ENERGY LEVELS INCREASE WHILE SURVIVABILITY DECREASES AS

TEMPERATURE RISES ACROSS DROSOPHILA SPECIES

BY

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ABSTRACT OF THE THESIS

Energy Levels Increase while Survivability Decreases as Temperature Rises
Across Drosophila Species

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In response to changes in temperature, organisms that are adapted to survive cold temperatures implement thermoregulatory systems that adjust their growth, locomotion, reproduction, and other physiological functions. We predict species that have been isolated in an extreme environment will have thermoregulatory and compensatory mechanisms increasing their tolerance to survive adverse conditions. For example, in ice worms, an increase in adenosine triphosphate (ATP) levels has been associated with cold tolerance. The recycling of Adenosine monophosphate or adenosine monophosphate (AMP) regulates adenosine diphosphate (ADP) levels. Thus, the degradation of AMP by AMP phosphatase (AMPP) and AMP deaminase (AMPD) controls the levels of ATP. We use *Drosophila* species endemic to different environments to study how changes in temperature affect them. *Drosophila* species thrive in different environments on the globe, thus providing a system to answer evolutionary questions about temperature adaptation. To test these mechanisms, we used *D. melanogaster* a temperate, widely distributed species, a *D. funebris* strain native to Alaska, and *D. mojavensis* a cactolaphilic species. We measured ATP levels, survivability and mobility of these flies at a diverse range of temperature points. Species-specific differences in tolerance to these abnormal temperatures were observed.

ACKNOWLEDGEMENTS

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INTRODUCTION

Adenosine-5'-triphosphate (ATP) is an essential molecule to all life on earth. ATP is a unit of intercellular energy transfer used in numerous cellular processes by enzymes and structural proteins for mobility, anabolism and cellular division. In eukaryotic organisms there are three main pathways to produce ATP- glycolysis, citric acid cycle/oxidative phosphorylation, and beta-oxidation. ATP is formed with the help of many enzymes and regulatory mechanisms through a series of phosphorylation starting with adenine (Fig. 1) (Napolitano and Shain 2005). Psychrophiles are extremophilic organisms that have adapted to live and prosper in cold environments as low as -15°C, while mesophilic organisms live in moderate temperatures around 20-45°C.

Some psychrophilic glacier species including, *Mesenchytraeus solifugus,* have increased levels of ATP compared to their mesophilic counterparts and that these levels of ATP increase further as temperatures decrease (Napolitano *et al.* 2004). This ability of *M. solifugus* to increase already relatively high levels of adenylates as temperatures lower may enhance their survivability on the glacier ice (Napolitano *et al.* 2004). To survive, psychrophilic organisms need to maintain adequate energy levels to continue their normal biological process at cold temperatures, where in mesophilic organisms would cease to function at that temperature (Belehradek 1935; Willis 1987). Psychrophiles have a compensatory mechanism to increase ATP levels and subsequently increase survivability in cold temperatures (Napolitano *et al*. 2004). In *E. coli* manipulation of AMP degradative genes were able to increase ATP levels and this increase of

ATP levels was able to increase cold tolerance of *E. coli* ten-fold when being stored at 0°C (Parry and Shain 2011).

Additional mechanisms to increase cold tolerance have been discovered in other organisms including membrane viscosity (Hazel, 1995), mitochondrial density (Johnston et al., 1988; Guderley, 1998; Johnston et al., 1998), metabolic rate (Peck, 2002), specific enzyme activities (Crockett and Sidell, 1990) and modulation of the cellular environment (Pˆrtner et al., 1998).

Thus, studying the mechanisms underlying cold tolerance in flies offers the promise of the extension of organ transplantation times. Currently, hearts can be preserved for 4-6 hours outside of a body, while liver, kidney, and pancreas can withstand 24-48 hours (Stringham *et al*. 1992). ATP is vital to the storage of viable organs and once the ATP present in an organ is depleted, the organ tissue will start necrosing and undergoing rigor mortis making it unsuitable for transplantation (Stringham *et al*. 1992).

Drosophila melanogaster is a powerful genetic model system to study mechanisms of cold tolerance. In addition, we take advantage of the many species of *Drosophila* endemic to different environments. Experimentation on the *Drosophila* includes three wild-type species representative of different environmental conditions, which include *D. melanogaster, D. funebris,* and *D. mojavensis*. *D. melanogaster* has the widest range of the three species living in temperate conditions and *D. melanogaster* is historically the most used and most well characterized species of all fruit flies. *D. funebris* is a species from a cold

habitat and the particular starin used for experimentation was isolated from Big Lake, Alaska. *D. mojavensis* is representative of desert environment and known to live and thrive in and around cacti*.*

We found species-specific differences in ATP levels, survivability and mobility. Specifically, we found that the largest percentage increase in ATP levels and mobility in *D. funebris* occurred between 9°C and 13°C while in *D. mojavensis* the largest increase occurred between 13°C and 23°C. The species with the largest increase in survivability at 13°C compared to 23°C was *D funebris*. D. melanogaster had a 25% death rate in the first six days of the survivability assay whereas, both D. funebris and D. mojavesis had no major die-off.

MATERIALS AND METHODS

Flies
The following *Drosophila* species were used in this study: *D. melanogaster* (wild-type OreR)*, D. funebris,* and *D. mojavensis* (The San Diego Stock Center)*. All flies were maintained on standard cornmeal media at the specified temperature for each experiment.

ATP Assay:
ATP concentration was determined using a Roche ATP Bioluminescence Assay Kit HSII and assay was performed as specified in user manual (Roche Applied Science, Germany). To test ATP concentration, third instar larvae were stored at desired temperature for 24 hours prior to experimentation. ATP levels were determined at 9°C, 13°C, 23°C, and 30°C. Ten third-instar larvae were homogenized in an eppendorf tube with a micropestle for 45 seconds in cell lysis reagent to release ATP into solution. The sample was then boiled for 5 minutes and centrifuged at 16060 x g for 3 minutes. The supernatant was then collected and centrifuged again at 16060 x g for 10 minutes. The supernatant is then placed immediately flash frozen in liquid Nitrogen and stored at -80°C until performing ATP concentration and Protein concentration assays.

For ATP assays, 50µL luciferase reagent, which contains a bioluminescence enzyme, was added to 50µL of a 1/1000 dilution of the homogenized larval solution. Luminescence was measured in a Glomax 20/20 luminometer with single auto injector (Promega). The intensity of luminescence produced correlates with the levels of ATP present in the sample. ATP levels were determined by comparison to a standard curve. The standard curve was

composed of 8 samples of known concentration of ATP (1.00x10^3 mg ATP/ml, 5.00x10^4 mg ATP/ml, 1.00X10^4 mg ATP/ml, 5.00X10^5 mg ATP/ml, 1.00X10^5 mg ATP/ml, 5.00X10^6 mg ATP/ml, 1.00X10^6 mg ATP/ml, 5.00X10^7 mg ATP/ml). The ATP concentration was then normalized against protein concentration determined by using a Bradford protein assay.

Protein Assay:
Protein concentration was determined by using a Bradford Protein Assay (Thermo Scientific Pierce). To measure protein concentration a 50µL sample of homogenized larval solution was mixed with 1.5mL of Coomassie reagent. The samples absorbance was measured at 595nm in a Beekman Coulter DV 530 UV/Vis spectrophotometer. The absorbance correlates to the protein concentration. The absolute concentration of protein was determined by comparing the level of absorbance to a standard curve. The standard curve consisted of nine samples of known concentration of bovine serum albumin (BSA) (2000µg/mL, 1500 µg/mL, 1000 µg/mL, 750 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 25 µg/mL, 0 µg/mL).

Survivability Assay:
Survivability was determined in adult flies at 23°C and 13°C. Flies less than 24 hours old were collected for the trials. Five males and five females were placed in a vial and kept in incubators at desired temperature. The vials were observed daily and the number of flies that remained alive in each vial was recorded. Vials are flipped regularly so that the progeny do not develop into adults and contribute to the results.

Mobility Assay:

Larval mobility was measured in third-instar larvae. Larvae were maintained at desired temperature for 24 hours prior to experimentation. The mobility of larvae was determined at the following temperatures, 9°C, 13°C, and 23°C. One larva was placed in the center of a temperature conditioned Luria Broth (LB) Ampicillin agar plate. Then 4μL of a liquid culture of ampicillin resistant *E. coli* was placed directly on top of the larvae. The larva was then allowed to crawl on the plate for 10 minutes. The path of larval crawling is reflected in the form of *E.* coli growth after an over night incubation at 37°C. The plates are then imaged and the distance traveled is measured using Image J software (Rasbend 1997).

RESULTS

ATP levels are reduced in lower temperature

Three species of *Drosophila* were used in the measurement of ATP levels, *D. melanogaster, D. funebris,* and *D. mojavensis*. ATP levels were measured at 4 temperatures: 9°C, 13°C, 23°C, and 30°C. When comparing intra-species differences in ATP levels, in *D. melanogaster* there is a significant increase in the level of ATP at 23°C as compared to 13°C (Fig. 2). In *D. funebris,* there is a significant increase in the levels of ATP at both 13°C and 23°C when compared to 9°C and 13°C, respectively (Fig. 2). In *D. mojavensis* there is a significant increase in the levels of ATP at both 13°C and 23°C when compared to 9°C and 13°C, respectively (Fig. 2). In *D. melanogaster* there is a significant decrease in the level of ATP at 30°C compared to 23°C (Fig. 2). Also, in *D. mojavensis* there is a significant decrease in the level of ATP at 30°C when compared to 23°C (Fig. 2).

Amongst the three species *D. melanogaster* had significantly lower ATP level than *D. funebris* at 13°C and 30°C and significantly lower ATP levels than *D. mojavensis* at all four temperature points (Fig. 2). Also, *D funebris* has significantly lower ATP levels at 9°C, 13°C, and 23°C compared to *D. mojavensis* (Fig. 2).

To compare the changes between temperatures we converted the data to percent change and we used the levels of ATP at 23°C as the 100%. Concentration of ATP for each fly species at 23°C is set to 100% and then the

values at 9°C, 13°C, and 30°C are given as a percent compared to 23°C. It can again be seen in all three species that the trend from 9°C to 13°C and from 13°C to 23°C is towards an increase in the of ATP, in contrast to the decrease from 23°C to 30°C (Fig. 3). In *D. melanogaster* when being compared to 23°C, the ATP level at 9°C, 13°C and 30°C are reduced by 74.7%, 58.4% and 41.5% respectively (Fig. 3). In *D. funebris*, when being compared to 23°C, the ATP level at 9°C, 13°C and 30°C are reduced by 85.5%, 34%, and 3.6% respectively (Fig. 3). In *D. mojavensis* when being compared to 23°C, the ATP level at 9°C, 13°C and 30°C are reduced by 73.7%, 48.3%, and 74.2% (Fig. 3).

Life span increases as temperatures decrease

The survivability assays for, *D. melanogaster, D. funebris,* and *D. mojavensis* were performed at 23°C and 13°C. The assays were performed at 23°C and 13°C. All three species were observed to have a longer life span at 13°C than at 23°C (Fig. 4 A-C). The median survival point (point at which 50% of the flies died) in *D. melanogaster* at 13°C was 70 days while at 23°C it was 37 days (Fig. 4A). The median survival point of *D. funebris* at 13°C is 74 days and at 23°C is 35 days (Fig. 4B). In *D. mojavensis* the median survival point at 13°C is 42 days while at 23°C is was 32 days (Fig. 4C). The longest lived *D. melanogaster* died at 152 days and 65 days at 13°C and 23°C, respectively. In *D. funebris* the oldest flies died at 153 days and 82 days for 13°C and 23°C, respectively. While, *D. mojavensis* lived for a much shorter amount of time at 53 days and 43 days at 13°C and 23°C, respectively.

Mobility is reduced as temperatures decrease

Mobility results were obtained using third instar larvae for, *D. melanogaster, D. funebris,* and *D. mojavensis* at three temperatures 9°C, 13°C, and 23°C. Experiments were performed on Luria Broth (LB) Ampicillin agar plate and using ampicillin resistant *E. coli* the path larvae crawled can be measured. For all three species the trend is towards an increase in mobility at 13°C compared to 9°C and at 23°C compared to 13°C (Fig. 5). In *D. melanogaster* there is a significant increase in the mobility of the flies at 13°C when compared to 9°C. In *D. funebris* there is a significant increase in mobility at 13°C and 23°C when compared to 9°C and 13°C respectively (Fig 5). For *D. mojavensis* there is a significant increase in mobility at 13°C and 23°C when compared to 9°C and 13°C respectively (Fig 5). For all three species there is no significant difference in mobility at 23°C, however, all species are significantly different from one another at both 9°C and 13°C. At both 9°C and 13°C, *D. melanogaster* has significantly higher mobility than both *D. funebris* and *D. mojavensis* (Fig. 5). At 9°C *D. mojavensis* is significantly higher mobility than *D. funebris*, while at 13°C *D. funebris* has a significantly higher mobility than *D. mojavensis* (Fig. 5).

To compare the changes between temperatues we converted the data to percent change and used the levels of ATP at 23°C as the 100%. Distance traveled for each fly at 23°C was set to 100%. The distance traveled at 9°C and 13°C are then given in a percent of the 23°C value for that species. For all three

species the trend is to have an increased mobility as temperature increases. In *D. melanogaster*, the distance traveled at 9°C and 13°C is reduced by 49.8% and 27%. In *D. funebris* the reduction at 9°C and 13°C is 90.6% and 34.8%. In *D. mojavensis*, the reduction is 84.4% and 62.2%.

DISCUSION

      The theory of evolution predicts that an organism living in an environment with stable temperature will adapt and better optimize its performance to that temperature range (Levins 1968). In addition when an organism is exposed to a different temperature it will adjust to the new conditions physiologically (Wilson and Franklin 2002). In the three species examined at the diverse spectrum of temperatures there are significant differences in ATP levels, survivability, and mobility both intra and inter species. There are also intriguing similarities and differences when comparing results from different assays in the same species.

ATP levels are reduced in lower temperature

      For all species examined, as the temperature increases towards 23°C the trend is for ATP levels to also increase (Fig. 2). A rise in temperature correlates to a rise in the levels of ATP in order to keep up with the increasing demands of energy (Fedorow *et al.* 1998; English and Storey 2000; Napolitano *et al*. 2004). However, between 23°C and 30°C all species examined display a trend towards a decrease in the ATP levels (Fig. 2). Many heat shock protocols involve exposing an organism to 30°C (Gietz *et al.* 1995). A decrease in the ATP levels of all *Drosophila* species may be due to the undo stress the flies are receiving at such a high temperature.

      The largest absolute and percentage decrease in ATP between two temperature points occurs in *D. mojavensis* from 23°C and 30°C (Fig. 2 and Fig. 3). This is a surprising result since *D. mojavensis* is a species that has adapted

to live in the extreme climate of the Mojave desert, which swings between cold temperatures at night and temperatures reaching 50°C during the day. Having to adapt and live in such low and high temperatures can explain the similar levels of ATP in both 9°C and 30°C, and may represent a mechanism to avoid movement when conditions are not optimal (Fig. 2).

Interestingly, the largest percentage increase in ATP levels between two adjacent temperature points in *D. funebris* occurs from 9°C to 13°C whereas for *D. melanogaster* and *D. mojavensis* the largest increase in ATP occurs from 13°C to 23°C (Fig. 3). Coming from a more consistently cold environment *D. funebris* is expected to be better adapted to lower temperatures and the ability to increase ATP levels at relatively lower temperatures would confer an advantage to the fly for survival and procreation at lower temperatures.

Life span increases as temperatures decrease

All the fly species examined have higher survival at 13°C as seen in the number of days required to reach the median survival point compared to 23°C. Studies have shown that lowering of core body temperature leads to an increase in the life span of that organism (Conti *et al.* 2006). However, there are species-specific differences in survivability. While, all species increased their median survival point, *D. funebris* had the greatest total number of days increase and the largest percentage increase from 23°C to 13°C.

At 13°C, 25% of *D. melanogaster* flies died in the first 6 days of the survivability trials. This is in contrast to *D. funebris* at 13°C, which does not hit the

25% death mark until 62 days into the trial. While, *D. melanogaster* is native to temperate climates, both *D. funebris* and *D. mojavensis* originate from relatively more extreme climates. Interestingly, reduction in temperature to 13°C did not improve considerably the survivability of *D. mojavensis*. The chronic exposure to 13°C may not be advantageous for a desert fly, which may need hot temperatures to survive.

The inverse relationship between ATP levels and survivability where flies at reduced temperatures have decreased ATP levels but increased life span may be explained by the mitochondrial free radical theory of aging (MFRTA). MFRTA proposes that aging is caused by free radicals generation during normal metabolic processes (Harman 1956). Originally thought that free radicals entered the body from an outside source, new evidence found that during normal mitochondrial respiration, oxygen could give rise to free radicals (Boveris and Chance 1973). Multiple components in the mitochondria produce free radicals including complex 1 (Kushnareva *et al.* 2002), complex 3 (Miwa and Brand 2005), glycerol 3-phosphate dehydrogenase (Tretter *et al.* 2007), and alpha-ketoglutarate dehydrogenase (Tretter and Adam-Vizi 2005). These free radicals cause oxidative damage to mitochondrial DNA and the amount of damage negatively correlates to life span (Sohal *et al.* 1995). Flies stored at decreased temperatures would have a reduced rate of metabolism, reduced ATP level and thus a reduced production of free radicals leading to the observed increase in lifespan.

**Mobility is reduced as temperatures decrease**

Mobility is an important aspect for larvae. Being mobile allows the larvae to find adequate food sources and suitable location to pupate. In all three species, distance traveled by the fly larvae increases as the temperature increases. ATP is used directly for movement of organisms (Campbell *et al.* 2006). The mobility for *D. melanogaster* is significantly higher than both *D. funebris* and *D. mojavensis* at 9°C and 13°C (Fig 5). Having a lower level of mobility would be advantageous for both *D. funebris* and *D. mojavensis* at lower than normal temperatures. Moving less and saving energy may be an advantage to species, adapted to live in these harsh environments. The graphs for percent mobility and percent ATP have very similar trends. For both percent ATP and mobility the largest increase occurs between 9°C and 13°C for *D. funebris*, while the largest increase for both percent ATP and mobility in *D. mojavensis* occurs between 13°C and 23°C (Fig. 5 and 6).

Interestingly, at 9°C, 13°C, and 23°C *D. mojavensis* has increased ATP levels over both *D. melanogaster* and *D.funebris,* however it does not have an increased mobility. This discrepancy can be explained by the ATP/ADP ratio. The chemiosmotic hypothesis proposes that fuel use and oxygen consumption are strongly dependent on ADP levels (Lardy and Wellman 1952, Chance and Willians 1955). Although there is a high level of ATP in *D. mojavensis* at 9°C, 13°C, and 23°C a lower level of ADP would make the ATP unavailable to be used in chemical reactions, and thus explains the lower than expected mobility at the same temperatures. This possible high ATP/ADP ratio may be a mechanism

that allowed *D. mojavensis* to conserve energy and adapt to live in desert conditions. In the future further analysis has to be performed to determine ADP levels in the selected species, to understand whether ATP/ADP ratio in the mechanism underlying mobility in different temperatures.

FIGURES

Figure 1
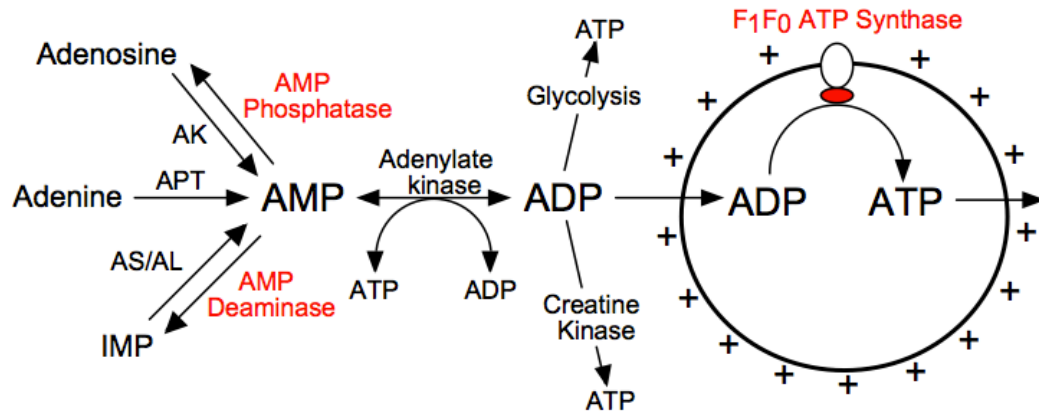
(Napolitano and Shain, 2005)

Figure 1: Overview of ATP production. The production of ATP is highly conserved pathway and is controlled and regulated predominatly by AMP degrative processes.
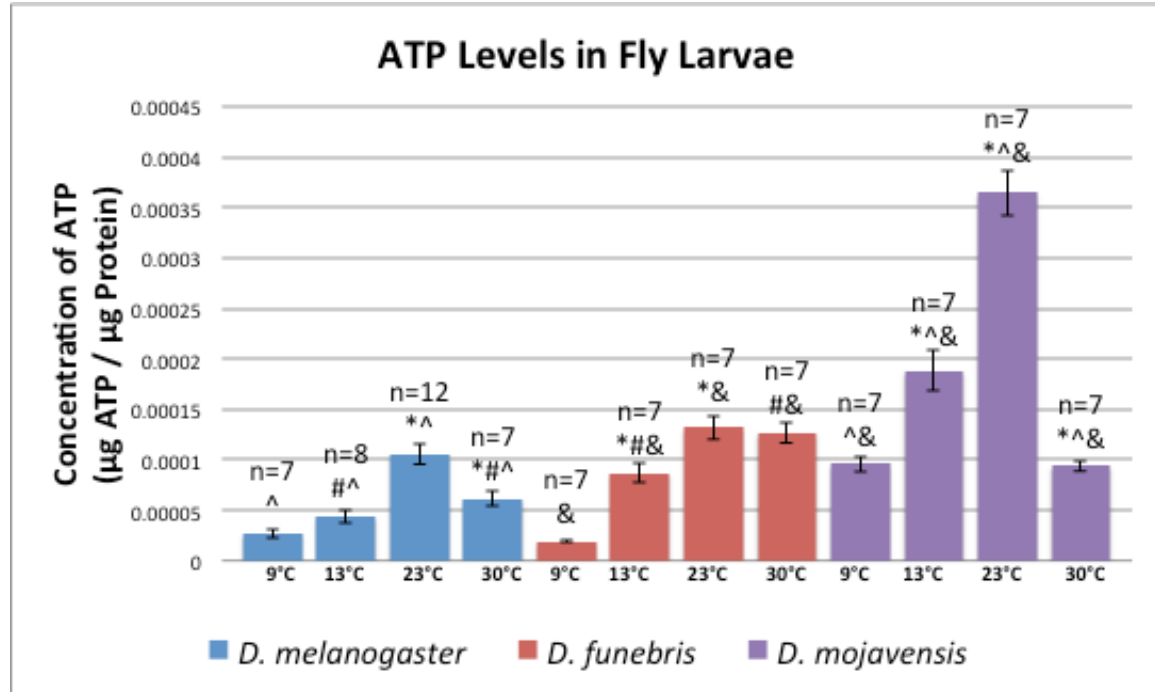
Figure 2

Figure 2: ATP Levels in Fly Larvae. ATP concentraton is shown in mgATP/µg Protein. The absolute concentration of ATP is shown for *D. melanogaster, D. funebris,* and *D. mojavensis* at 9°C, 13°C, 23°C, and 30°C. An * indicates that there is a significant difference in the level of ATP between that temperature and the temperatures directly lower. A # indicates there is a signigicant difference between *D. melanogaster* and *D. funebris* at that temperature. A ^ indicates there is a significant difference between *D. melanogaster* and *D. mojavensis* at that temperature. A & indicates there is a significant difference between *D. funebris* and *D. mojavensis* at that temperature. The cutoff of significance *p*=0.05.
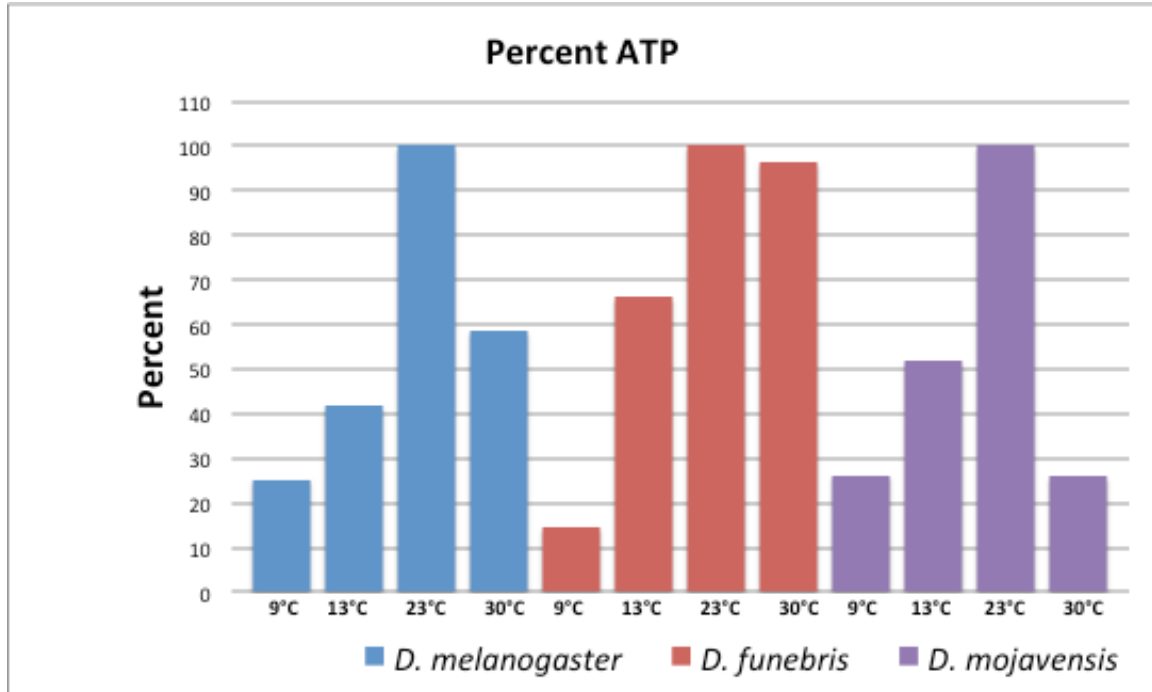
Figure 3

Figure 3: Percent ATP. Percent ATP is shown for *D. melanogaster, D. funebris,* and *D. mojavensis* at 9°C, 13°C, 23°C, and 30°C. Percent ATP is shown so that the ATP level of all three flies at 23°C has been set to 100%.
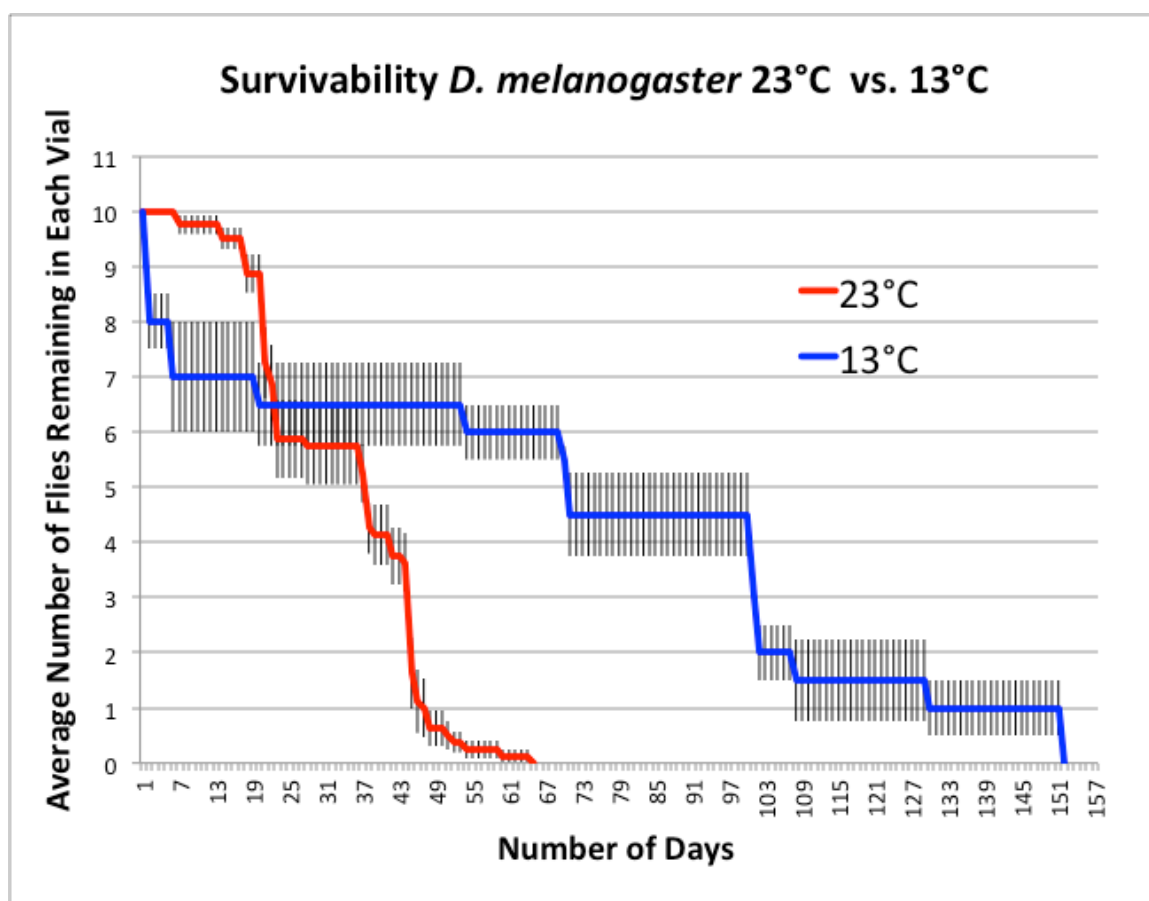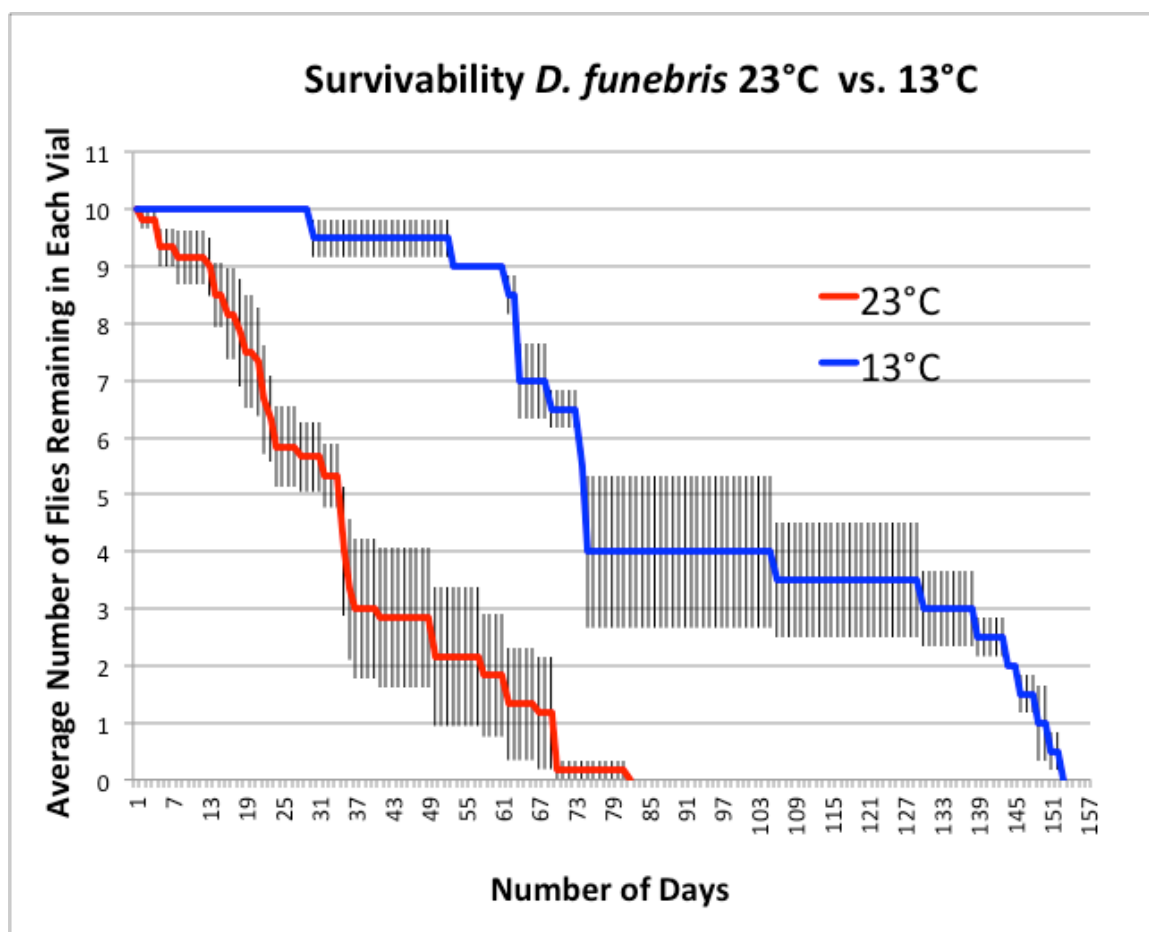
Figure 4

Figure 4A

Figure 4B

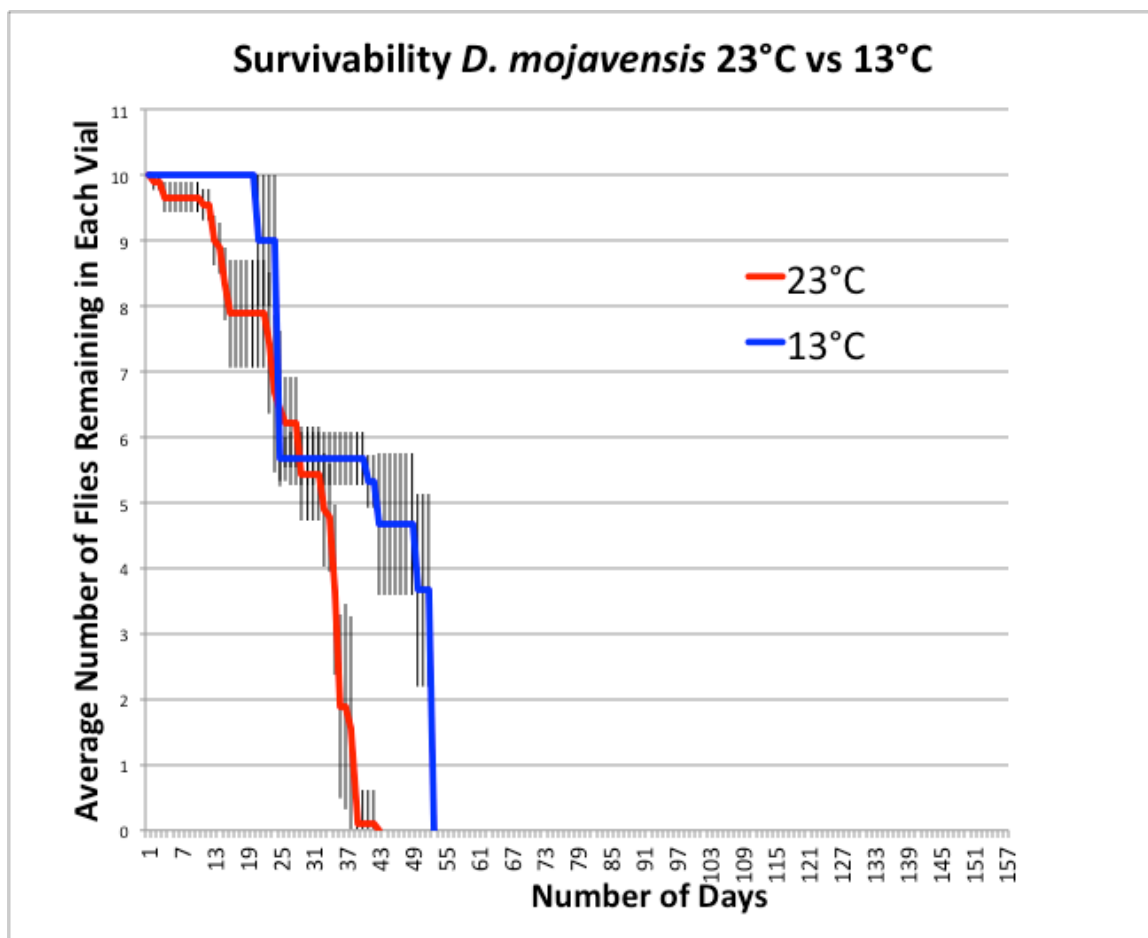Survivability *D. funebris* 23°C vs. 13°C

Figure 4C

Figure 4: Survivability. (4A) The survivability of *D. melanogaster* at both 23°C and

13°C. (4B) The survivability *of D. funebris* at 23°C and 13°C. (4C) The

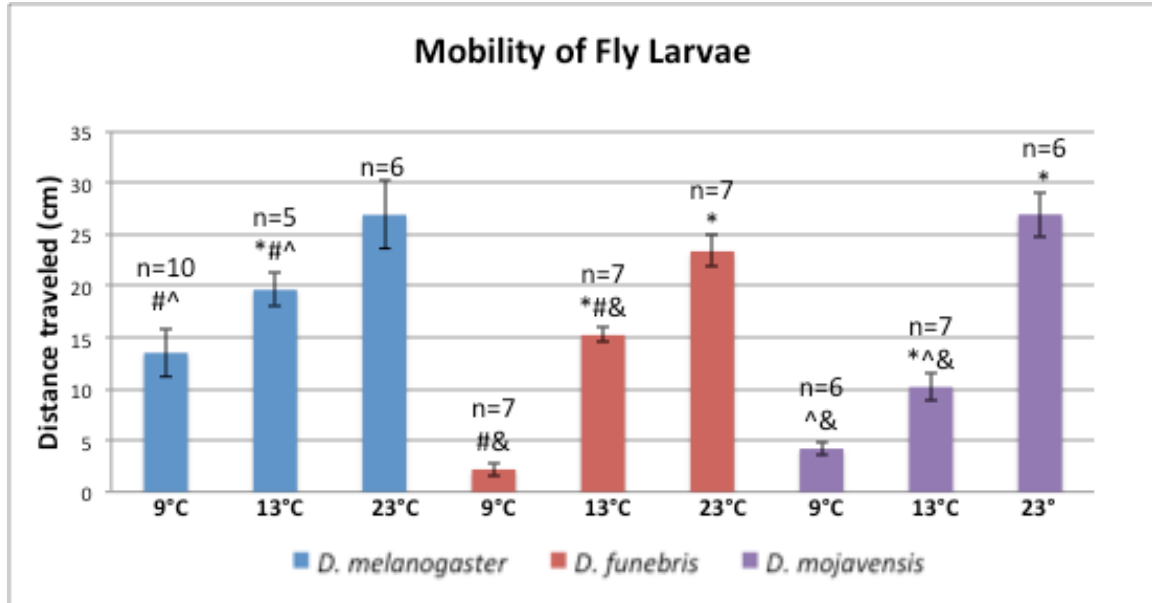survivability of D. mojavensis at 23°C and 13°C.

Figure 5

Figure 5: Mobility of Fly Larvae. Mobility is shown in centimeters. Mobility is shown for *D. melanogaster, D. funebris,* and *D. mojavensis* at 9°C, 13°C, and 23°C. An * indicates that there is a significant difference in mobility between that temperature and the temperatures directly lower. A # indicates there is a significant difference between *D. melanogaster* and *D. funebris* at that temperature. A ^ indicates there is a significant difference between *D. melanogaster* and *D. mojavensis* at that temperature. A & indicates there is a significant difference between D. funebris and D. mojavensis at that temperature.
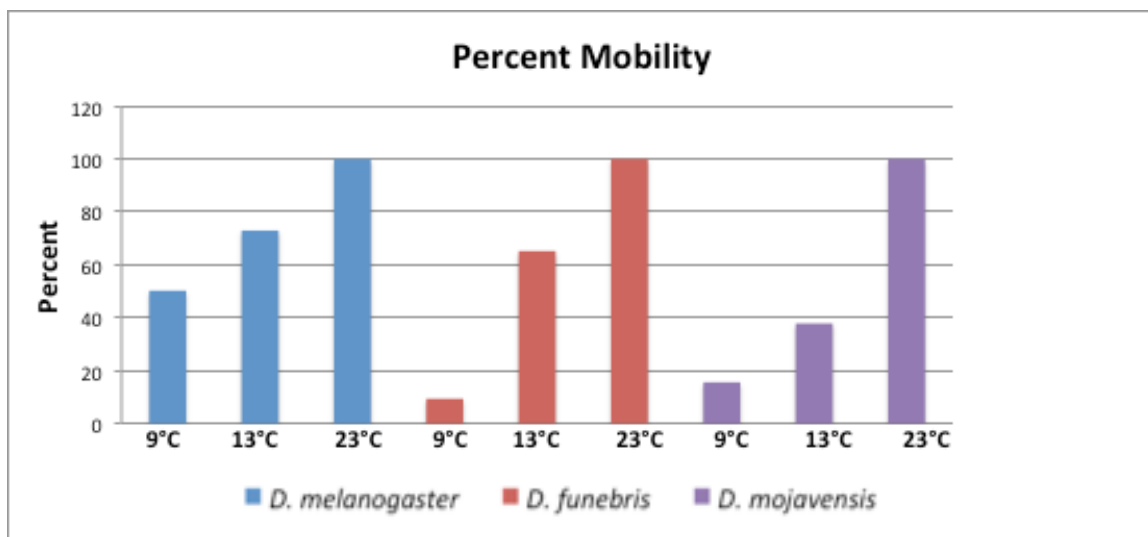
Figure 6

Figure 6: Percent Mobility. Percent mobility is shown for *D. melanogaster, D. funebris,* and *D. mojavensis* at 9°C, 13°C, and 23°C. Percent mobility is shown so that the distance traveled of all three flies at 23°C has been set to 100%.

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