University of Nevada, Reno

Isolation and Characterization of an Isoprenoid Transporter Protein in *Manduca sexta*

A thesis submitted in partial fulfillment
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By

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We recommend that the thesis
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Abstract

Metabolic regulation and transport is a fundamental process in all eukaryotic organisms. Metabolites synthesized throughout the cell must be used in various locations to ensure proper cell function. Isoprenoids are metabolite molecules implicated in cell growth, energy production, and act as precursor to steroids, cholesterol, and other derived compounds in living organisms. Within the isoprenoid pathway, isopentenyl pyrophosphate (IPP) was found to be transported by a specific membrane protein molecule in plants (Bick and Lange, 2003). To date, inhibition of this pathway is used in mammalian drug therapy where isoprenoid utilizing enzymes are the candidates of inhibition accomplished by a class of drugs called stains, though many fundamental properties of this pathway await elucidation. The identification and characterization of an IPP transport protein in *Manduca sexta* would provide a model system for the study of IPP transport in animals as well as to allow characterization of mitochondrial metabolically derived compounds, which have not been possible to study. By isolating mitochondria from *M. sexta*, this study aims to purify the unknown transport protein for detailed characterization of isoprenoid pathway. These results may allow inhibition and manipulation of the isoprenoid pathway in insects possibly allowing the discovery of new insecticides.

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Introduction

Insecta is one of the largest classes of animals on earth comprising over 1 million classified species representing approximately 90% of all multicellular, eukaryotic living organisms. Insects are found in a vast array of environments, including harsh environments such as oceans and the arctic (Erwin, 1982). Throughout history, insects have been considered as a leading cause of disease due to their ability to carry and transfer pathogens form one area to another. Many epidemics throughout time have been associated with insect transmission as a root cause such as the Black plague during the medieval times to malaria in the Modern world (Garrett, 2012). However, an insect's ability to carry and transfer pathogens has been shown to be a less important factor than is popularly believed, and disease transmission has been associated at higher levels to the epizootic cycle between flies, rats, and humans (Goddard, 2000). While the majority of diseases have been shown to be associated with other routes of transmission, insects are still the primary cause of many diseases that are detrimental to human health such as malaria, dengue fever, Lyme disease, river blindness, elephantiasis, and West Nile Virus. Along with the possibility of carrying diseases, insects are also known for causing agricultural destruction resulting in reduced crop yields from invasive insect species. Over the last several decades, the use of insecticides has increased dramatically in attempting to control the massive population of insects and the negative effects they cause on resources. By controlling the population size of insects, improvements to human's health and prevention of infectious disease transfer can be achieved. As the utilization of commonly used insecticides is the primary means of achieving this goal, insects are acquiring resistance to the chemicals used much like bacteria acquire

resistance to antibiotics through adaption and evolution. Therefore, novel methods of insecticide discovery and use are a fundamental and key area of research to control insect population and maintain crop yields within the ever-progressing urban development. (Schooley, 2005). Currently, the insecticides used only target a narrow range of species calling for research into insecticides that are not only environmentally safe but can effect a large range of species. As the need of improved insecticides to decrease the negative effects to human health and development increases, the use of targeted metabolic inhibition has become a focal point of the research field. The identification and characterization of new metabolic pathways lends itself to the ability to create targeted inhibitors of insect development that can reduce their detriment on agriculture as well as human health. Diuretic hormones, water retention, and metabolic conversion of molecules required for development have all been presented as candidate targets to provide novel ways of insect population control. Refining this research focus to precursor molecules involved in growth and repair would provide a targeted insecticide that interrupted development early on in an insect's life cycle before the adults could reproduce or become agricultural pests.

Isoprenoids serve as conserved metabolites in cell growth, repair, and energy production in living organisms. Within the isoprenoid pathway, the common early molecule, isopentenyl pyrophosphate (IPP), has been characterized as precursor compound for steroids, cholesterol, bile acids, and a wide variety of other derived compounds. According to previous studies, the characterization of cytosolic IPP synthesis has been well defined and the important roles of IPP within the living organisms are well understood. However, it has been shown that the compound is primarily used in the

cytosol (Lange et al., 2000). Recently, it has been shown that the IPP precursor molecule in plants is transported into the mitochondrion by a membrane transport protein providing the only evidence that the molecule does not move by simple diffusion. This study however did not isolate the location of transport nor determine if this mechanism was conserved in other phyla or even species (Bick and Lange, 2003). Currently, there is no understanding on how IPP is transported from the cytosol into the mitochondria to serve a vital function in organisms. Therefore, the identification and characterization of the IPP transport protein and its mechanism in *Manduca sexta* would elucidate the metabolic production of isoprenoids within the mitochondria of animals. It is also believed that the characterization of this pathway and identification of transport proteins presents itself as a novel and method for production of insecticides.

Methods

Dissection of *Manduca sexta* and Mitochondrial Preparation For Competitive Binding Studies

Twelve 5th instar staged *Manduca sexta* were dissected in 1 hour (Cioffi, 1983) and the fat body was isolated and homogenzied with chilled, aerated homogenization buffer (250 mM sucrose, 5 mM EDTA, 5 mM Tris, 5 mM KF, 10 mM imidazole, and 100 µg/ mL streptomycin at pH 7.1) in a 5 mL Potter- Elvehjem homogenizer (Yamamoto, 1969). The homogenized preparations were centrifuged at 5,000 x g for 10 minutes. The supernatant was recovered and centrifuged again at 10,000 x g for 10 minutes. The cytosolic supernatant was recovered once more and centrifuged at 16,000 x g for 15 minutes. The mitochondrial pellet was washed with 1 mL homogenization buffer, vortexed, and centrifuged at 10,000 x g for 10 minutes. The homogenizer buffer

was removed and the pellet was resuspended with saved cytosolic supernatant (Godin, 2014). The Protein quantification of the mitochondrial preparations was determined using the EZ-Q protein assay (Invitrogen Cat R- 33200).

IPP Competitive Analog Studies

200 µL aliquots of mitochondrial preparations were placed in 15 Eppendorf tubes with 2.5 µL of 0.4 nmol/µL [^{14}C] IPP and varying concentrations of 0.2 nmol/µL of DMAPP (Table 1). These samples were vortexed, incubated, terminated by centrifugation at 10,000 x g for 10 minutes at room temperature, and analyzed with Liquid Scintillation Analysis (Warner, 2014).

Table 1. Concentration and volumes of [^{14}C] IPP and DMAPP. DMAPP = Dimethylallyl pyrophosphate, IPP = Isopentenyl pyrophosphate

[^{14}C] IPP 0.4nmol/ µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL	2.5 µL
DMAPP 0.2nmol/ µL	5 µL	15 µL	50 µL	150 µL	500 µL

Liquid Scintillation Analysis

After incubation, the reaction was terminated by centrifuging at 10,000 x g for 10 minutes. 100 µL of cytosolic supernatant were removed from each sample and placed in the corresponding labeled liquid scintillation counting vials. The remaining samples were centrifuged at 10,000 x g for another 10 minutes. After centrifugation, the cytosolic supernatant was extracted and discarded. The mitochondria pellet was suspended in 200 µL of homogenization buffer, vortexed and centrifuged at 16,000 x g for 10 minutes. This procedure was repeated twice and the supernatant was discarded each time. Finally, the washed mitochondrial pellet was resuspended in 200 µL of homogenization buffer and

vortexed. 100 µL of mitochondrial pellet was placed into corresponding labeled liquid scintillation counting vials. All samples placed in the LSC tubes were filled with 5 mL of Ultima Gold LSC cocktail and the samples were analyzed with liquid scintillation counting for 1 minute per vial (Warner, 2014).

Dissection of *Manduca sexta* and Mitochondria Isolation

Ten 5th instar staged *Manduca sexta* were dissected in 1 hour (Cioffi, 1983) and the fat body was isolated and suspended in chilled, aerated homogenization buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris – HCl at pH 7.5, 10 mM imidazole, 0.25 mM AEBSF protease inhibitor, and 100 µg/mL streptomycin) and subjected to homogenization in a 5 mL Potter- Elvehjem homogenizer (Yamamoto, 1969). The homogenized preparations were aliquoted into 1.5 mL Eppendorf tubes and centrifuged at 1,000 x g for 10 minutes to remove tissue debris. The supernatant was recovered and centrifuged at 5,000 x g for 10 minutes to remove cellular debris. The supernatants was again recovered and placed into a new Eppendorf tube and centrifuged at 16,000 x g for 15 minutes. The cytosolic supernatant was removed and saved; the mitochondrial pellet was washed in 1 mL of homogenizer buffer. The supernatant from mitochondrial pellet was discarded and the mitochondrial pellet was resuspended in cytosolic supernatant. Mitochondria samples were stored at -20^{o} C until ready to use.

Mitochondrial Isolation By Sucrose Gradient

Purified mitochondria were isolated by sucrose density gradient centrifugation. Sucrose gradients were made in 4-mL high- speed centrifuge tubes (Beckman Coulter, USA) with 1 mL of 1.5 M sucrose buffer (1.5M sucrose, 10 mM Tris base, and 0.1 mM EDTA, pH 7.6) overlaid with 1.6 mL of 1.0 M sucrose buffer (1.0 M sucrose, 10 mM

Tris base, and 0.1 mM EDTA, pH 7.6). The mitochondrial sample was centrifuged at 16,000xg for 30 minutes. The supernatant was discarded and mitochondrial pellet was resuspended in 1.6 mL of 1x MS buffer (12.5 mM Tris- HCl, pH 7.5, 525 mM mannitol, 175 mM sucrose, and 2.5 mM EDTA). The mitochondria suspension was layered on top of the sucrose gradient and centrifuged again at 40,000 x g for 30 minutes at 4^{o}C. The cloudy mitochondria white band between the gradient was gently removed with an 18 gauge needle and syringe and, transferred to 2 mL Eppendorf tube. The mitochondria band was centrifuged at 16,000 x g for 15 minutes. The supernatant was discarded and the pellet was resuspended in two- dimensional (2D) solubilization buffer (8M urea, 2 M thiourea, 4% (w/v) CHAPS, and AEBSF protease inhibitors) (Stimpson, 2015).

Membrane and Soluble Protein Fraction

The mitochondria samples extracted from sucrose gradient were placed in 20 mM Hepes for 3 minutes on ice and an equal volume of 2x PBS buffer was added after incubation. The mitochondrial samples were centrifuged at 125,000 x g for 3 hours at 4^{o}C; the supernatant was removed and saved as soluble fraction. The membrane pellet was resuspended in 1x PBS buffer and centrifuged at 125,000 x g for 3 hours at 4^{o}C. The supernatant was removed and combined with soluble fraction. The solubilized membrane pellet was suspended in 2D solubilization buffer. Both combined supernatants and mitochondrial pellet fractions were concentrated by dialysis with an additional 10 µL of β-mercaptoethanol to help remove solute in sample (Stimpson, 2015)

Gel Electrophoresis Preparation of Supernatant and Mitochondrial Pellets

The dialyzed samples of combined supernatants and mitochondrial pellets were prepared for gel electrophoresis to confirm the presence of proteins. 30 µL of samples

were combined with an equal volume of 1:20 ratio of 2- mercaptoethanol and Laemmlli buffer to solubilize proteins in two different sets of Eppendorf tubes. The first set of samples were vortexed and the second set of samples were vortexed and then boiled at 95^{o}C for 10 minutes prior to loading the samples in gel for analysis (Godin, 2014).

Analysis by gel electrophoresis

20 µL of standards and 40 µL of each sample were loaded into the Bio- Rad Mini-Protean TGX 4-20% Tris- HCl precast gels (Cat. 456 – 10943) using the Mini- Protean TGX gel protocol. Samples were run at 200 V for 40 minutes and washed with deionized water for 1-2 minutes, repeated twice. The protein gel was stained with 50 mL of BioSafe^{TM} Coomassie for 3 hours while shaking at 50 RPM. It was destained with deionized water for 5 hours while shaking at 50 RPM and water was replaced every hour (Godin, 2014).

Results

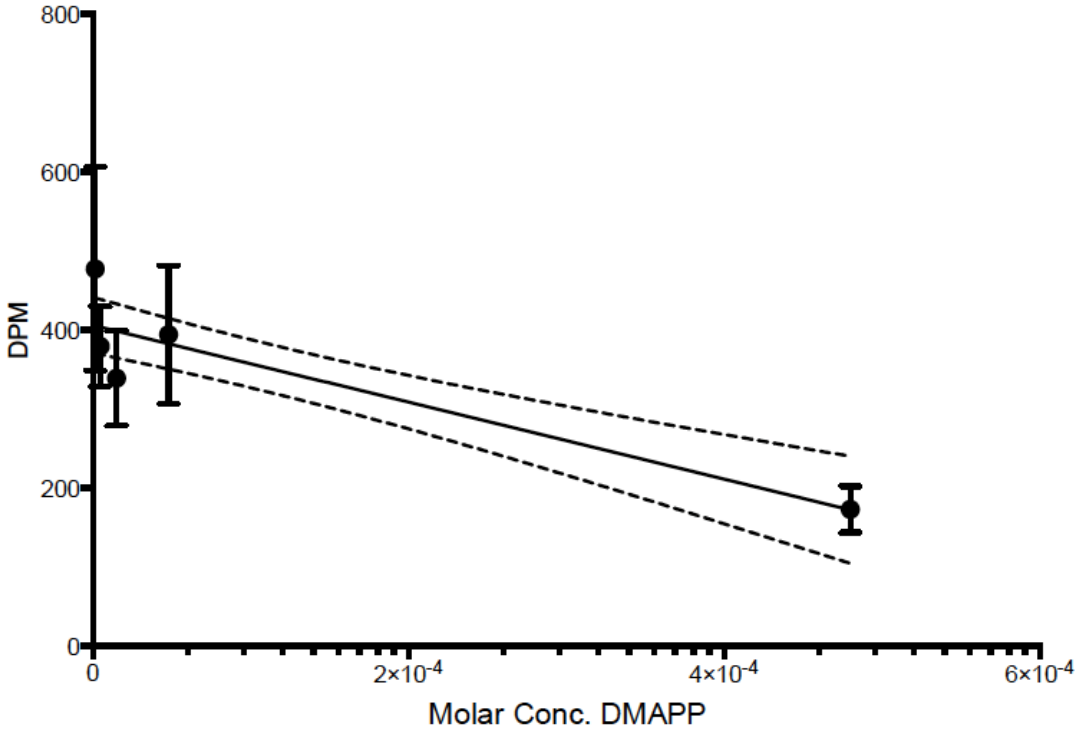

Figure 1. Competition study of radioactively labeled Isopentenyl Pyrophosphate and Dimethylallyl Pyrophosphate for bioavailability of the radio- analog. 200 µL aliquots of mitochondrial preparations were incubated for 10 minutes with 2.5 µL of 0.4 nmol/ µL [^{14}C] IPP and varying concentrations of 0.2 nmol/µL of DMAPP (Table 1). Samples were analyzed with Liquid Scintillation Analysis.

Figure 2. Gel Electrophoresis of dialyzed combined supernatant and mitochondria pellet. 10 wells of the gel were used to determine the presence of protein. 20 µL of standard were loaded onto lane 1 and 6. 40 µL of each sample was loaded into lanes 2, 3, 4, 5, 7, 8, 9, and 10. Lane 2 contains mitochondria pellet and lane 3 contains combined supernatant from the same fat body (mitochondria samples) unboiled. Lane 4 contains mitochondria pellet and lane 5 contains combined supernatant from the same fat body, mitochondria samples, unboiled. Lane 7 contains mitochondria pellet and lane 8 contains combined supernatant from the same fat body (mitochondria sample) as lane 2 and 3 but boiled in 92ºC for 10 minutes. Lane 9 contains mitochondria pellet and lane 10 contains combined supernatant from the same fat body (mitochondria sample) as lane 4 and 5 but boiled in 92ºC for 10 minutes.

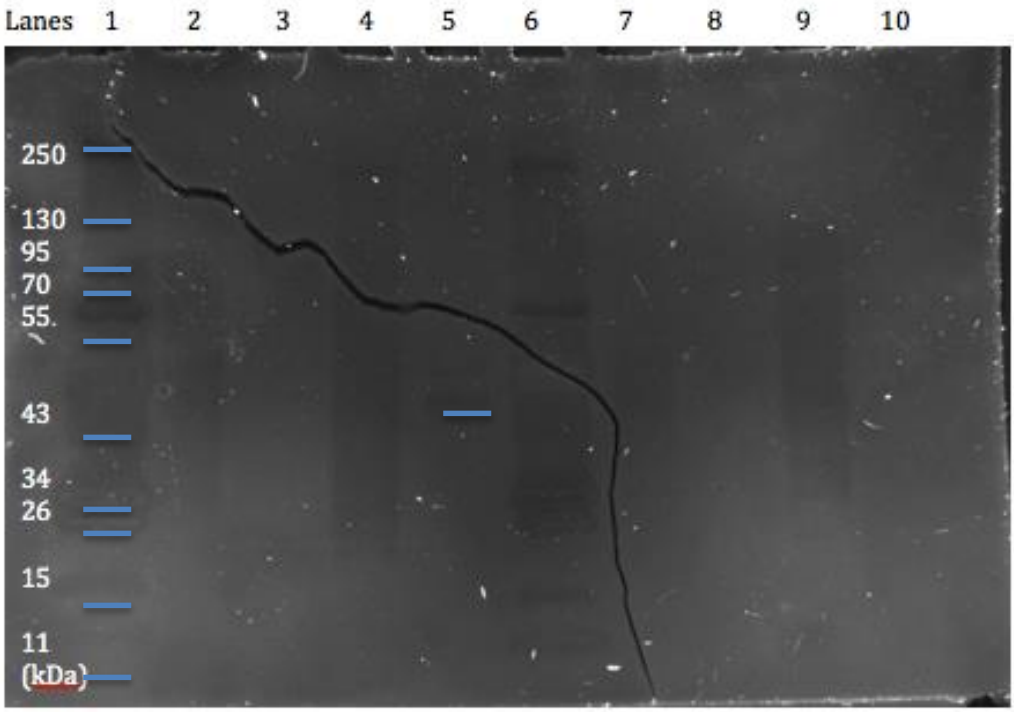

Table 1. Protein concentration of isolated mitochondrial pellet preparations. 1 µL of various concentrations (0.02 mg/mL, 0.10 mg/ mL, 0.50 mg/mL, 1.0 mg/ mL, 1.5 mg/ mL, 2.5 mg/ mL, and 5.0 mg/mL) of stock solution of ovalbumin were spotted onto the protein assay paper stabilized by 96- well microplate cassette in triplicate followed by 1 µL of each sample in triplicate to confirm presence and concentration of protein. EZ-Q protein assay paper was used to quantify proteins in the 2- D gel. This table is representative of each preparation where n=3.

Samples	**Concentrations of Protein (mg / mL)**		
Isolated Mitochondria Pellet	2.11	1.99	2.08

Table 2. Protein concentration of the dialyzed combined supernatant and mitochondria pellet. 1 µL of various concentrations (0.02 mg/mL, 0.10 mg/ mL, 0.50 mg/mL, 1.0 mg/ mL, 1.5 mg/ mL, 2.5 mg/ mL, and 5.0 mg/mL) of stock solution of ovalbumin were spotted onto the protein assay paper stabilized by 96- well microplate cassette in triplicate. Following by 1 µL of each sample in triplicate to confirm presence and concentration of protein. EZ-Q protein assay paper was used to quantify protein in the 2-D gel. This able is representative of each preparation where n=3.

Samples	**Concentrations of Protein (mg/ mL)**		
1. Dialyzed mitochondria pellet	0.070	0.077	0.065
2. Dialyzed combined supernatant	0.061	0.072	0.063
3. Dialyzed mitochondria pellet	0.091	0.093	0.090

4. Dialyzed combined supernatant	0.068	0.061	0.061

Discussion

DMAPP (dimethylallyl pyrophosphate) and IPP (isopentenyl pyrophosphate) are isomeric metabolites used in organisms for cell growth, repair, and energy production. To study the transport of IPP, the use of the radio labeled metabolite was necessary in this project. A competition study was conducted, it was concluded that [^{14}C] IPP competes with DMAPP for transportation into the mitochondria making it available for ubiquinone biosynthesis (Figure 1).

The isolation of mitochondria via centrifugation was successful, cellular debris was removed from the preparations and mitochondria pellet was extracted from the fat bodies from *M. sexta*. The protein concentration of the samples was determined via EZ-Q assay (Table 1). Protein concentration of isolated mitochondria pellet contained an average of 2.06 mg / mL protein per preparation. This demonstrates that there is adequate protein within the purified mitochondria pellet to proceed with isolation of the transport protein. The sucrose gradient produced a round white band observed between two different concentrations of sucrose as previously described. The white band was carefully extracted from the sucrose gradient, which shows that this is successful in purifying and removing cytosolic membrane proteins. The extracted white band containing mitochondria pellet was washed and resuspended to remove more cytosolic membrane proteins. The mitochondria pellet was successfully recovered from the 120,000 x g spin in the ultracentrifuge for 1 hour at 4^{o}C, which further purified and eliminated cytosolic proteins. The samples were dialyzed to remove solutes such as urea to eliminate any

faults in quantifying protein. The protein concentration of combined supernatant from the washes containing the membrane and soluble protein fractions and the mitochondria pellet were determined by EZ-Q assay (Table 2. Protein Concentration of the Dialyzed Combined Supernatant and Mitochondria Pellet). Sample 3 of the dialyzed mitochondria pellet had an average of 0.091 mg/mL and sample 4 of the combined supernatant had an average of 0.063 mg/mL, the protein concentrations were relatively low and it might explain why no distinctive band was observed from the gel electrophoresis besides the standards (Figure 2). The low protein concentration could be due to loss in dialysis, which it removed some of the proteins. Lane 2, 4, 7 and 9 contained mitochondria pellet samples showed a faint big smear band. Lane 3, 8, and 10 contained combined supernatant showed no bands. Lane 5 containing combined supernatant sample showed a faint band at approximately 50 kDa, which shows that there is protein activity within the samples. However, the protein activity should have appeared in the mitochondria pellet and this could be due to not having enough sample care during purifying the samples with centrifugation procedure. Ultimately, by isolating and purifying the mitochondria proteins we will be able to identify the IPP transporter protein. Although, no definite visible protein band appeared on the gel electrophoresis, through the EZ-Q assay it was determined that there was protein within the combined supernatant and mitochondrial pellet after wash. Due to our failure to identify the protein size by gel electrophoresis, isolation and purification procedures must be repeated with extra care technique to ensure any loss of protein. After protein is successfully identified with gel, samples will be reconstituted into liposomes and assayed for the ability of each liposome to transport IPP. Those liposomes that transport IPP will be further purify by HPLC with Strong Anion-

Exchange and Hydrophobic Exchange Column to elute proteins impurities. This should isolate the IPP transporter protein. The IPP transporter protein is predicted to be in either the outer or the inner mitochondrial membrane. After, the transporter protein is successfully identified; the purified protein will be reconstituted and placed into a liposome. A time course transport protein study will be conducted with [^{14}C] IPP to confirm the translocation of IPP from cytosolic to the mitochondria. Lastly, characterization of the IPP transporter protein, will allow new development of way to modify and establish new methods of insecticides to improve human health. It will also allow mining genomes of animals to locate the orthologous transport protein in animals. This is of huge medical important, as ubiquinone is an all important redox electron carrier vital for respiration in all animals. It has a short life, and it continuing synthesis from IPP is required for life in all animals and plants, as well as most bacteria and fungi. These results will form the basis of an RO1 application.

Literature Cited

Bick J.A. and Lange M. (2003) Metabolic cross talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: unidirectional transport of intermediates across the chloroplast envelope membrane. Archives of Biochemistry and Biophysics. 146- 154.

Cioffi, M. and Wofersberger, M.G. (1983) Isolation of separate apical, lateral, and basal plasma membrance from cells of an insect epithelium. A procedure based on tissue organization and ultrastructure. Tissue Cell. 781-803.

Erwin, T.L. (1982). Tropical forests: their richness in Coleoptera and other arthropod species. The Coleopterists Bulletin 36, 74-75.

Garrett, A. M. (2012) The effect of the corticotropin releasing factor- like diuretic hormone, Manse –DH, on the molecular mechanism of activation of vacuolar – ATPase in Manduca sexta Malpighian tubules. University of Nevada, Reno. 1 – 10.

Goddard, J. (2000). Infectious Diseases and Arthropods (Totowa: humana Press Inc).

Godin, Brandon (2014). Isolation and Identification of an Isoprenoid Transport Protein using an Isopentenyl Pyrophosphate Analog Photoprobe.

Lange, B.M., Rujan, T., Martin, W., Croteau, R., (2000). Isoprenoid biosynthesis: The evolution of two ancient and distinct pathways across genomes. Proc. Natl. Acad. Sci. USA 97, 12172 – 13177.

Yamamoto, R.T. (1969). Mass rearing of the tobacco hornworm. II. Larval rearing and pupation. J. Econ. Entomol. 62, 1427-1431.

Schooley, D. A., Horodyski, F.M., and Coast, G.M. (2005) Hormones controlling homeostasis in insects. In Comprehensive Molecular Insect Sciene. L.I. Gilbert, Insect Endocrinology. Elseviver, 366 - 429.

Stimpson, S.E. (2015). Optimal isolation of mitochondria for proteomic analyses. 475. 1-3

Warner, D (2014). IPP Competitive Analog Studies.