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EFFECTS OF POLYMORPHISM IN THE HYPOXIA SIGNALING PATHWAY ON OXYGEN DELIVERY AND MITOCHONDRIAL INTEGRITY

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ABSTRACT

Oxygen delivery to tissues is of paramount evolutionary importance in aerobic organisms because it allows for the production of energy that can be used for fitness-related processes such as growth and reproduction. Insects deliver oxygen to tissues through tube-like networks of tracheae and tracheoles whose size and branching is increased during development by the action of the hypoxia inducible factor (HIF) pathway. A key transcription factor in the hypoxia inducible pathway is HIF 1-α, which is regulated by the substrate of the TCA cycle enzyme, *succinate dehydrogenase* (SDH). Previous work has shown that human loss-of-function mutations in the gene encoding *succinate dehydrogenase subunit d*, *Sdhd*, constitutively activate the hypoxia inducible pathway, and cause the development of vascularized tumors. Here we show that in lowland populations of Glanville fritillary butterflies (*Melitaea cinxia)* subtle allelic variation in the *Sdhd* gene which does not result in complete loss of SDH activity is associated with differences in tracheal abundance and mitochondrial integrity. Both of these traits are related to a molecular marker for the activation level of the hypoxia inducible pathway. These physiological traits presumably involve fitness tradeoffs, thus allowing the alleles to be maintained in populations over time. These results could also be applicable to other species, including humans, providing new insight into how organisms sense and respond to changes in oxygen, which in turn affects their development of important physiological traits.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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Chapter 1

Introduction

The transport and delivery of oxygen from the external atmosphere to internal organs and tissues is essential for all aspects of the life of aerobic organisms, including their development and ability to sustain high levels of activity. Humans have lungs and blood vessels to accomplish this whereas insects have a tracheal network. The tracheal network is a continuous series of connected tubes in which larger tracheae branch into thinner branches known as tracheoles that deliver oxygen to tissues and organs (Centanin et al, 2010). A greater degree of tracheal branching can improve delivery of oxygen to the tissues. The development of this vitally important branched tracheal network occurs during embryogenesis and the growth of the network continues through the early adult stage. Specific groups of ectodermal cells invaginate and form sac-like structures that sprout branches. These branches migrate toward other branches and fuse with them to eventually form a continuous tubular network (Ebner et al, 2002). The *breathless* (*btl*) and *branchless* (*bnl*) loci in *Drosophila* help control the cell signaling and migration necessary for tracheal morphogenesis. *Bnl* encodes a protein similar to a fibroblast growth factor (FGF) while *btl* encodes an FGF-like receptor molecule (Katsuma et al, 1996) (Sutherland et al, 1996). The cells expressing *bnl* signal the migration of the cells with *btl* receptors causing the branches on these different cells to join together. (Ebner et al, 2002). Once a *bnl-btl* tracheal cell cluster is formed, *bnl* expression is turned off for that cluster and then turned on for other cells nearby, so that they will form clusters and elongate the branched trachea that is being formed.

Tracheae play an important role in insect physiology because of their function in oxygen delivery. It is important for the insect to have just the right amount of oxygen. Too little

oxygen is detrimental because the cells of the body won't have enough oxygen to fuel activity, growth, and reproduction. Tissue hypoxia can occur when an insect undergoes periods of vigorous activity such as flight that depletes oxygen, or when there is less oxygen available in the atmosphere to begin with (hypoxic atmosphere) (Harrison et al, 2012). This can result in slowed or abnormal development of the insect. Too much oxygen (hyperoxia) is also problematic because when oxygen delivery exceeds tissue demands there can be production of reactive oxygen species (ROS) such as superoxides, $O_2^{\cdot -}$ and hydroxyl radicals, $OH^{\cdot}$. The highly reactive nature of these oxygen species allows them to cause structural alterations to DNA such as insertions and deletions, as well as modulating the activity of regulatory proteins responsible for processes such as cell differentiation and proliferation (Wiseman and Halliwell, 1996). These changes in DNA and protein structure due to ROS activity can be highly toxic and damaging to the cells.

Thus, tracheae determine the potential tradeoff between how much oxygen the insect's tissues can consume during maximal activity versus how much excess oxygen is being delivered to the tissues during rest. Efficient delivery of the necessary amount of oxygen by tracheae affects fitness- related processes such as flight and reproduction. Thus, the dynamic formation of tracheae and the regulation of tracheal formation are of great evolutionary importance. Genetic changes that result in the formation of more tracheae for better oxygen delivery may be selected for in populations located in certain hypoxic environments, such as high elevations (Hoback and Stanley, 2001).

The tracheal formation process is dynamic in order to adjust to variation in internal conditions such as insect body size and shape, as well as external conditions such as atmospheric oxygen levels in order to match the insect's demands for oxygen with the supply of oxygen that is available in the atmosphere. This regulation is accomplished by transcription factors that are part of the hypoxia inducible pathway. The key transcription factor in this pathway is the hypoxia

inducible factor (HIF), a heterodimer that exists in several isoforms, and is conserved across animal species (Gorr et al, 2004). In conditions where tissue oxygen levels are low (hypoxia) HIF is activated by the binding of the HIF-1α subunit to the HIF-1β subunit (Gorr et al, 2006). The activated HIF is translocated to the nucleus, where it binds to hypoxia response elements in the promoter region of target genes and initiates transcription of these genes.

Target genes of HIF can affect metabolic responses to hypoxia in a variety of ways. HIF can increase the transcription rate of genes encoding enzymes of glycolysis and glucose transporters, resulting in an increased level of glycolysis (Kim et al, 2006). HIF also activates transcription of the gene encoding pyruvate dehydrogenase kinase-1 (PDK-1). PDK-1 phosphorylates and inhibits the activity of pyruvate dehydrogenase (PDH), the enzyme complex responsible for the conversion of pyruvate to acetyl-CoA for use in the citric acid cycle of aerobic respiration. By inhibiting PDH, and activating glycolytic enzymes, HIF helps cells adapt to lower levels of oxygen by increasing anaerobic respiration and decreasing aerobic respiration. This decrease in aerobic respiration during insufficient oxygen levels is important in avoiding the formation of reactive oxygen species (ROS) that are highly reactive and form when oxygen levels recover after hypoxia is relieved or intense exercise ends (i.e. ischemia-reperfusion injury).

A HIF-mediated increase in the transcription of genes such as the *btl* and *bnl* loci in *Drosophila* increases the formation and branching of tracheae to increase oxygen delivery to tissues and organs (Ebner et al, 2002). Similarly, in humans, hypoxic conditions result in the activation of genes involved in the formation of erythropoietin which allows for the synthesis of erythrocytes. HIF also turns on genes involved in the formation of new blood vessels (angiogenesis). The upregulation of angiogenesis is particularly relevant for the growth of solid tumors that create hypoxic local environments and need a greater delivery of oxygen and nutrients to maintain growth. This is why many cancer treatments for tumors work to inhibit angiogenesis in tumors (Semenza, 2003). Thus, the hypoxia inducible pathway is a critical

functional module that is conserved across many species, because if an organism's cells can't respond to oxygen stresses then the organism will not survive.

In order to sense and respond to changes in oxygen levels, insects such as *Drosophila* have enzymes with characteristic prolyl-4-hydroxylase domains (PHD) that act as oxygen sensors (Acevedo, 2010). These enzymes are members of a dioxygenase super family that can incorporate a hydroxyl group into a molecule in the presence of 2-oxoglutarate and oxygen. (Gorr, 2006). In normal atmospheric oxygen conditions (normoxia-21% O_2), PHD hydroxylates two proline residues in HIF-1α. This results in the binding of HIF-1α to the Von Hippel-Landau (VHL) protein, a recognition site of an E3 ubiquitin ligase complex that promotes proteasomal degradation of HIF-1α protein (Gorr et al, 2006). In hypoxic conditions, PHD activity is inhibited and HIF-1α is not hydroxylated, and therefore it is not proteasomally degraded, and instead accumulates. HIF-1α is translocated to the nucleus where it binds to HIF-1β and initiates nuclear transcription of hypoxia response elements. HIF-1β (also known in vertebrates as aryl hydrocarbon nuclear translocator, ARNT) is not regulated by oxygen levels as HIF-1α is, but it is constitutively expressed and is always present in the nucleus. Thus, PHD is a key enzyme regulating HIF activity which in turn determines the extent of hypoxia signaling in an organism.

The *Drosophila* PHD locus, *fatiga*, encodes three isoforms of PHD: PHD 1, PHD 2, and PHD 3 (Acevedo et al, 2010). PHD 2 and PHD 3 are oxygen and Fe(II) dependent, and act to shut off HIF activity when oxygen levels return to normal. PHD 1 activity is oxygen independent so it may constantly hydroxylate HIF and constitutively regulate hypoxia signaling. Since PHD 2 and 3 are also Fe (II) dependent, they can be inhibited and hypoxic conditions can be imitated using transition metals such as Co^{2+} and chelators such as DFO and desferrioxamine (Gorr, 2006).

HIF can also be regulated by factors other than PHD. A transcription factor known as FIH-1 (Factor Inhibiting HIF-1α) can also inactivate HIF-1α during normal oxygen levels by hydroxylating an asparagine residue near the C-terminal of HIF-1α (Gorr et al, 2006). The

transcription factor, NF-kB, can function as a transcriptional regulator of HIF-1α. In the absence of NF-kB there is no transcription of the HIF-1α gene. (Dery et al, 2005). HIF activity can also be increased when phosphorylated by the p42/p44 mitogen activated protein kinases (MAPK) (Richard et al, 1999). Non-hypoxic stimuli such as cytokines and vascular endothelial growth factors can also activate p42/p44 MAPK and increase HIF activity even when oxygen levels are normal (Richard et al, 2000). These forms of regulation are in addition to the established view of HIF regulation by PHD, which is the form of regulation we focus on in this paper.

Since the hypoxia inducible pathway is of great evolutionary importance due to its effect on delivery of oxygen and the energy demands of an organism, the effect of polymorphisms in genes that control the HIF pathway are of particular interest. One such gene is *Sdhd* (succinate dehydrogenase subunit d). Succinate dehydrogenase (SDH) is a heterotetrameric enzyme that participates in both the citric acid cycle and respiratory electron transport chain. In the citric acid cycle, succinate dehydrogenase oxidizes succinate to fumarate while reducing the electron carrier FAD to $FADH_2$. SDH is also able to function in the electron transport chain because its C and D subunits are integral membrane proteins that anchor succinate dehydrogenase to the membrane (Nelson and Cox, 2008). Succinate dehydrogenase , also known as Complex II or succinate:oxidoreductase in the mitochondrial respiratory chain, has subunits A and B that contain iron-sulfur centers that function to pass electrons from $FADH_2$ to reduce ubiquinone to ubiquinol. Mutations in *Sdha*, *Sdhb*, *Sdhc*, or *Sdhd* result in a loss of SDH function and the formation of paraganglioma (Hartmut et al, 2004) (Favier et al, 2005). Paraganglioma can be characterized by vascularized tumors in the head and neck region, particularly the carotid body which acts as a sensor for oxygen levels in the blood (Baysal, 2003). These mutations can also result in tissue damage due to the production of ROS (Yankovskaya et al, 2003).

Loss-of-function mutations in genes encoding for SDH subunits cause constitutive activation of the HIF pathway. Because SDH is nonfunctional, it is unable to convert succinate to

fumarate. Succinate accumulates and rapidly leaves the mitochondrion through the action of dicarboxylate carriers (King et al, 2006). Once in the cytosol, succinate inhibits the action of PHD, allowing the dimerization of HIF-1α to HIF-1β and transcription of the hypoxia response elements (Selak et al, 2005). A loss of function of fumarate hydratase, the enzyme that converts fumarate into malate, also results in activation of the HIF pathway due to accumulation of fumarate that leaves the citric acid cycle and inhibits PHD (Pan et al, 2007). Thus, accumulation of both the succinate and fumarate metabolites can activate the HIF pathway, even if there is not a hypoxic environment.

The purpose of this study is to examine whether allelic variation in *Sdhd* and hypoxia signaling has an effect on important developmental traits such as tracheal formation, mitochondrial integrity, and flight performance in the Glanville fritillary butterfly, *Melitaea cinxia*. *M. cinxia* has been a model organism for population ecology studies (Hanski et al, 1994) (Kuussaari et al, 1996). *Melitaea cinxia* was also useful for this study because of genomic data available from previous work that has been done in our laboratory to sequence its transcriptome (Vera et al, 2008). The effect of mutations in *Sdhd* that result in complete loss-of-function of SDH has already been documented but we are interested in more subtle allelic variation in *Sdhd* that results in varying levels of SDH activity. In Glanville fritillary butterflies, there is an indel polymorphism in the 3´-

UTR of Sdhd that is associated with differences in dispersal and flight metabolic rate (Wheat et al, 2011). These traits are likely to be affected by oxygen supply to the tissues, and hence we hypothesized that this polymorphism in *Sdhd* may affect hypoxia signaling. Differences in hypoxia signaling may in turn influence important physiological traits such as tracheal formation and mitochondrial integrity. Polymorphism in *Sdhd* may also be important in a broader evolutionary context because natural selection may favor certain physiological traits such as

greater tracheal formation in certain environments, and the alleles that favor these traits may be maintained over time in natural populations.

Chapter 2

Methods

Butterfly Sampling

Melitaea cinxia butterflies used in this study were from two large populations in Provence, in 2010, and another population from near Viladrau, Spain in 2011. The French butterflies were collected as eggs from mated females in two populations and were reared to adults in the lab. The Spanish butterflies were caught as recently emerged adults in the wild. Some of the Spain adults (N = 134) were weighed to obtain a starting mass then marked with individually unique numbers (Sharpie pen) on their wings. The butterflies were then released at the site where they were captured and we returned to the sites to re-capture butterflies periodically (usually every other day) over a time period of about two weeks. This was done in order to quantify how long the butterfly was still flying around and using body resources from when it was first captured to when it was re-captured. If the butterfly was re-captured it was weighed and then destructively sampled. Body parts were saved for DNA and a sample of flight muscle was preserved in fixative for microscopy to examine mitochondrial phenotypes. In some cases, butterflies were re-captured multiple times before finally being sampled destructively, particularly when the butterfly was re-captured very quickly (1-2 days) after it was first released.

Sdhd Allele Characterization and Genotyping

Sdhd indel genotype was determined from clone-verified fragment sizes of a portion of the 3´-UTR from PCR amplification of either genomic DNA or cDNA using fluorescently labeled

forward (5´-/56-FAM/--ACTTAATGAAAAGYGTGATTG-3´) and pig-tailed reverse (5´-GTTTCTTTGTTAAAAGGTCTTGAGTTCG-3´) primers (Marden et al, 2012).

Flight Experiments

In order to assess flight performance and metabolic rate, the butterflies were flown in a respirometry chamber which consisted of a 1 L plastic jar with a screw-on lid. CO_2 – free air was flowed through the chamber at a rate of 0.95 L/minute. Lab-reared male butterflies from the French populations (N=28, 2-4 days old) were placed in the chamber and stimulated to fly by shaking or tapping the chamber. The peak amount of rate of CO_2 emission and total CO_2 emitted by the butterflies during flight were recorded during a ten minute period of continuous flight in a normoxic (21 % O_2) atmosphere. The butterflies were allowed to recover for 30 minutes and then were flown again in a hypoxic atmosphere (14% O_2) in which the peak amount of rate of CO_2 emission and total CO_2 emitted were again measured just as in the normoxic flight trial. The butterflies were then dissected and muscle samples were preserved in fixative or frozen in liquid nitrogen in order to obtain flight muscle for microscopy and analyses of gene expression.

Confocal Microscopy for Analysis of Tracheal Morphology

Thoracic dorsal longitudinal flight muscle sections were obtained through dissection and were preserved in fixative (gluteraldehyde/paraformaldehyde) in order to analyze the extent of tracheal development in the butterflies. The samples were prepared for confocal microscopy using a standard Calcofluor staining protocol. Calcofuor was chosen because it stains the chitin that is present in the walls of tracheae (Zimech et al., 2005). Some of the frozen muscle samples were embedded in OCT Cryomatrix and sectioned into 10 µm slices using a Thermo-Shandon Cryostat. Others were embedded in paraffin and sectioned into 7 µm slices using a Thermo Fisher Finesse Paraffin microtome. The sections from the Cryostat were placed on slides and dried at 40°C on a hotplate. Once dry, the slides were fixed with periodate-lysine-paraformaldehyde (PLP) fixative for 90 minutes at room temperature. The cryostat sections were washed 3 times for

5 minutes each in Sorensen's phosphate buffer. The paraffin embedded sections were de-waxed using a Thermo Gemini auto-stainer. Both the sections were stained for one hour in the dark with a solution of 0.1% Calcofluor stain, 0.1% Triton, and 2% BSA in 0.1 M Sorensen's phosphate buffer. The sections were then washed 3 x 5 min in Sorensen's phosphate buffer. There were no observable differences in the level of staining between the samples sectioned with the Cryostat and the samples sectioned with the paraffin microtome. This preparation protocol was successful for 27 of the 28 butterflies from the flight experiments, so confocal images were obtained for 27 individuals.

Coverslips were sealed to the slides using fluorogel and the sections were viewed using an Olympus Fluoview FV1000 confocal microscope. A laser with wavelength of 405 nm was used for illuminating the samples. A DAPI dye setting was chosen on the imaging software to obtain confocal images. In order to attain effective contrast between the tracheae of interest and the background tissue when capturing images, the voltage of the photomultiplier tube (PMT) was adjusted to limit autofluorescence (making the background tissue very dark), and emphasize the tracheae from the surrounding tissue.

Stacked images of the 10 μm thick slices of flight muscle (myofibrils) were obtained for each individual butterfly. These stacked tracheal images were analyzed using Image J. In the software, contrast and brightness were adjusted to achieve greater brightness of the tracheae relative to the background tissue. The freehand selection tool was used to select various subregions within each confocal image that contained fluorescently stained tracheae of approximately equal brightness. The selected subregions were chosen so that all of them together would encompass just the area within each image that contained tracheae, while excluding areas with only flight muscle. The area of each subregion was recorded in pixels to give a selection area measurement. The threshold tool was used on each selected area in order to select only the fluorescently stained tracheae from the background based on their different pixel values. The

selected tracheae were converted to a binary image and the area of these selected tracheae (in pixels) was measured. The tracheal area measurement was divided by the selected area measurement for each section to give a proportional tracheal image for each section. This protocol was carried out for at least 3 selected subregions for each of five individual sections that made up the stacked image for each individual, allowing for a single value of mean cross-sectional area of tracheae for each butterfly that was used in statistical analyses.

TEM for Analysis of Mitochondrial Morphology

Dorsal longitudinal flight muscle samples were prepared for transmission electron microscopy (TEM) from 21 out of the 28 butterflies used in the respirometry experiments and examined for mitochondrial morphology. These samples were prepared for TEM using a standard protocol provided by the Penn State EM facility in order to obtain cross-sectional images of the flight muscle and their mitochondria. (Bozzola et al., 1999) (Dykstra, 1992). The thorax of each individual was dissected and muscle samples were placed in a solution of 1.5% glutaraldehyde, 2.5 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 with 4% sucrose as a primary fixative. The samples were then incubated for two hours at room temperature. The samples were washed 3 times in 0.1 M phosphate buffer and then incubated in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.2 for one hour at room temperature. The samples were then washed once in 0.1 M phosphate buffer and twice in water. The samples were subjected to en bloc staining in 2% aqueous uranyl acetate for one hour.

The stained samples were dehydrated through a graded ethanol series, and then with acetone. A three step process was used to infiltrate the samples with Spurr's resin. First, the samples were placed in a 50:50 acetone/Spurr's resin solution overnight. Then the samples were infiltrated twice with 100% Spurr's resin for four hours each. The samples were polymerized for 24 hrs at 60°C. Samples were cross-sectioned into 70 nm thick slices using an Ultracut E Leica Ultramicrotome with a Diatome diamond knife. The sections were post stained with a standard

2% Uranyl Acetate/lead staining protocol and washed with water to remove any staining artifacts (Venable and Coggeshall, 1965).

TEM images of 5,000 X magnification were obtained from random views of flight muscle for each individual using a JEOL JEM 1200 EXII. The NIH imaging program, ImageJ, was used to determine the size and structural integrity of the mitochondria in each of the TEM images. For each TEM image, mitochondria were selected individually using the lasso tool and the brightness range was normalized for each selected mitochondrion so that the darkest pixels of were black and the lightest pixels were white (i.e. levels of 0 and 256 on the brightness scale). The area (μm^2) and inverse average optical density were then measured for each selected mitochondrion. The inverse average optical density is a measure of how tightly packed the crista membranes are in the mitochondrion. A higher value for the inverse average optical density indicates a more damaged mitochondrion.

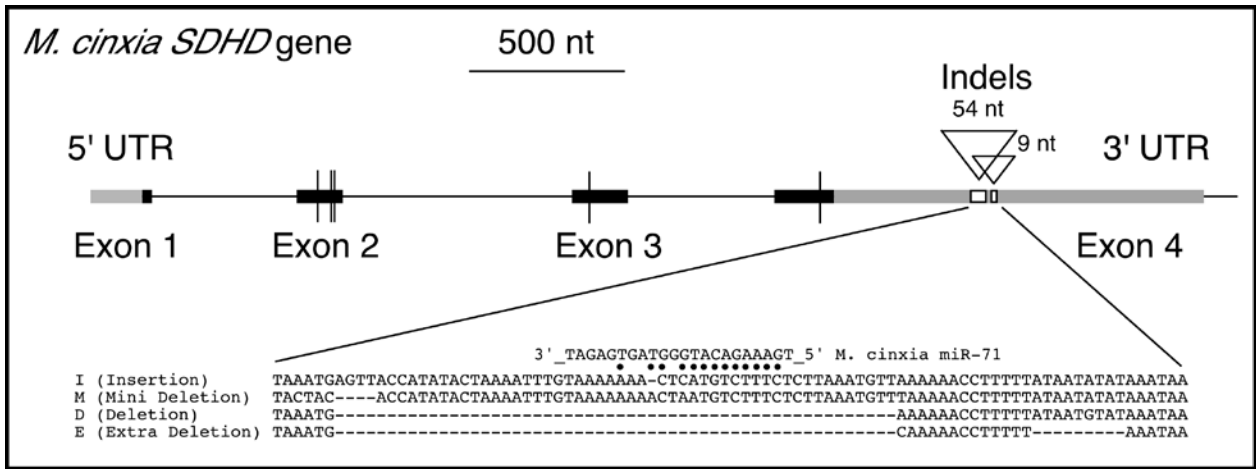
Statistical Analysis of Data

Continuous variables from the flight performance experiments, confocal microscopy, and TEM, along with categorical data from the genotyping were analyzed using mixed model analyses in JMP. Each of these mixed model analyses will be described in detail in the results section below.

Chapter 3

Results

Figure 1: Genotyping Results of 3' UTR of *Sdhd*



The *Sdhd* gene in Glanville fritillary butterflies contains an indel polymorphism that is 492 nucleotides from the stop codon at the 3' end in the untranslated region (UTR) of the mRNA (see Figure 1) Sequencing and cloning revealed four different alleles for this indel polymorphism: a deletion (D) and extra-deletion (E) lacking 53 and 62 bases in the polymorphism, and two insertion alleles (I and M) separated by a four nucleotide mini-deletion in the M allele. *Sdhd* genotype was determined for all of the butterflies in the flight experiments that were then prepared for TEM and confocal microscopy so that the individuals' physiological traits such as metabolic rate, proportion of flight muscle areas containing tracheae, and mitochondrial integrity could be compared to *Sdhd* genotype

Figure 2: Difference in Average Proportion of Tracheal Area of Flight Muscle for M allele vs. non-M allele butterflies

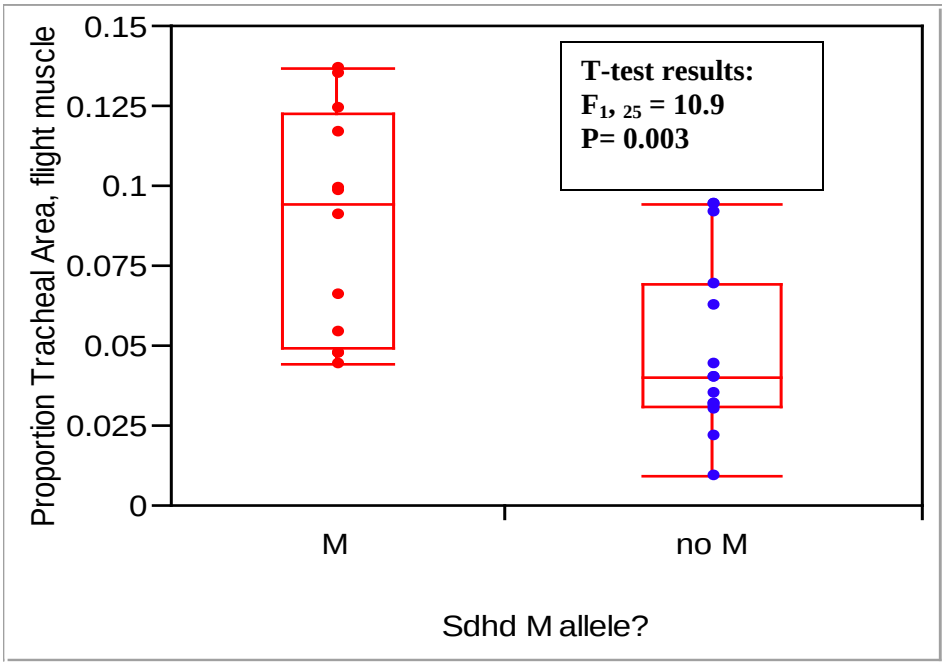
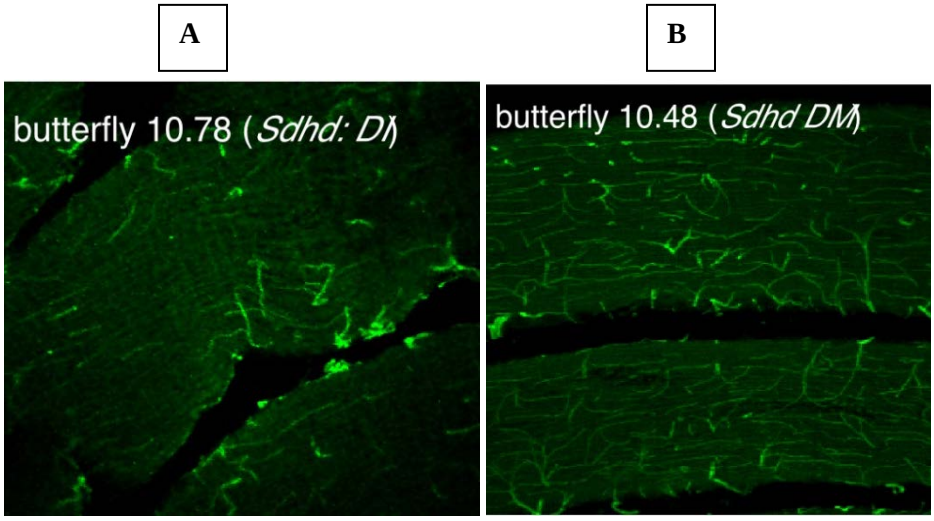


Figure 2 shows the results of the Image J analysis of the confocal microscopy images obtained from the flight muscle of 27 French population butterflies that underwent the flight experiments. The genotype of each butterfly was compared to the average proportion of flight muscle area that contained tracheae. There is a statistically significant difference in the average proportion of flight muscle that contains oxygen-carrying tracheae for M allele butterflies vs. non-M allele butterflies (P = 0.003). Butterflies with the M allele have a greater degree of tracheal formation than butterflies without the M allele.

Figure 3A and 3B: Confocal Microscopy Images of M allele (A) vs. non-M allele (B) butterfly flight muscle



Figures 3A and 3B show a comparison of confocal images from two different *M. cinxia* individuals from the French population. Figure 3A shows a butterfly without the M allele (DI genotype for the 3' UTR of *Sdhd*) while Figure 3B shows a butterfly with the M allele (DM genotype). The DM butterfly appears to have a much higher density of branching, bright-stained tracheae than does the DI butterfly on the right. Thus, these images serve as a visual confirmation for the statistically significant difference found in proportion of flight muscle-containing tracheae seen in Figure 2.

Table 1: Summary of Mixed Model for Proportion of Tracheal Area of Flight Muscle (R^2 = 0.31)

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Sdhd M allele?	1	1	0.01043982	10.4619	0.0035
Body mass (mg)	1	1	0.00027998	0.2806	0.6012

Table 1 shows the results of a mixed model analysis with the average proportion of flight muscle area containing tracheae as the response variable. This model accounts for butterfly body mass because larger butterflies may have a larger tracheal density simply because of their size,

and not just because they have the M allele. Body mass itself, controlling for the *Sdhd* allele of the butterfly does not have a statistically significant effect on the proportion of tracheal area (P = 0.601). However, there is still a statistically significant difference in the proportion of flight muscle that contains tracheae between M allele and non-M allele butterflies (P = 0.0035) after accounting for body mass.

Table 2: Summary of Mixed Model for Peak CO_2 Rate in Normoxia (21% O_2) (R^2 = 0.34)

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Proportion Tracheal Area, flight muscle	1	1	3.2711569	6.3952	0.0184
Body mass (mg)	1	1	2.2718170	4.4415	0.0457

Table 2 shows the results of a mixed model analysis of the data collected during the flight experiments with the 27 butterflies from the French *M. cinxia* population. In this mixed model, the peak rate of CO_2 production (mL/hour) during the flight period in normoxic conditions (21% O_2) was the response variable in order to give a sense of the metabolic rate of each butterfly during periods of exercise-related oxygen stress. A greater rate of CO_2 production indicates that the butterfly is flying more vigorously and undergoing cellular respiration to produce more CO_2 as a waste product. There is a significant positive association (P = 0.018) between the proportion of flight muscle area containing tracheae and the peak rate of CO_2 production during the flight period in normoxia while controlling for the body mass of the butterflies. As seen in Figures 2 and 3, and Table 1, the M allele is significantly correlated with having an increased area of tracheae in flight muscle, which is why the proportion of flight muscle area containing tracheae was used as a variable in this model instead of whether or not the butterfly had the M allele. There is also a statistically significant positive association (P = 0.046) between the body mass of the butterflies and the peak rate of CO_2 production with larger butterflies producing more CO_2.

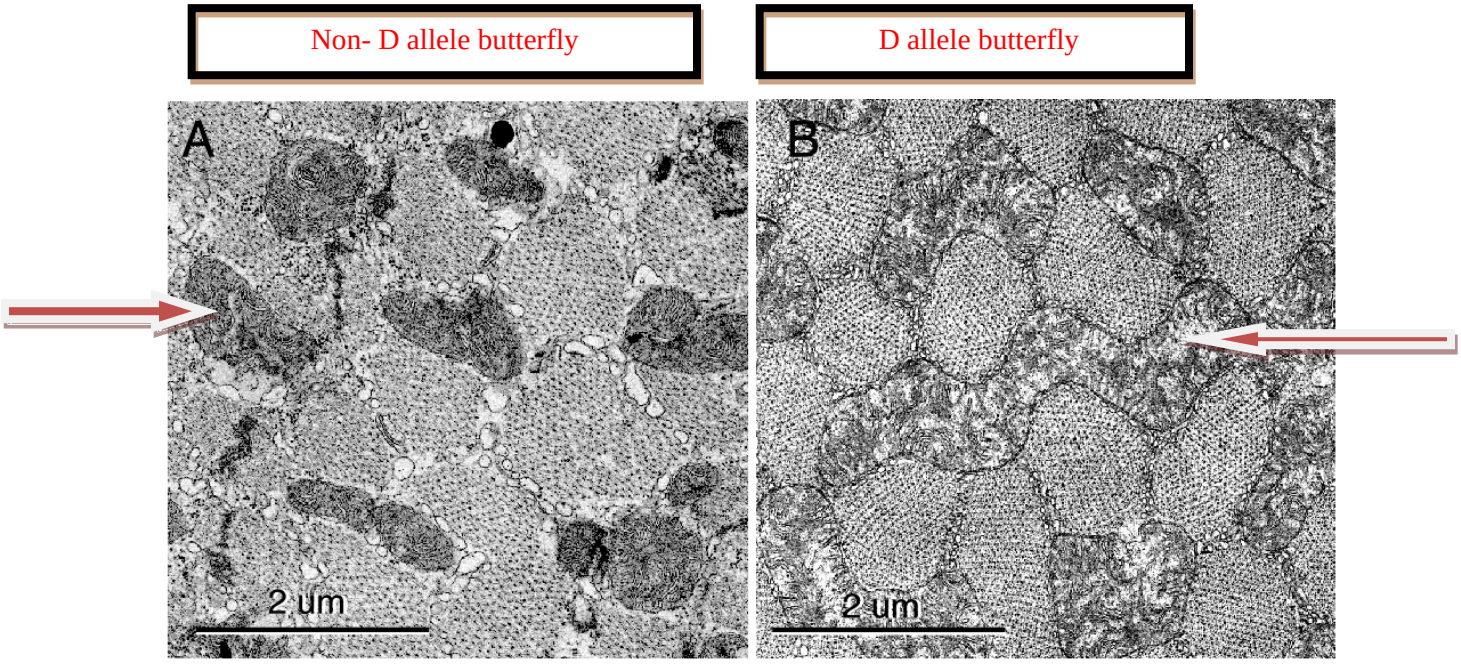
Table 3: Summary of Mixed Model for 2010 French *M. cinxia* Mitochondrial Integrity (R^2 =0.26)

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Body mass (mg)	1	1	144.25606	3.8635	0.0650
Sdhd D allele?	1	1	192.82292	5.1643	0.0355

Table 3 shows the results of the mitochondrial TEM images obtained for 21 of the *M. cinxia* individuals from the French population that underwent the flight experiments. In this model, the average inverse optical density of the mitochondria was the response variable. Inverse optical density is a pixel measurement of the TEM images of the mitochondria, with a higher value indicating a less dense mitochondria that has undergone some kind of damage, lessening its intactness (crista membrane packing). This model shows that body mass of the butterfly does not have a statistically significant effect on mitochondrial integrity (P = 0.065) when controlling for the genotype of the butterfly. There is a statistically significant positive association between a butterfly having the D allele in its genotype and having a higher average inverse optical density (P = 0.036) when controlling for body mass. This indicates that butterflies with the D allele suffer more damage to their mitochondria than butterflies that don't have the D allele.

Figures 4A and 4B: Comparison of Mitochondria for D Allele (A) vs. non-D allele Butterflies (B)



Figures 4A and 4B are a comparison of TEM images of flight muscle obtained from two different *M. cinxia* butterflies from the French population that underwent the flight experiments. Figure 4A shows the mitochondria of a butterfly that does not have the D allele in its *Sdhd* genotype while Figure 4B shows the mitochondria of a butterfly that does have the D allele. The arrow in 4A is pointing to a mitochondrion with dense, intact cristae (little to no white space within the mitochondrion) while the arrow in 4B is pointing to the white space in the mitochondrion, indicating less dense cristae. This greater amount of inner white space indicates the mitochondria of the D allele butterfly are more swollen and damaged, providing a visual representation of the results seen in Table 2.

Table 4: Summary of Mixed Model for 2010 French *M. cinxia* Mitochondrial Size (R^2 = 0.33)

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Residuals sum integrated CO2	1	1	0.80873937	6.9319	0.0164

Table 4 shows the results of a mixed model analysis with residual mitochondrial size after accounting for tracheal area as the response variable. There was a statistically significant positive association between residual total CO_2 production (adjusted for body mass and from both the normoxic and hypoxic flight conditions) and mean mitochondrial size. Butterflies from the French population that emitted more CO_2 had more swollen mitochondria when we controlled for tracheal abundance.

Table 5: Summary of Mixed Model for 2011 Spanish *M. cinxia* Mitochondrial Integrity (R^2 = 0.68)

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Mass at Time of Sampling	1	1	511.67825	13.4977	0.0063
days elapsed first to last	1	1	279.46052	7.3720	0.0264
starting mass	1	1	349.23503	9.2126	0.0162

Table 5 shows the summary of mixed model analysis with 2011 adult Spanish *M. cinxia* with average inverse optical density of the butterflies' mitochondria as the response variable. There is a statistically significant positive association (P =0.0063) between the mass of the butterfly at the time it was sampled (just before being dissected) and the average inverse optical density of the butterfly's mitochondria, with butterflies having a larger mass at the time of sampling having more damaged mitochondria. There is a statistically significant negative association (P = 0.026) between days elapsed from when the butterfly was first captured and marked and when the butterfly was caught again and destructively sampled, and the average inverse optical density of

the butterfly's mitochondria. Butterflies that were out in the wild longer before we finally caught them again had less damaged mitochondria than butterflies that were quickly captured again after a shorter time. Finally, there was also a statistically significant negative association ($P = 0.0162$) between the starting mass of the butterflies when they were first captured and marked, and the average inverse optical density of their mitochondria. Butterflies that had a high mass when they were initially captured had a low inverse optical density, indicating mitochondria that were more intact.

Chapter 4

Discussion

This study of lowland populations of Glanville fritillary butterflies sought to determine whether polymorphism in *Sdhd* results in different levels of hypoxia signaling that can affect the development of tracheae, and related traits such as mitochondrial integrity and peak metabolic rate. Previous work has shown that mutations in *Sdhd* resulting in complete loss of SDH enzyme function activate the hypoxia inducible pathway and cause the formation of vascularized tumors (Hartmut et al, 2004) (Favier et al, 2005). However, we hypothesized that subtle allelic variation in the 3' UTR of *Sdhd* (not resulting in complete loss of enzyme function) would be associated with variable levels of hypoxia inducible pathway activation and hence variation of hypoxia-related physiological traits. The results obtained from the French and Spanish populations of *M. cinxia* support the initial hypothesis that there is a relationship between polymorphism in *Sdhd*, activation of the hypoxia inducible pathway, and the development of physiological traits. One of these physiological traits is the formation of tracheae that allow for delivery of oxygen to tissues for fitness-related activities. Figure 2 shows that butterflies with the M allele in the 3'UTR region of the *Sdhd* gene have a greater fractional cross-sectional area of tracheae in their flight muscles than butterflies that do not have the M allele, and this relationship was significant when we controlled for the body mass of the butterflies (Table 1). We hypothesize that this greater level of tracheal formation is due to higher constitutive activation of the hypoxia inducible pathway during flight muscle development, even though the individuals were from a lowland population that did not experience a hypoxic environment. This greater level of tracheal formation also

allowed M-allele butterflies to have a higher metabolic rate in normoxia during the flight experiments when controlling statistically for body mass, as seen in Table 2. Tracheal abundance was used as a proxy for M-allele in the mixed model analysis seen in Table 2 since it was shown that M-allele butterflies had the highest tracheal abundance This greater tracheal abundance, associated with the M-allele and lower SDH activity (which shows reduced SDH enzyme activity, Marden et al, 2013) allowed for greater oxygen delivery and reduced hypoxic stress after periods of flying. Quantitative PCR (qPCR) data (Marden et al, 2013) obtained after the butterflies flew in both normoxia and hypoxia showed that M-allele butterflies had a lower level of *Phd*, a hypoxia responsive gene responsible for shutting down hypoxia signaling. This lower level of *Phd* expression indicates a lower level of flight-induced hypoxic stress. These qPCR data support the hypothesis that M-allele butterflies have a more efficient oxygen delivery system that reduces hypoxic stress during periods of intense exertion. A more efficient oxygen delivery system is an important fitness-related trait that could be evolutionarily advantageous, resulting in the M-allele being maintained in *M. cinxia* populations.

The reason for the pseudo-hypoxia signaling in the M allele butterflies resulting in greater levels of tracheal formation is not certain, but one possible mechanism is by microRNA (miR) regulation in the 3'UTR of the *Sdhd* gene, which contains a putative miR target (Marden et al, 2013). MiRs are known to bind to the 3'UTR region of mRNA sequences and regulate translation of the mRNA sequence (Filipowicz et al, 2008). Previous studies have also shown that the human *Sdhd* gene contains a binding site in its 3' UTR for a miR known as *miR-210* (Puissegur et al. 2011). Structural modeling of the secondary structure of the mRNA for each *Sdhd* allele done in our lab using the CONTRAfold program (Do et al, 2006) showed that the M-allele lacks a secondary structure "hairpin" loop that other alleles, such as the I allele have, resulting in the availability of six additional bases that can bind to the complement *miR-71* bases.

We found measurable levels of *mir-71* expressed in flight muscle of the butterflies sampled for confocal microscopy and TEM (Marden et al, 2013).

The binding of miR-71 to this site could be responsible for regulating the levels of SDH enzyme activity. An enzyme assay of SDH activity also performed in our lab showed that M allele butterflies have a lower level of SDH activity. A lower level of SDH activity could result in the accumulation of succinate which as shown in previous literature can inhibit PHD and activate the hypoxia inducible pathway (Selak et al, 2005). *MiR-71* has been shown in *C. elegans* to play a role in longevity and DNA damage responses to toxic oxygen radicals, with higher levels of *miR-71* allowing for a greater longevity (Pincus, 2011). This development of oxygen radicals often occurs due to mismatches between oxygen delivery and cellular oxygen needs. Since tracheae are responsible for oxygen delivery, variation in binding of *miR-71* to the indel region and its regulation of translation of SDH enzyme could be the link between *Sdhd* polymorphism and varying degrees of tracheal formation that affects oxygen delivery. This is just a proposed mechanism so more work will need to be done to elucidate the role *miR-71* may play in regulating SDH activity.

Another important physiological trait shown to be affected by polymorphism in *Sdhd* is mitochondrial integrity. The butterflies used in the flight experiments were flown to exhaustion in both normoxic and hypoxic conditions. This strenuous flight activity depleted the butterflies' supply of ATP. This loss of ATP can result in imbalances of key ions such as K^+ that are involved in osmoregulation of the mitochondria, resulting in movement of water into the compartment between the cytosol and matrix which causes mitochondrial swelling (Kaasik et al, 2007) (Halestrap, 1994). Transmission electron micrographs of flight muscle obtained from the French *M. cinxia* (Figures 4A and 4B) show that D-allele butterflies have mitochondria with significantly more swelling and damage than non D-allele butterflies even after controlling statistically for body mass (Table 3.) Butterflies with a greater total output of CO_2 during the flight experiments

had larger mitochondrial size (more swollen) when we accounted for the proportion of flight muscle containing tracheae (Table 4). For a given tracheal cross-sectional area, butterflies with an increased output of CO_2 may be experiencing a greater mismatch between the rate oxygen is delivered to tissues and the rate at which it is used for cellular aerobic respiration, resulting in ATP depletion and the formation of reactive oxygen species that are highly damaging to tissues (Wiseman and Halliwell, 1996).

Mitochondrial swelling is generally a sign of cell damage related to ischemia, or lack of oxygen flow to tissues (Blomberg and Hagber, 2006). In the Introduction it was stated that reactive oxygen species (ROS) can form during periods of recovery following hypoxic stress (such as intense exercise) and cause oxidative damage to tissues. In this process, known as ischemia-reperfusion injury, when blood returns to tissues after a period of ischemia an inflammatory response is initiated in which oxygen radicals are produced. These ROS could cause damage and swelling to the mitochondria. In addition, a complex interaction of the oxygen radicals, mitochondrial membrane potential, Ca^{2+}, and pH can open mitochondrial permeability transition pores (PTPs) (Honda et al, 2006). If these PTPs remain open long enough they can trigger apoptosis, resulting in cell death. This could be a possible explanation for why the D-allele butterflies have more swollen and damaged mitochondria than non-D allele butterflies. D-allele butterflies have a lower level of tracheal abundance than M-allele butterflies so they do not have as extensive an oxygen delivery network than can help prevent hypoxic stress during periods of intense activity. Thus, during the recovery period following intense flight in normoxia, the D-allele butterflies may have been more susceptible to ischemia-reperfusion in which ROS were produced and damage to the mitochondria occurred. Although it is not completely clear as to the exact mechanism by which D allele butterflies have more swollen and damaged mitochondria, it's likely that higher SDH activity associated with the deletion in the 3'UTR of *Sdhd* affects hypoxia

signaling and creates mismatch between oxygen delivery and demand that can produce damaging reactive oxygen species.

An interesting result was seen in the data from the Spanish population of butterflies that were captured as adults in the wild. Butterflies with a higher mass at the time of dissection also had mitochondria with less dense cristae, indicating their mitochondria were more damaged (Table 5). It is possible that larger butterflies have greater metabolic demands just due to their larger size. However, higher mass at initial capture was significantly related to more intact mitochondria had a low inverse optical density, indicating mitochondria that were more intact (Table 5) perhaps indicating that larger initial size allows better repair so that damage did not accumulate. Also, there was an inverse relationship between mitochondrial integrity and the number of days elapsed from when a butterfly was first captured to when it was sampled destructively. That is, butterflies that were out in the wild longer before they were recaptured had less damaged mitochondria than butterflies that were recaptured sooner. This trend is not easily understood. One possible explanation is that the butterflies with less intact mitochondria are less likely to survive long enough to be captured at later times, and that the reason other butterflies were around long enough to be captured at a later date is because of their more robust mitochondria. Although the interpretation of these data is not clear, the results illustrate some of the life-history trade-offs related to the physiological variables we have been examining in these studies. We might expect larger butterflies to be more physiologically robust and thus have greater fitness, but this larger mass could come at the cost of placing metabolic demands on mitochondria which are essential for energy production, and this can negatively affect fitness. Although these results seem to be contradictory, they hint at factors that create stabilizing selection for an optimal body size and the fact that other environmental factors such as habitat and nutrient supply may help shape the physiological response to the genetic polymorphism.

Overall, this study helps illustrate that the way the body senses and responds to oxygen is a fundamental feature of aerobic organisms, and this study has helped provide insight into the role polymorphism in *Sdhd* plays in this process. Genetic polymorphisms affecting the hypoxia inducible pathway present a large mutational target for alleles that create quantitative variation in oxygen metabolism and physiology. Our results have revealed that subtle regulatory changes in the 3'UTR of *Sdhd* can result in many different phenotypes involving important traits such as tracheal development, mitochondrial integrity, and metabolic rate. However, there are many more questions to be answered related to this regulatory process of the hypoxia inducible pathway involving *Sdhd*. One major question is what are the possible tradeoffs associated with the different *Sdhd* alleles. If the M allele is associated with greater tracheal abundance and an increased metabolic rate, and the D-allele is associated with an increase in mitochondrial damage then why would any allele other than the M allele be maintained in populations? Other data from the flight experiments not presented in this thesis showed that there were only two DM heterozygote individuals in the French population and none in the Spanish population but that these two French butterflies had the greatest total metabolic rate (total CO_2 production) during the normoxic and hypoxic trials. It is possible that if the D allele is combined with the M allele there is a heterozygote advantage in which the butterfly gains the benefit of an increased metabolic rate for flying and other fitness-related activities, without the downside of increased mitochondrial damage associated with just having the D allele.

However, the small number of DM individuals in the population was a limitation in investigating this possible heterozygote advantage. What would be ideal would be to repeat these flight experiments with a sample containing more DM butterflies in order to get a better idea of why the D allele may be maintained in populations. Another related limitation of the study is that the presence of ROS was never exactly tested for, but instead mitochondrial damage was used as an indicator of the presence of ROS. Future work could be done in which assays for catalase and

superoxide dismutase activity, two anti-oxidizing enzymes involved in the conversion of oxygen radicals into oxygen and water, could give a better indicator of ROS activity following periods of hypoxic stress (Mishra and Delivoria-Papadopoulous, 1988). These enzyme assays could help provide support for the hypothesis that D-allele butterflies with more mitochondrial damage have an increased production of ROS following hypoxic stress.

Another exciting future direction for these results would be to determine if alleles in the 3' UTR of *Sdhd* are common in other species and whether they have the same effect on the hypoxia inducible pathway and the development of related physiological traits. Previous work has been done in humans with rare *Sdhd* mutations resulting in complete loss of SDH function that cause vascularized tumors, but it would be interesting to measure the metabolic rate of highly trained athletes and correlate it with measures of their SDH activity and *Sdhd* genotype. By investigating the role of *Sdhd* in the hypoxia inducible pathway of humans we could gain insights into human health and development that could be very beneficial.

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ACADEMIC VITA

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Education

B.S., Biology, 2013, The Pennsylvania State University, University Park, PA

Student Marshal for Penn State Department of Biology at Spring 2013 Eberly College of Science Graduation Ceremony

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Honors and Awards

- Evan Pugh Senior Scholar Award, Penn State University, April 2013
- Evan Pugh Junior Scholar Award, Penn State University, March 2012
- Hill Memorial Biology Fund Award, Penn State University, May 2011
- President Sparks Award, Penn State University, March 2011
- President's Freshman Award, Penn State University, March 2010
- College of Science Undergraduate Research Grant, Penn State University, October 2010
- Academic Excellence Scholarship, Schreyer Honors College at Penn State University, 2009-2013

Activities

- Penn State Triathlon Club Vice-President, August 2011-May 2012
- Penn State Newman Catholic Student Association Service Chair, August 2010-May 2011
- Schreyer Honors College Orientation Mentor, August 2010

Research Experience

Penn State Department of Biology: Marden Lab Group, University Park, PA

- Performed molecular biology techniques such as PCR and gel electrophoresis

- Prepared and stained tissue samples for confocal microscopy, and transmission electron microscopy
- Obtained images using confocal and TEM microscopes and analyzed the images using the NIH software Image J
- Conducted field research in Spain during Summer 2011

Research Interests

I have broad interests in ecology and evolutionary biology. Specifically, I am interested in physiological ecology or how organisms develop adaptations that are evolutionarily advantageous to their specific environment. In the lab I work in we look at how genetic mutations in the gene encoding the enzyme succinate dehydrogenase effect important physiological traits such as oxygen delivery and mitochondrial integrity.

Professional Presentations

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Publications and Papers

Marden, J. H., Fescemyer, H.W., Schilder, R.J., Doerfler, W.R., Vera, J.C., Wheat, C.W. "Genetic Variation in HIF Signaling Underlies Quantitative Variation in Physiological and Life-History Traits within Lowland Butterfly Populations." *Evolution*. **67**, 1106-1116 (2013).