Characterization of protein expression profile during development of the Puerto Rican monarch butterfly *Danaus plexippus megalippe f. portoricensis* A. Clark, 1941 (Lepidoptera: Danainae)

by

Ramón A. Rosario Rodríguez

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

UNIVERSITY OF PUERTO RICO
MAYAGÜEZ CAMPUS
2015

Approved by:

_____	_____
Ana V. Vélez Díaz, MS Member, Graduate Committee	Date
_____	_____
Carlos J. Santos Flores, PhD Member, Graduate Committee	Date
_____	_____
Carlos A. Acevedo Suárez, PhD President, Graduate Committee	Date
_____	_____
Teodoro M. Ruíz López, PhD Graduate School Representative	Date
_____	_____
Nanette Diffoot Carlo, PhD Chairperson of the Department	Date

Abstract:

Danaus plexippus megalippe, a non-migratory sub-species of the monarch butterfly, is a resident species in Puerto Rico. Molecular studies in monarch butterflies are fairly scarce and a protein profile has never been documented. Given that it has been petitioned to place the monarch butterfly in the Endangered Species Act, understanding its development has become a priority. We optimized an SDS-PAGE protocol in order to compare the protein profiles of the three developmental stages of *Danaus plexippus megalippe* and performed BCA protein assays to determine the amount of protein per cell. We found that both the larvae and the adult have near identical protein profiles and show two prominent bands at approximately 40 and 70 kDa. The pupa shows several other bands but also consistently expresses the 40 and 70 kDa bands. Based on the size of lepidopteran proteins that have been described, these bands could represent antitrypsin and a Na+/K+ ATPase subunit. However, further studies are necessary in order to characterize these protein bands. In BCA assays the pupa has significantly more protein than the larvae or the adult, which is consistent with the changes happening due to metamorphosis. In addition, we found that adults who eclose the pupa but never stretched their wings and died, presented high variability in their protein profiles.

Resumen

La sub especie residente, no migratoria, de la mariposa monarca en Puerto Rico es *Danaus plexippus megalippe*. Hay muy pocos estudios moleculares acerca de las mariposas monarcas y nunca se ha documentado un perfil proteico de esta. Dado que se ha solicitado incluir a la mariposa monarca en el Acta de Especies en peligro de Extinción (ESA por sus siglas en inglés), entender el desarrollo de la mariposa monarca se ha vuelto una prioridad. Optimizamos el protocolo de Electroforesis de poliacrilamida (SDS-PAGE) para poder comparar el perfil proteico de las tres etapas del desarrollo de las mariposas monarca e hicimos un ensayo de cuantificación de proteínas (BCA) para determinar la cantidad de proteína por célula. Encontramos que las larvas y los adultos tienen un perfil proteico casi idéntico en el cual se pueden observar dos bandas prominentes, de 40 y 70 kDa, consistentemente. La pupa muestra una variedad mayor de bandas, sin embargo también podemos observar consistentemente las bandas de 40 y 70 kDa. Basándonos en el peso molecular de las proteínas que se han descrito en lepidópteros, estas bandas podrían ser antitripsina y una sub unidad de la ATPasa de sodio y potasio. Sin embargo, se deben realizar otros experimentos para caracterizar estas bandas de proteínas. En los ensayos de BCA, la pupa demuestra tener más proteína que las otras dos etapas. Esto va en acorde con los cambios que ocurren dentro de esta durante la metamorfosis. Finalmente encontramos que, los adultos que salían de la pupa pero no lograban estirar sus alas y morían, presentaban una alta variabilidad cuando se observaban sus perfiles proteicos y la concentración de sus proteínas.

To Zeny and Luna

I love you both

Always and forever

Table of contents

Introduction

The monarch butterfly, *Danaus plexippus*, is a species of the family Nymphalidae (subfamily Danainae) that is commonly known for its long migration across North America. On August 2014, a petition was filed by the Xerces Society, the Center for Biological Diversity, and Dr. Linda Bower to protect the monarch butterfly under the Endangered Species Act (ESA) (1). It has been shown that the continuous loss of milkweed (2), the monarch butterfly's host plant, and the toxic effects from transgenic crops (3-4) have contributed largely to a drastic population decrease. In Puerto Rico, we can find a non-migratory sub-species, *D. p. megalippe,* with a color variation unique to the island. During winter, the migratory sub-species *D .p. plexippus* can also be found on the island.

Although there are some molecular studies of butterflies (5-7), they are rare for monarch butterflies (8-9). In 2011, the *Danaus plexippus* genome was sequenced (10) and that sparked a renewed interest in genetic studies of the species (11). With a sequenced genome, and groups of their proteins described (some thanks to their existence in other arthropods, such as *Bombyx morii),* a protein profile of monarch butterflies could promote new studies that could help preserve the species a possibility.

Our study aims to standardize protein extraction protocols for monarch butterflies, quantify proteins using Bicinchoninic acid assays (BCA assay) and create a protein profile using Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). We believe that a better understanding of the proteins that are involved in monarch butterfly development is crucial for the species' survival. Proteins identified using these methods could help avoid

exposing the butterfly to proteins that will have a toxic effect on them (3) or explain which processes are affected by parasites (12-13) and how the species responds when infected. Thus, we aim to compare protein profiles between the three developmental stages of the monarch butterfly (larvae, pupae and adults) to better understand the biochemical processes and changes that occur during its life cycle.

Literature Review

The monarch butterfly (*Danaus plexippus)* belongs to the family Nymphalidae (subfamily Danainae). It is a milkweed butterfly from North America whose range has broadened to include New Zealand, Australia, the Caribbean, the Canary Islands, Azores and Madeira, and, occasionally, Western Europe and the United Kingdom (*14*). It is known for its long migrations across the United States and Mexico (*15*). Monarch butterfly sub-species are separated into migratory winter butterflies (such as the sub-species *plexippus*) and non-migratory summer butterflies (such as the sub-species *megalippe*). The migratory behavior makes winter sub-species larger and stronger than the non-migrating summer sub-species (*8*). *Danaus plexippus megalippe* has three forms (color variations based on brightness and spot patterns), that were previously considered to be separate sub-species: *leucogyne, tobagi*, and *portoricensis* (*16*). The form *portoricensis* is the one being evaluated in this study. Physical differences may be found between *Danaus plexippus plexippus* and *megalippe* sub-species according to their developmental stage. The larva, or caterpillar, of the sub-species *plexippus* has a yellow band in the middle of each thick black band (Fig 1) that the *megalippe* sub-species lacks. The pupae for both sub-species are virtually identical and, since they vary in size from one individual to another, they are almost impossible to distinguish from one another. The adult, or butterfly, of the *plexippus* species is consistently larger and flies more acrobatically than its smaller and more gentle flying *megalippe* counterpart (*17*).

Figure 1. The larvae of *Danaus plexippus megalippe* (left) and *Danaus plexippus plexippus* (right) have a slightly different band pattern. The *megalippe* sub-species has thicker black bands while the *plexippus* sub-species has thinner black bands broken by a thinner yellow band.

Insects have two common types of metamorphosis. Insects from the subclass Pterygota such as grasshoppers, crickets, and cockroaches have incomplete metamorphosis and their young (called nymphs) look like the adult but without wings. On the other hand, butterflies, moths, and flies, from the subclass Endopterygota, undergo complete metamorphosis. There are four stages in complete metamorphosis: egg, larva, pupa and adult. The young, also known as larvae, look very different from the adult and also have a different diet.

Eggs are laid by the adult female butterfly on a host plant; a species-specific plant that will serve as food for the larvae. In *D. p. megalippe*, host plants are commonly milkweeds (Asclepidae), plants from the genus *Asclepias* (Fig 2) as well as *Calotropis procera* (*18*-19). After the eggs hatch, the larva, also called a caterpillar, eats and grows constantly. It will shed its skin 5 times before pupating. The time before each shed is called an instar, the first of these being the newborn larva and the last being the stage before it pupates. Monarch butterflies have a

larva that starts as roughly the size of a pinhead and later grows up to two inches long after several weeks. When the caterpillar is fully grown, and in its fifth instar, it will attach itself to the host plant with a silk disc and shed its skin one more time revealing an emerald green exoskeleton that will harden and become its pupa, also known as chrysalis. Apoptosis, or programmed cell death, occurs inside the cocoon and transforms the larvae cells into the components needed to later form the legs, wings, eyes and other parts of the adult butterfly. Once the metamorphosis is complete the pupa will become translucent and reveal the adult butterfly inside, which will subsequently eclose. In contrast to the larva, the adult butterfly has long legs, antennae and compound eyes (Fig 2). After stretching its wings the adult butterfly will begin the final stage in its life cycle, in which it will look for a mate for reproduction, locate the host plants on which it will lay its eggs and, in some sub-species, migrate (*20*).

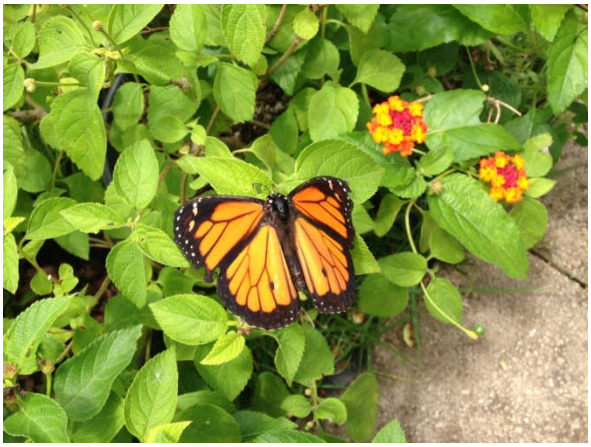

Figure 2. A male *Danaus plexippus megalippe f. portoricencis* on *Asclepias curassavica.*

The host plant of the monarch butterfly larvae is commonly a member of the genus *Asclepias*, most of which carry toxins called cardenolides (*21*). These compounds are heart arresting steroidal toxins. Some herbivores have developed the capacity to sequester cardenolides while greatly reducing their harmful effects (*22*). For example, during the larval stage, the monarch sequesters cardenolides, from the milkweed plant, to use as a method of self-defense against predators (*22-23*).

Although molecular studies of insects have become a popular topic among entomologists, they are commonly focused on DNA studies and aim to improve taxonomy or to show population genetics (*24-26*). A variety of other studies focus on species that affect agriculture (*27-28*). Although studies that use protein profiles in insects are available (13, 29-30), comparison of protein profiles among different stages of development of insects have not been published so far. A protein profile study may pave the path to the description of the monarch's proteins. This would help to understand how human factors, such as Bt maize pollen (4), are negatively affecting the monarch in a physiological level.

Most work regarding monarch butterflies is based on describing their behavior and how it affects their environment. For example, there have been studies on how they mediate pollen dispersion (*3*), yearly migration (*8*, *11*, *17*, *31*), or the differences in behavior between winter and summer butterflies (*8*). Some researchers have proposed changes in monarch classification based on morphological data(11, *16*). In 2011, the *Danaus plexippus* genome was sequenced (*10*) and that has sparked a renewed interest in genetic studies (*11*). Although there are a few molecular studies in butterflies (*5-7*), they are rare for monarchs (*8-9*).

On August 2014, a petition was filed by the Xerces Society, the Center for Biological Diversity, and Dr. Linda Bower to protect the monarch butterfly under the Endangered Species Act (ESA) (*1*). It has been shown that the continuous loss of milkweed plants(*2*) and the lethal effects from transgenic Bt maize crops (*3-4*) have contributed largely to a drastic population decrease. Several organizations, such as the Xerces Society and the Center for Biological Diversity, have presented census data documenting a 90% population decline (*32-33*). Thus, having a well-rounded understanding of the monarch butterfly, in aspects other than just its behavior, has become vital for its conservation.

In 1993, protein fingerprinting was developed allowing scientists to document the differences in proteic compositions of different organisms, (*34*). Sodium dodecyl sulfate polyacrylamide gel electrophoresis, or SDS-PAGE, is a commonly used technique that separates proteins according to their size (length of the polypeptide chain) while neutralizing the effect of their charge (*35*). By adding SDS (an anionic detergent), proteins are denatured and a net negative charge is imparted to the linearized proteins. This negative charge makes molecular weight the only factor that will influence the protein's migration through the polyacrylamide gel. Thus a protein profile is generated based on the migration pattern of the bands observed in the polyacrylamide gel. The patterns of bands on the gel are referred to as SDS-PAGE protein profile. This technique has been used in medicine (for detection of prostate cancer (*36*), or brain tumors (*37*)), for differentiating species (*38*), and in the food industry (*39*).

Protein fingerprinting and genetic studies have also been used as a defining factor in the differentiation of several species, both eukaryote and prokaryote (*9*, *22-23*, *26*, *38*). It has been

shown that physical parameters such as temperature (*40*), salinity (*41-42*), seasonal changes (*43*) can be factors that stimulate positive selection towards different traits. Positive selection towards butterflies who resist different types of weather or that do not have to migrate is a main cause of the differentiation of the sub-species of the monarch butterfly (*8*).

Recent studies involving butterfly proteins have focused on a cytotoxic protein called pierisin, found in butterflies of the family Pieridae (*5-6*, *44-47*). This protein, that has pro-apoptotic activity, is expressed during the chrysalis stage. This protein's cytotoxic capabilities have been tested on human cancer cell lines, and it has yielded positive results suggesting that it may be investigated as possible cancer treatment (*5*). No studies on pierisin have been done on the Western Hemisphere and it is unclear if other butterfly families have proteins with functions similar to pierisin during metamorphosis.

The present study aims to promote the characterization of monarch butterfly proteins in an effort to strengthen conservational efforts and also to raise awareness of the possible uses of monarch proteins in the medical field. Proteins identified using these profiles could help avoid exposing the butterfly to chemicals that will cause a lethal effect on them (3) or explain which processes are affected by parasites (12-13) and how the species responds when infected.

Materials and methods

Butterflies

The bulk of our monarch specimens (*Danaus plexippus megalippe f. portoricensis*) were bred in the Juan A. Rivero Zoo in Mayagüez. These were provided, in their different developmental stages, by the biologist Joalisse Mendoza. Additional specimens were collected from the Jardín Tanamá Butterfly Garden at the University of Puerto Rico - Utuado Campus, thanks to the collaboration of Dr. Marisol Dávila. Adults that had fed and adults that had just eclosed were used in order to avoid any bands produced by proteins found in the gut after feeding.

Protein extraction

No previous protocol or studies have been published regarding protein extraction or sample preparation on butterflies for SDS-PAGE. The samples were macerated and filtered through a 0.1 mm filter using 1X PBS buffer to dilute them. For caterpillars and pupae, the entire organism was used, and for adult butterflies we used only the abdomen. We modified a published protein extraction protocol (*48*), by counting the number of cells per sample with a hemocytometer (Fisher Scientific; 0.1 mm deep), to ensure reproducibility in our experiment. Each sample was comprised of three individuals of the same developmental stage, and contained 10^7-1.5 X 10^7 cells. We mixed each sample with 100 µl of either 6X SDS sample buffer (7 ml 4x TrisCl/SDS pH 6.8, 3.0 ml 3.8 g glycerol, 1 g SDS [1% w/v], 0.5 M β - mercaptoethanol, 1.2 mg bromphenol blue, add H2O to 10 ml) or radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS)

and 100 µl of protease inhibitor cocktail from Roche. The samples were left on ice for two hours and then centrifuged at 13,000 rpm for 15 minutes. The pellet was discarded and the samples were stored at -4°C.

BCA Protein Assay (Pierce Biotechnology protocols)

BCA assay (Pierce) was performed according to the manufacturer's instructions (*49*). BCA Assay is a modification of the Biuret protein assay. Copper ions are reduced in the presence of proteins, from Cu^{2+} to Cu^{1+}. The amount of copper reduced is proportional to the concentration of protein in the sample. When the BCA reagent is added to the solution, color starts to change from green to purple as the reagent interacts with the reduced copper ions, darker hues indicating higher protein concentration.

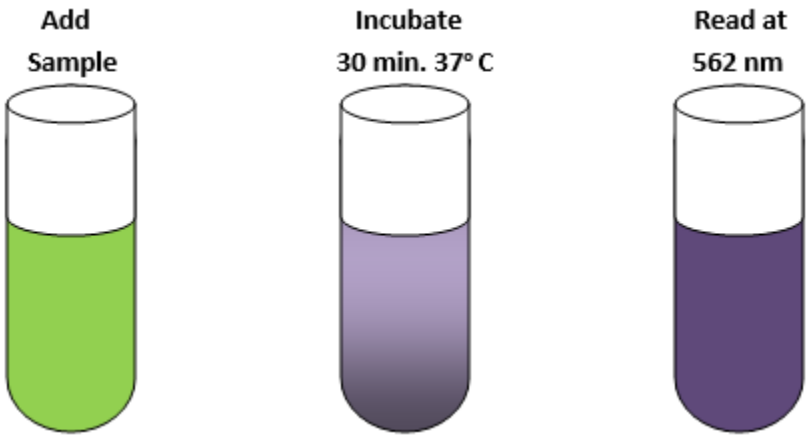

Figure 3. BCA Assay

A spectrophotometer is used to read the samples at 562 nm. The absorbance of our samples was then compared to a standard curve generated using known concentrations of Bovine Serum Albumin (BSA). A Multiskan Ascent microplate reader from Thermo Scientific was used to scan the samples.

SDS-PAGE

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed using standard protocols (*50*). We heated 30 µl of each sample containing 5 µl of sample buffer. Gels contained 10% acrylamide and were typically run at 90-100 V using the Bio Rad Mini Protean Tetra System electrophoresis chamber. The gels were stained using Coomassie® blue.

Results

Protein profile comparisons between the different stages of insects have not been published so far. We consistently found two bands present in all three developmental stages of the monarch butterfly. These bands corresponded to 40 and 70 kDa. The pupae also consistently showed several bands that were not detectable in the larvae or the adult.

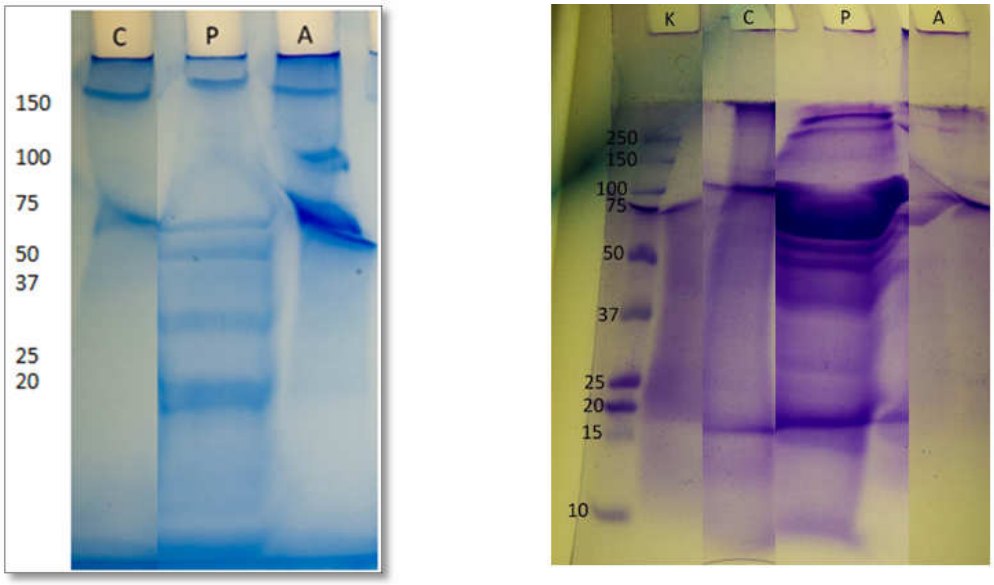

Figure 4. Protein profile of caterpillar (C), pupa (P) and adult (A) *D. p. megalippe*. Each lysate contains 10^7 (left) and 1.5 x 10^7 (right) cells total from a pool three individuals. The lysates were prepared with SDS sample buffer and were run on 10% SDS-PAGE. Molecular weight markers (in kilodaltons) are indicated on the left.

Table 1. BCA Assays in healthy specimens mean values ± standard deviation (N=4)

	SDS			RIPA		
Developmental Stages	**Larvae**	**Pupae**	**Adult**	**Larvae**	**Pupae**	**Adult**
Concentration (µg/µl)	54.95±3.45	42.99±6.86	46.02±0.67	7.61±0.41	41.97±8.09	7.21±0.3

BCA protein assays performed with samples lysed with SDS sample buffer show that adults and pupae have almost identical protein concentration but larvae samples have approximately 1.3 times more protein. When compared with RIPA buffer, SDS sample buffer extracts a higher amount of protein (which is consistent with SDS-PAGE results). Also, BCA Assays show that larvae and adults have almost identical protein concentrations but, the pupae has approximately 4.5 times more protein. Each concentration measurement represents four different experiments.

Non Viable adults (NVA), are adults that did not survive after eclosion from the pupa and never stretched their wings. SDS-PAGE and BCA protein assays of these adults showed similar protein profiles and concentration to the pupa but showed a high degree of variability.

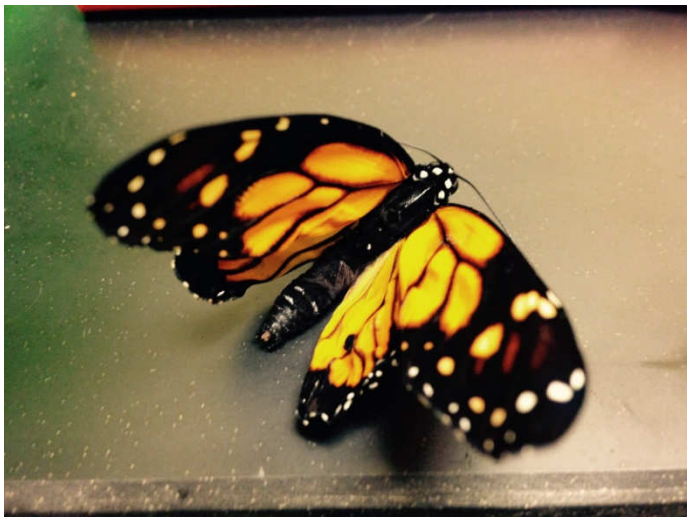

Figure 5. *Danaus plexippus megalippe* Non Viable Adult (NVA).

Table 2. BCA Assay in Non Viable adult specimens

	SDS			RIPA		
Non Viable adults	**NVA1**	**NVA2**	**NVA3**	**NVA1**	**NVA2**	**NVA3**
Concentration (µg/µl)	44.58	49.1	46.87	25.2	31.11	10.56

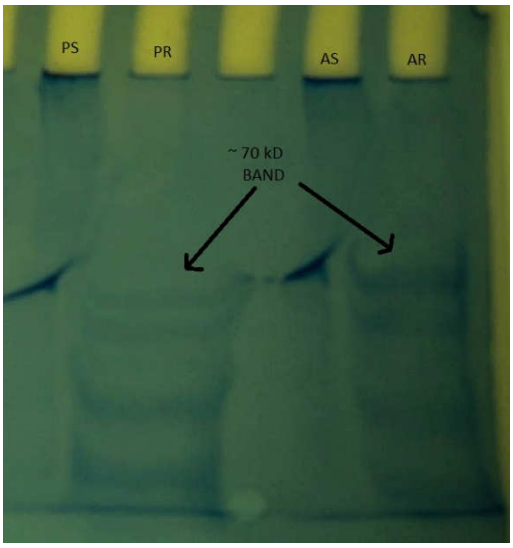

Figure 6. Lysates from 10^7 cells from *D. plexippus* pupa (PS and PR) and adults who could not stretch their wings (AS and AR) were prepared with both SDS sample buffer (AS and PS) and RIPA buffer (AR and PR). Samples were run on 10% SDS-PAGE. RIPA buffer better resolution in both pupa and the adult butterfly in contrast to the samples lysed with SDS. A very similar patterns of bands can be observed in both pupae and the NVA.

Discussion

Due to a paucity of protein profiling and BCA assays in insects, fine tuning the protocols was an important part of our experiments. During the fine tuning of the protein extraction process, we used complete adults, thorax, head and wings, but found that the proteins found in those parts were too low in concentration to be detectable using Coomassie® blue.

When comparing the molecular weight of the two bands that repeatedly appeared on our SDS-PAGE to several proteins that have been previously found on monarch butterflies or other Lepidoptera, there were several possible identities for the proteins. The higher molecular weight of some of the proteins encountered here could be due to methylation. For the 40 kDa band we believe that the protein corresponds to the Casein kinase II alpha subunit which has a molecular weight of 41.8 kDa and has been described in *Danaus plexippus*. There are two other proteins that have been described in the order Lepidoptera and that are also close to the 40 kDa molecular weight. The first of which is the Prothoracicotropic hormone (PTTH), which is found in the brain and induces molting or pupation (*51-52*),with a molecular weight of 37.51 kDa. The other possibility is that it is an antitrypsin (serin 1), a protease inhibitor from the serpin family, with a molecular weight of 47.92 kDa.

For the 70 kDa band, a potential candidate is the Na+/K+ ATPase alpha subunit, with a molecular weight of 86.68 kDa, which has been described in *Danaus plexippus*. Just as with the 40 kDa band, two other proteins have been described in the family Lepidoptera that are close to 70 kDa. The first of these proteins being Trehelase-2, a glycoside hydrolase enzyme, with a molecular weight of 78.576 kDa (*53*). The second protein is FTZ-F1, a transcription factor, with a molecular weight of 66 kDa (*12*). Further investigation is necessary in order to determine if

any of these are the proteins found in our 40 kDa and 70 kDa bands, such as using Edman degradation or other protein sequencing techniques.

Silver staining detects as little as 1ng of protein, while Coomassie® blue has a detection range of >100ng. Thus silver staining could be used to look at our protein profiles by electrophoresis. This staining technique could show bands or proteins that are in too low of a concentration for Coomassie® blue to detect. Silver staining in protein profiles of two mosquito species (*T. splendens* and *A. darlingi*) show more bands than our protein profiles but also consistently show bands at ~70 kDa and ~40 kDa (*13, 29*). Hymenopteran protein profiles, using silver staining, also show different patterns of bands than those shown on our samples (*30*).

BCA assays of healthy individuals show that the pupa has more protein than both the adult butterfly and the larvae. One possible explanation is that the protein concentration of the pupae is higher due to the amount of changes that the butterfly is undergoing throughout this stage. To determine if the different developmental stages rendered the same amount of protein we did a One-way Anova to compare protein extraction among stages (larvae, pupae, and adults) and found significant differences for SDS sample buffer ($F= 7.79$, $p= 0.01$) as well as for RIPA buffer ($F = 72.69$, $p < 0.0001$). When looking at the mean values we can show that larvae rendered more protein for SDS sample buffer data and that pupae produced a higher protein concentration for RIPA buffer.

It was noticed by the butterfly garden's staff that adults would sometimes eclose but were not able to stretch their wings and died. We called these individuals Non Viable adults (NVA). This condition could be due to infection with *Ophryocystis elektroscirrha* an obligatory protozoan parasite, from the family Ophryocystidae, that causes similar symptoms during mild

infections (*54*, *55*). Further studies need to be done in order to identify the presence of this protozoan. During BCA assays and SDS-PAGE of these NVA we found that the bands in the electrophoresis and the protein concentration from the BCA assays were similar to those of the pupal stage but also showed a high degree of variability. The remnants from their pupal stage might have still been present, perhaps due to their inability to complete metamorphosis or that proteases, such as serine proteases found in *D. plexippus*, had not been deactivated or were defective.

Conclusions

1. Larvae and adults of *Danaus plexippus megalippe* have near identical protein profiles that consistently show two bands at ~40 and ~70 kDa. The pupa also shares these bands but also presents other bands that are not present in the adults or larvae.
2. RIPA buffer produces samples with a lower protein concentration, but better resolution in gels.
3. 6X SDS sample buffer proved to be more effective at extracting proteins but also gave a cruder sample that had a higher concentration of proteins during BCA assays.
4. Based on a reported molecular weight of 41.8 kDa by Shuai Zhan et al. 2011, the ~40 kDa band could represent the casein kinase II alpha subunit.
5. Based on a reported molecular weight of 86.68 kDa by Shuai Zhan et al. 2011, the ~70 kDa band could represent the Na+/K+ ATPase alpha subunit.
6. Non-Viable adults were not able to complete metamorphosis successfully. They can produce pupae-like protein profiles but their profiles can be very variable. Their protein concentration can be close to the concentration of pupa proteins but is also variable.

Future studies

Using *Danaus plexippus megalippe* specimens from different countries might show a different protein profile than the Puerto Rican form. A further study could also take into account other sub-species of *Danaus plexippus*, such as the migratory plexippus sub-species and its four generations.

Characterize the proteins found in our 40kDa and 70kDa bands using proteases such as chemotrypsin and then comparing the findings to other reported butterfly proteins. Markers could be used to identify the proteins by CVB ELISA or gene profiling.

A large part of our project focused on fine tuning the protein extraction process for the SDS-PAGE. A few tests were run with *Ascia monuste eubotea* but due to a rapidly dwindling supply of the specimens in the zoo, any further SDS-PAGE runs were not possible. Thus, it is suggested to test the fine-tuned protocol developed herein with several other butterfly families.

Acknowledgments

I would like to thank everyone who made it possible for me to reach the end of this journey. To my mentor, Carlos A. Acevedo Suárez, who guided me in my personal and scientific growth from my first day as a graduate student, for being a mentor and friend like no other, who taught me to never say "I don't think I can do that", without whom I would not be the scientist I am today, and who made sure that this "wibbly wobbly timey wimey" adventure would be amazing. The Juan A. Rivero Zoo, and its personnel, who provided the bulk of the specimens used for our work and also the University of Puerto Rico - Mayaguez Campus for providing the laboratory and materials we worked with. To Katherine Carrero, who helped me remember all the techniques that I learned as an undergrad but thought I would never use. To the University of Puerto Rico - Utuado Campus, its Jardín Tanamá Butterfly Garden and Dr. Marisol Dávila for providing additional specimens. To Magaly Zapata, who made sure that my samples were always cold and sterile. To Ana V. Vélez, who taught me to love all branches of science, helped me find a newfound love for human anatomy, and trusted I could teach cell physiology "without letting anyone drink the chemicals". To Carlos Santos, who trusted I could do anything I wanted (even when I tried to work with aquaculture), and followed my academic journey from beginning to end. To Donato Seguí, who was a mentor and a friend, and who always backed me up, even in my wildest of decisions. To Jeanine Vélez, who manages to laugh whenever we remember that the general biology lab had a few fire incidents (mostly my hair), but somehow still trusted me to teach courses under her coordination. And to everyone in the Biology Department who helped me move forward, thank you.

I had amazing friends who helped me stay on track and helped me smile whenever things were not going my way. To Zullaylee Ramos, who dragged me along whenever I didn't want to move forward (even after she moved to Washington), and with whom I had late night FGS (frustrated graduate student) meetings to ensure one another that everything was alright and we were exaggerating. To Arlene Megill, who somehow ended up working with me in molecular biology, managed to ignore everything I said (or at least said she did) so that things actually worked the way we wanted them to work, and for helping me finish this thesis' photos. To Cecillanne Tirado and Jesús Seguí, who helped me discover that "sometimes the one who puts salt on the cake, doesn't know how to do it". To Manuel Martínez, José Cruz, Emmanuel Martínez, Fernando Cartagena, Efraín Muñíz and Gabriela Martínez, all of whom have helped me in every step of my journey and, although my scientific ramblings may have been a foreign language to them, they made sure that, at the end of the day, everything was fine in my world (even if that meant staying up late playing games to let up stress when we all had work or class the next day). To the Association of Biology Graduate Students, AEGB (which I lovingly revived), and the Association of Biology Students, AEB (that adopted me), and all their respective members who supported me throughout my time as a graduate student, thank you. And to all of my friends and fellow students whom I didn't mention, thank you so much for being there for me.

I would also like to thank my pillars, my family. To my grandmother Victorina Pérez, for remembering that scientists need a grandmother's meal from time to time in order to function. To Margarita "Gary" Nieves, who has been supporting me since I was little and has been

nothing less than a second mother, I love you. To my sister Andrea Rosario, my biologist partner-in-crime who has made me proud day after day, walking my footsteps and making me proud to be her role model. To my aunts Carmen Andújar and Flor Rosario, both their husbands and my cousins, for supporting me and cheering me on. To my wonderful mother Lourdes J. Rodríguez Andújar who has, from my birth, told me to follow my dreams, supported my every decision and been proud of all my achievements, Mom, I hope I'm making you proud! My father, Ramón E. Rosario, my inspiration, my role model and the one who fights against all adversities to make sure I can keep moving forward, I hope this makes you proud! My incredible wife Zenaida Calero Nieves, the one who hears my every complaint, cheers me up when I'm down, never lets me stop moving forward, and has the luck to have me and all my moods home every night (Note: Sarcasm), I love you so much and thank you for helping me through every step of this journey, this victory is yours as well! And last, but not least, my adorable, loving, playful daughter Luna Bella Rosario Calero; you are my shining star, every step forward is one that I take for you, for your smile and for every smile you help me have, I love you with all my heart.

Thank you for being with me through this journey and I hope you will still be with me for my next adventures!

Literature cited

1. Center for Biologicl Diversity, The Xerces Society, Lincoln Brower, and the Center for Food Safety, “PETITION TO PROTECT THE MONARCH BUTTERFLY (DANAUS PLEXIPPUS PLEXIPPUS) UNDER THE ENDANGERED SPECIES ACT,” (available at http://www.biologicaldiversity.org/species/invertebrates/pdfs/Monarch_ESA_Petition.pdf).

2. J. M. Pleasants and K. S. Oberhauser, Milkweed loss in agricultural fields because of herbicide use: effect on the monarch butterfly population. *Insect Conserv. Divers.* **6**, 135–144 (2013).

3. L. C. Hansen Jesse and J. J. Obrycki, Field deposition of Bt transgenic corn pollen: lethal effects on the monarch butterfly. *Oecologia*. **125**, 241–8 (2000).

4. K. Oberhauser and E. Rivers, Monarch butterfly (Danaus plexippus) larvae and Bt maize pollen: a review of ecological risk assessment for a non-target species. *AgBiotechNet*. **5** (2003) (available at http://t.monarchlab.org/Lab/app/upload/pdf/CON-1 Oberhauser and Rivers Bt Pollen Risk Assessment 2003.pdf).

5. K. Koyama, K. Wakabayashi, Presence in Pieris rapae of cytotoxic activity against human carcinoma cells. *Cancer. 87, 1259-1262* (1996).

6. M. Watanabe, T.Nakano, B. Shiotani, Y. Matsuhima-Hibiya, M. Kuichi, F. Yukuhiro, T. Kanazawa, K. Koyama, T. Sugimura and K. Wakabayashi, Developmental stage-specific

expression and tissue distribution of pierisin-1, a guanine-specific ADP-ribosylating toxin, in Pieris rapae. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **139**, 125–31 (2004).

7. H. Zhu, Q. Yuan, A. D. Briscoe, O. Froy, A. Casselman, and S. M. Reppert, The two CRYs of the butterfly. *Curr. Biol.* **15**, R953–4 (2005).

8. H. Zhu, R. J. Gegear, A. Casselman, S. Kanginakudru, and S. M. Reppert, Defining behavioral and molecular differences between summer and migratory monarch butterflies. *BMC Biol.* **7**, 14 (2009).

9. M. Hay-Roe, Effects in reciprocal crosses of Danaus erippus and Danaus plexippus (Lepidoptera: Danainae), supported by differentiation of cuticular hydrocarbons, establish their status as separate species. *Biol. J. Linn. Soc.* **91**, 445–453 (2007).

10. Zhan, S., Merlin, C., Boore, J. L., and Reppert, S. M. (2011). The monarch butterfly genome yields insights into long-distance migration. *Cell*, *147*(5)

11. S. Zhan, W. Zhang, K. Niitepold, J. Hsu, J. Fernandez Haeger, M. P. Zalucki, S. Altizer, J. C. De Roode, S. M. Reppert and M. R. Kronforst, The genetics of monarch butterfly migration and warning colouration. *Nature*. **514**, 317–321 (2014).

12. C. K. Ohno, H. Ueda, and M. Petkovichl, The Drosophila Nuclear Receptors FTZ-Flx and FTZ-F1l Compete as Monomers for Binding to a Site in the fushi tarazu Gene. *Mollecular Cell. Biol.* **14**, 3166–3175 (1994).

13. N. Jariyapan, W. Choochote, A. Jitpakdi, and P. A. Bates, Salivary gland of Toxorhynchites splendens Wiedemann (Diptera: Culicidae): ultrastructural morphology and electrophoretic protein profiles. *J. Med. Entomol.* **41**, 569–574 (2004).

14. Monarch Watch, (available at http://monarchwatch.org/).

15. L. Brower, Monarch butterfly orientation: missing pieces of a magnificent puzzle. *J. Exp. Biol.* **199**, 93–103 (1996).

16. D. Smith, G. Lushai, and J. Allen, A classification of Danaus butterflies (Lepidoptera: Nymphalidae) based upon data from morphology and DNA. *Zool. J. …*, 191–212 (2005).

17. S. Altizer, and A. K. Davis, Populations of monarch butterflies with different migratory behaviors show divergence in wing morphology. *Evolution (N. Y).* **64**, 1018–1028 (2010).

18. L. Goehring and K. Oberhauser, Effects of photoperiod, temperature, and host plant age on induction of reproductive diapause and development time in Danaus plexippus. *Ecol. Entomol.*, 674–685 (2002).

19. C. A. Dixon, J. M. Erickson, D. N. Kellett, and M. Rothschild, Some adaptations between Danaus plexippus and its food plant, with notes on Danaus chrysippus and Euploea core (Insecta: Lepidoptera). *J. Zool.* **185**, 437–467 (2009).

20. A. R. Pérez-Asso, J. A. Genaro, and O. H. Garrido, *Butterflies of Puerto Rico - Las Mariposas de Puerto Rico* (2009)

21. C. B. Zehnder and M. D. Hunter, Interspecific variation within the genus Asclepias in response to herbivory by a phloem-feeding insect herbivore. *J. Chem. Ecol.* **33**, 2044–53 (2007).

22. A. A Agrawal, G. Petschenka, R. A. Bingham, M. G. Weber, and S. Rasmann, Toxic cardenolides: chemical ecology and coevolution of specialized plant-herbivore interactions. *New Phytol.* **194**, 28–45 (2012).

23. A. Knight and L. P. Brower, The influence of eastern North American autumnal migrant monarch butterflies (Danaus plexippus L.) on continuously breeding resident monarch populations in southern Florida. *J. Chem. Ecol.* **35**, 816–23 (2009).

24. E. Gaublomme, K. Maebe, K. Van Doninck, H. Dhuyvetter, X. Li, and K. Desender, Loss of genetic diversity and increased genetic structuring in response to forest area reduction in a ground dwelling insect: a case study of the flightless carabid beetle Carabus problematicus (Coleoptera, Carabidae). *Insect Conserv. Divers.* **6**, 473–482 (2013).

25. S. De Mandal, L. Chhakchhuak, G. Gurusubramanian, and N. S. Kumar, Mitochondrial markers for identification and phylogenetic studies in insects – A Review. *DNA Barcodes*. **2**, 1–9 (2014).

26. G. D. Edgecombe, V. Vahtera, S. R. Stock, A. Kallonen, X. Xiao, A. Rack, and G. Giribet, A scolopocryptopid centipede (Chilopoda: Scolopendromorpha) from Mexican amber: synchrotron microtomography and phylogenetic placement using a combined morphological and molecular data set. *Zool. J. Linn. Soc.* **166**, 768–786 (2012).

27. B. Escriche, A. C. Martinez-Ramirez, M. D. Real, F. J. Silva, and J. Ferre, Occurrence of three different binding sites for Bacillus thuringiensis-endotoxins in the midgut brush border membrane of the potato tuber moth,phthorimaea operculella (zeller). *Arch. Insect Biochem. Physiol.* **26**, 315–327 (1994).

28. A. R. War, M. G. Paulraj, T. Ahmad, A. A. Buhroo, B. Hussain, S. Ignacimuthu and H. C. Sharma, Mechanisms of plant defense against insect herbivores. *Plant Signal. Behav.* **7**, 1306–20 (2012).

29. C. K. Moreira, M. T. Marrelli, S. P. Lima, and O. Marinotti, Analysis of salivary gland proteins of the mosquito Anopheles darlingi (Diptera: Culicidae). *J. Med. Entomol.* **38**, 763–767 (2001).

30. M. P. Dani, E. H. Richards, R. E. Isaac, and J. P. Edwards, Antibacterial and proteolytic activity in venom from the endoparasitic wasp Pimpla hypochondriaca (Hymenoptera: Ichneumonidae). *J. Insect Physiol.* **49**, 945–954 (2003).

31. J. Rawlins and R. Lederhouse, Developmental influences of thermal behavior on monarch caterpillars (Danaus plexippus): an adaptation for migration (Lepidoptera: Nymphalidae: Danainae). *J. Kansas Entomol. Soc.* (1981) (available at http://www.jstor.org/stable/25084171).

32. Monarch Watch, Bring Back The Monarchs (2014), p. http://monarchwatch.org/bring–back–the–monarchs/ca.

33. L. N. Smith, Monarch Butterfly's Reign Threatened by Milkweed Decline. *Natl. Geogr. Mag.* (2014), (available at http://news.nationalgeographic.com/news/2014/08/140819-monarch-butterfly-milkweed-environment-ecology-science/).

34. D. J. C. Pappin, P. Hojrup, and A. J. Bleasby, Rapid identification of proteins by peptide-mass fingerprinting. *Curr. Biol.* **3**, 327–332 (1993).

35. A. Rath, M. Glibowicka, V. G. Nadeau, G. Chen, and C. M. Deber, Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 1760–1765 (2009).

36. B. Adam, Y. Qu, J.W. Davis, M. D. Michael, M. A. Clements, L. H. Cazares, O. J. Semmes, P. F. Schellhammer, Y. Yasui, Z. Feng and G. L. Wright, Advances in Brief Serum Protein Fingerprinting Coupled with a Pattern-matching Algorithm Distinguishes Prostate Cancer from Benign Prostate Hyperplasia and Healthy Men. *Cancer Res.* **62**, 3609–3614 (2002).

37. J. Liu, S. Zheng, J. Yu, J. Zhang, and Z. Chen, Serum protein fingerprinting coupled with artificial neural network distinguishes glioma from healthy population or brain benign tumor. *J. Zhejiang Univ. Sci. B*. **6**, 4–10 (2005).

38. I. Berber, C. Cokmus, and E. Atalan, Characterization of Staphylococcus species by SDS-PAGE of whole-cell and extracellular proteins. *Microbiology*. **72**, 42–47 (2003).

39. M. Etienne, M. Jerome, J. Fleurence, H. Rehbein, R. Kundiger, and R. Mendes, Identification of fish species after cooking by SDS-PAGE and urea IEF: A collaborative study. *J. Agric. Food Chem.* **48**, 2653–2658 (2000).

40. L. McAlister and D. B. Finkelstein, Heat shock proteins and thermal resistance in yeast. *Biochem. Biophys. Res. Commun.* **93**, 819–824 (1980).

41. K. Mitsui, F. Ochi, N. Nakamura, Y. Doi, H. Inoue, and H. Kanazawa, A novel membrane protein capable of binding the Na+/H+ antiporter (Nha1p) enhances the salinity-resistant cell growth of Saccharomyces cerevisiae. *J. Biol. Chem.* **279**, 12438–47 (2004).

42. R. Munns and M. Tester, Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* **59**, 651–81 (2008).

43. J. C. Wingfield, C. M. Vleck, and M. C. Moore, Seasonal changes of the adrenocortical response to stress in birds of the Sonoran Desert. *J. Exp. Zool.* **264**, 419–28 (1992).

44. T. Kanazawa, T. Kono, M. Watanabe, Y. Matsushima-Hibiya, T. Nakano, K. Koyama, N. Tanaka, T. Sugimura and K. Wakabayashi, Bcl-2 blocks apoptosis caused by pierisin-1, a guanine-specific ADP-ribosylating toxin from the cabbage butterfly. *Biochem. Biophys. Res. Commun.* **296**, 20–25 (2002).

45. M. Yamamoto, T. Nakano, Y. Matsushima-Hibiya, Y. Totsuka, A. Takahashi-Nakaguchi, Y. Matsumoto, T. Sugimura and K. Wakabayashi, Molecular cloning of apoptosis-inducing

Pierisin-like proteins, from two species of white butterfly, Pieris melete and Aporia crataegi. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **154**, 326–33 (2009).

46. M. Yamamoto, A. Takahashi-Nakaguchi, Y. Matsuhima-Hibiya, T. Nakano, Y. Totsuka, S. Imanishi, J. Mitsuhashi, M. Watanabe, H. Nakagama, T. Sugimura, And K. Wakabayashi, Nucleotide sequence and chromosomal localization of the gene for pierisin-1, a DNA ADP-ribosylating protein, in the cabbage butterfly Pieris rapae. *Genetica*. **139**, 1251–8 (2011).

47. J. H. C. Orth, B. Schorch, S. Boundy, R. French-Constant, S. Kubick and K. Aktories, Cell-free synthesis and characterization of a novel cytotoxic pierisin-like protein from the cabbage butterfly Pieris rapae. *Toxicon*. **57**, 199–207 (2011).

48. T. M. Michael, R. Green, and J. Sambrook, *Molecular Cloning, A laboratory manual* (Cold Spring Harbor Laboratory, Fourth Ed., 2012).

49. Thermo Scientific, Pierce BCA Protein Assay Kit. *Instr. Man.* **0747** (2011).

50. K. Struhl, F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology, 5th ed., 2002.

51. C. M. Williams, Physiology of Insect Diapause II. Interaction between the pupal brain and prothoracic glands in the metamorphosis of the giant silkworm, Platysmata Cecropia. *Biol. Bull.* **93** (1947).

52. C. M. Williams, Physiology of insect diapause IV. The brain and prothoracic glands as an endocrine system in the cecropia silkworm. *Biol. Bull.*, 120–138 (1947).

53. R. P. Gibson, T. M. Gloster, S. Roberts, R. A. J. Warren, I. Storch de Gracia and A. Garcia, Molecular basis for trehalase inhibition revealed by the structure of trehalase in complex with potent inhibitors. *Angew. Chem. Int. Ed. Engl.* **46**, 4115–9 (2007).

54. S. Altizer and K. Oberhauser, Effects of the Protozoan Parasite Ophryocystis elektroscirrha on the Fitness of Monarch Butterflies (*Danaus plexippus*). *J. Invertebr. Pathol.* **88**, 76–88 (1999).

55. S. Altizer, Migratory behaviour and host – parasite co-evolution in natural populations of monarch butterflies infected with a protozoan parasite. *Evol. Ecol.* **3**, 611–632 (2001).