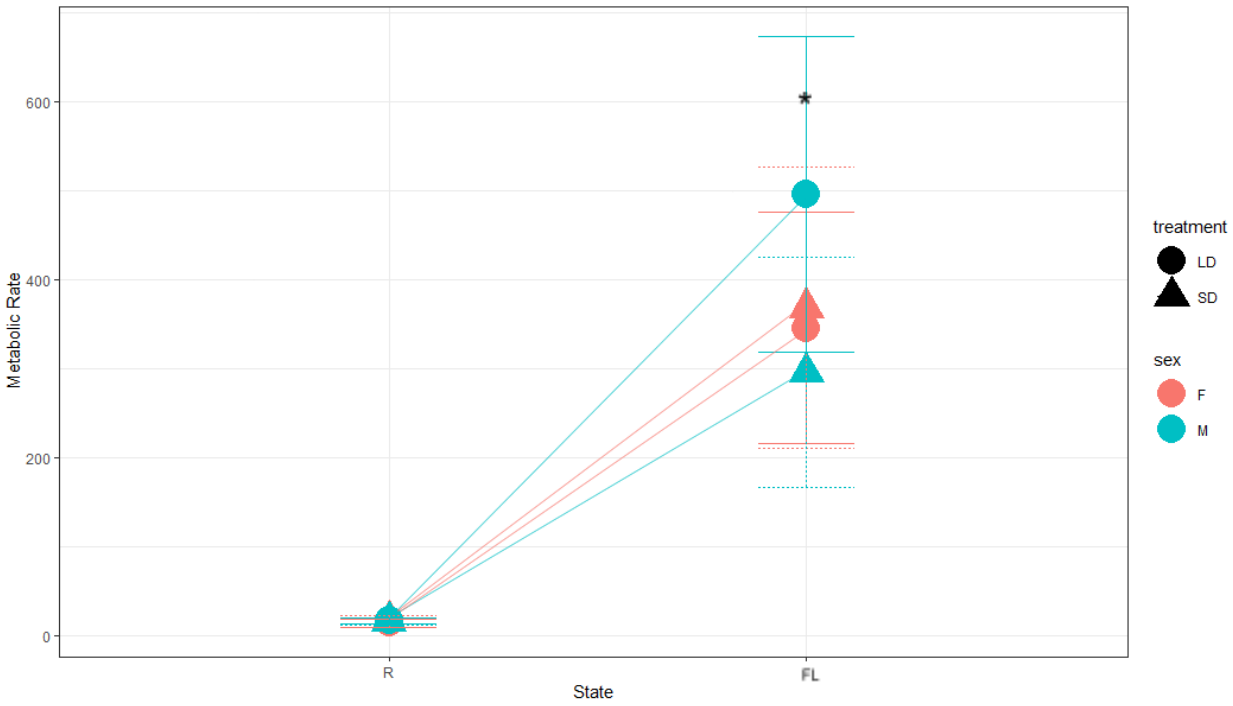

Figure 14. At rest (R), there was no difference in the metabolic rate of the true armyworm moths, *Mythimna unipuncta*, regardless of the sex (M: Male, F: Female) or long day (LD) and short day (SD) treatment. However, there was a significant increase in the flying (FL) metabolic rate of the male LD moth compared to the male SD moth. Data presented are mean ± SD, N=15 for each sex and treatment combination. * indicates a significant difference between treatments within the males and ° indicates a significant difference between treatments within females.

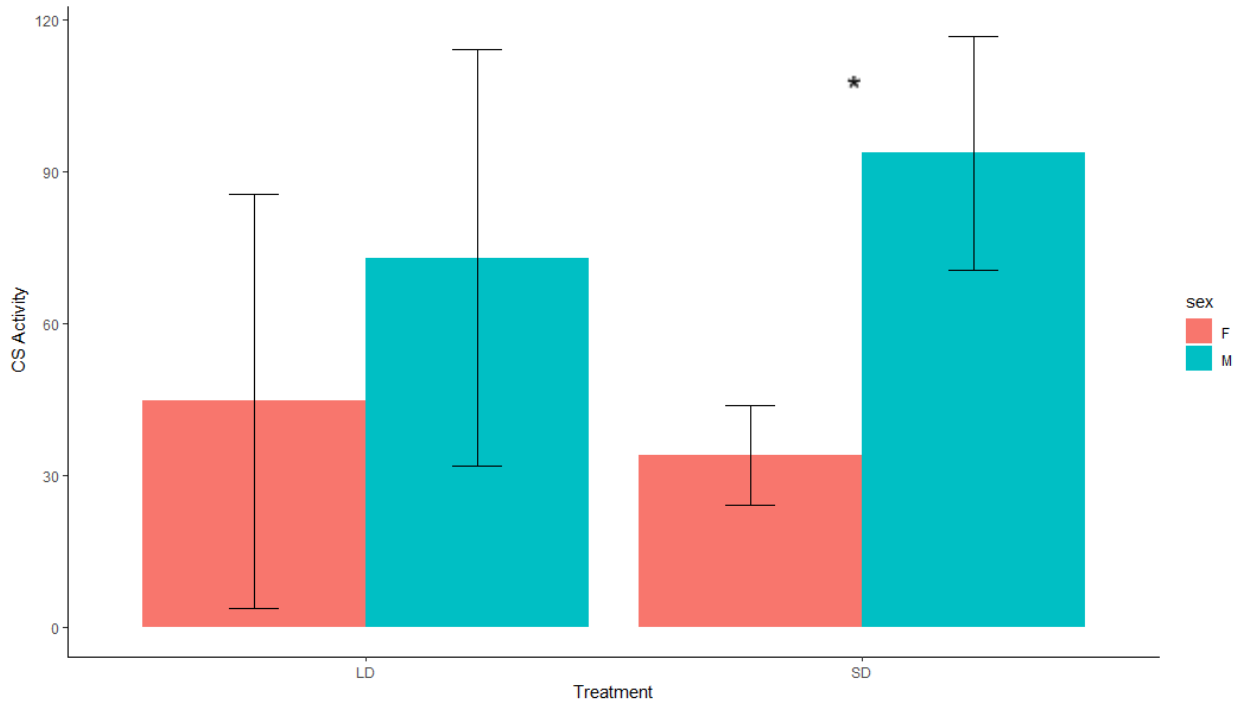

Figure 15. There was a significant difference in citrate synthase (CS) activity between sexes in the true armyworm moths, *Mythimna unipuncta*. The short day (SD) males (M) had significantly higher CS activity compared to the long day (LD) females (F) and the SD females. Data presented are mean ± SD, N=15 for each treatment and sex combination.* indicates a significant difference between treatments and ° indicates a significant difference within sexes.

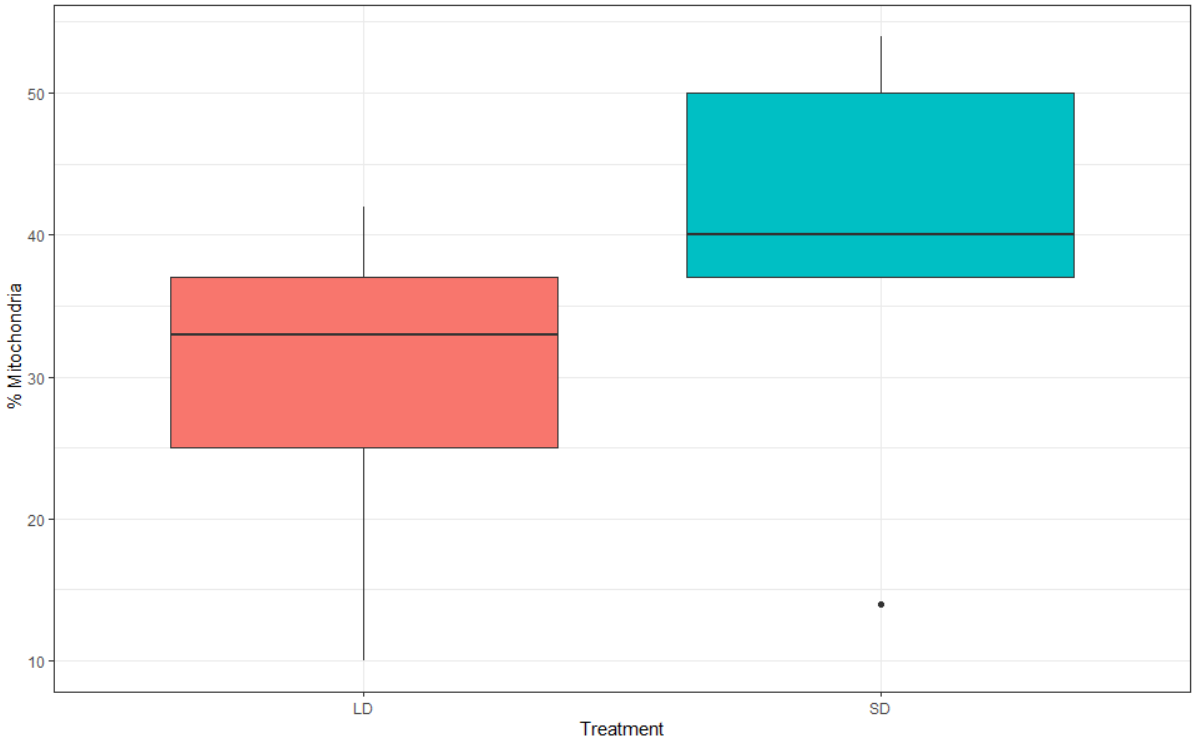

Figure 16. There was no significant difference in the percent mitochondrial volume density in the thorax between the long day (LD) and short day (SD) treatments of the true armyworm moths, *Mythimna unipuncta*. Data presented are median, first and third quartiles and the minimum and maximum, N=8 for each treatment and sex combination.

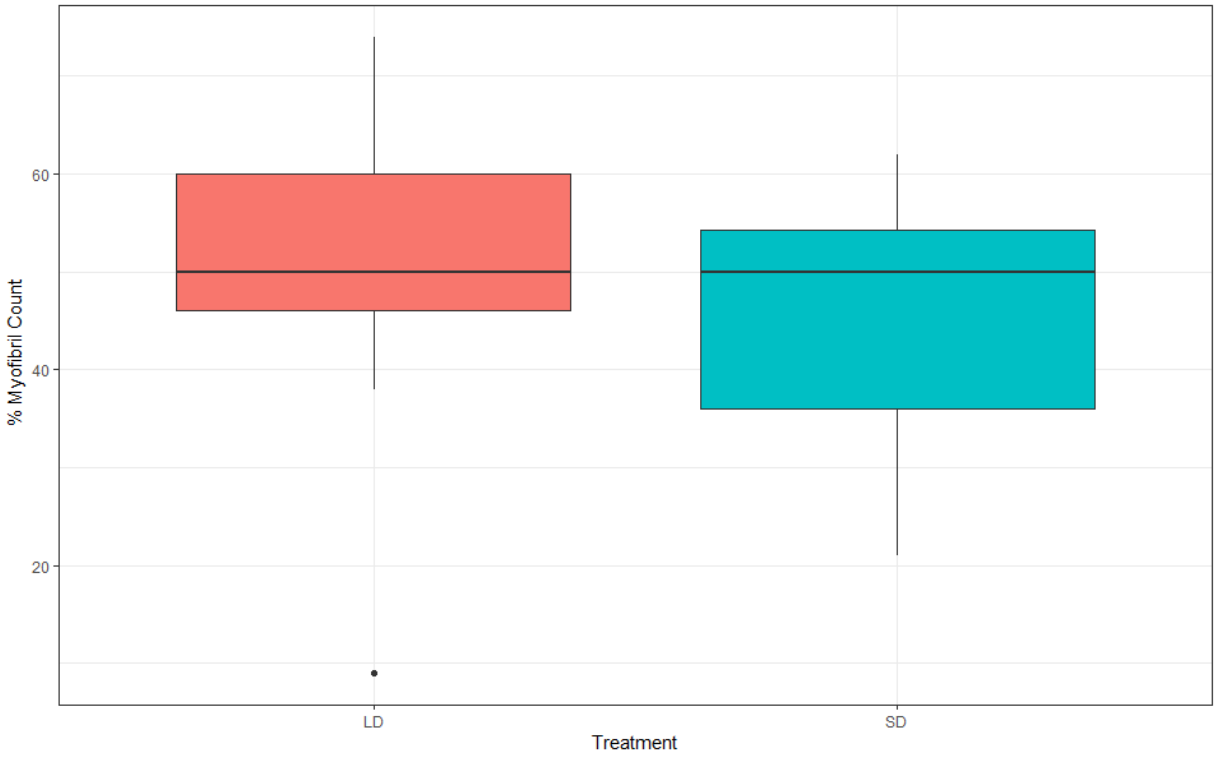

Figure 17. There was no significant difference in the percent myofibril volume density in the thorax between the long day (LD) and short day (SD) treatments of the true armyworm moths, *Mythimna unipuncta*. Data presented are median, first and third quartiles and the minimum and maximum, N=8 for each treatment and sex combination.