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Storage and Utilization of Hexamerin Proteins in the Pitcher Plant Mosquito, *Wyomyia Smithii*

Gangadasu E.C.V. Reddy

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STORAGE AND UTILIZATION OF HEXAMERIN PROTEINS IN THE PITCHER
PLANT MOSQUITO, *WYEOMYIA SMITHII*

by

GANGADASU E.C.V. SAGAR REDDY

(Under the Direction of William S. Irby)

ABSTRACT

The acquisition of hexamerin proteins in the pitcher plant mosquito, *Wyeomyia smithii* shows a North-South cline in the southeastern United States. Adult female *W. smithii* in North Carolina are completely autogenous and larger in size (based on wing length). They are anautogenous in Florida and smaller in size. The adult females in Georgia are intermediate in size with both autogenous and anautogenous behavior.

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS- PAGE) of adult female *Wyeomyia smithii* revealed a specific temporal pattern of utilization of hexamerin proteins for egg protein, vitellogenin, synthesis. *W. smithii* from North Carolina utilize majority of stored hexamerins in 30 hours after emergence with simultaneous increase in vitellogenins. Those from Georgia retain hexamerins for up to 42 hours and vitellogenins build up after 36 hours. Florida subpopulation show delayed utilization of hexamerin reserves with delayed egg laying (vitellogenins accrue rapidly after 60 hours).

Male pupae store less protein relative to females and utilize them within 6 hours after emergence into adults.

INDEX WORDS: Autogeny, Hexamerin, *Wyeomyia smithii*

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PLANT MOSQUITO, *WYEOMYIA SMITHII*

by

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A Thesis Submitted to the Graduate Faculty of Georgia Southern University in
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MASTER OF SCIENCE

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DEDICATION

To my wife, son, and parents.

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CHAPTER 1

INTRODUCTION

Blood feeding behavior in mosquitoes is responsible for their role as vectors of pathogens for many known human and animal diseases. An important factor affecting disease transmission patterns is the likelihood of mosquitoes in different species and populations to feed on blood. This is evident in the broad spectrum of blood-feeding behaviors that have been discovered in different mosquitoes, ranging from those that never take blood meals to produce eggs to those that only produce eggs as a result of blood-feeding. Autogeny is defined as production of eggs (or at least deposition of some yolk) without ingestion of protein by adult female mosquitoes. Autogenous mosquitoes do not require exogenous protein from a blood meal for initial egg laying, but they may ingest blood for subsequent cycles of egg laying. Anautogeny is egg production after blood feeding. Autogenous behavior was first reported in the mosquito *Culex pipiens* (Clements 1963, 1992; Spielman 1971). Since then, a variety of species have been found to exhibit this behavior, expressed as either an environmentally determined behavior (facultative autogeny), or as a genetically determined behavior (obligatory autogeny), or as a combination of both factors (reviewed in Clements, 1992).

Autogenous behavior can be obligatory (e.g., *Aedes atropalpus*) or facultative (e.g., *Deinocerites cancer*). Despite the availability of a vertebrate host for blood feeding, an obligatory autogenous species of mosquito will not feed on its host for initial egg laying. They depend on body proteins accumulated during larval

stages (Van Handel, 1976). Subsequent egg laying may or may not require a blood meal. A facultative autogenous species of mosquito will search for a suitable host for blood feeding after their emergence from the pupal stage. If they fail to encounter a host within a given time frame, they lay the first batch of eggs solely based on larval reserves. The eggs are smaller in size compared to a blood-fed female (O' Meara, 1985).

Reproduction and fecundity in insects are influenced by larval nutrition (Engelmann, 1970; O' Meara, 1987; Clements, 1992; Wheeler, 1996). Protein and lipid are important components of yolk in mosquito and other insect eggs (Clements, 1992). To facilitate yolk formation, female *Ochlerotatus atropalpus*, an obligatory autogenous mosquito, accumulates more proteins and fats than their counterpart males at all levels of larval nourishment, demonstrating a sex-influenced expression of metabolic processes. Poor nourishment of female larvae led to the emergence of smaller sized adults with decreased egg laying capacity. Rich larval diets led to the emergence of large sized adults with more egg laying abilities (Telang, 2004).

Autogeny offers a survival advantage at places where the probability of finding a host is diminished. It requires a shift in acquisition of nutritional reserves for egg production from adult to larval stages. The key adaptation that permits the expression of autogeny is the ability to accumulate these nutritional reserves in the form of storage proteins, or hexamerins.

Hexamerins belong to an expanding family of hemocyanins of arthropods and tyrosinases such as phenoloxidases. Hexamerins are high molecular weight

(~ 500 K Da) proteins composed of six polypeptide subunits each with molecular weight ranging from 72 – 90 K Da (Burmester et al., 1998). Depending on the properties of the subunits, insect hexamerins can be broadly divided into four categories: aromatic amino acid rich arylphorins; methionine-rich, female-specific hexamerins; riboflavin-binding hexamerins; and juvenile hormone-suppressible hexamerins (reviewed in Kanost et al., 1990). Based on the amount of aromatic amino acid content (Phe + Tyr), higher dipteran arylphorins are of two types: proteins belonging to the Larval Serum Protein-1 (LSP-1) subclass with high aromatic (>20%) and high methionine (>4%) content like blowfly calliphorin and LSP-1 of *Drosophila melanogaster*; and proteins belonging to the Larval Serum Protein-2 (LSP-2) subclass with only slightly elevated aromatic amino acid (15%) content and average methionine (2.5%) content like LSP-2 of *Drosophila melanogaster* (reviewed in Telfer and Kunkel, 1991). Hexamerin-1 (Hex-1) and Hexamerin-2 (Hex-2) belong to the LSP-1 and LSP-2 subclasses respectively. In some Lepidoptera such as Luna moths (*Actinias luna*), female pupae are shown to have more abundant overall methionine content compared to males (Pan and Telfer, 1996). Hex-2 is the primary hexamerin of mosquito larval hemolymph (lower dipterans) compared to higher dipterans where Hex-1 (or calliphorin or LSP-1) is the primary hexamerin of hemolymph. For example, in a lower dipteran, the yellow-fever mosquito *Aedes aegypti*, Aa Hex-2 is the primary hexamerin of late larval hemolymph (Korochkina et al., 1997).

Hexamerins serve as nutritional reserves during non-feeding stages of moulting and metamorphosis (Reviewed in Levenbook, 1985) and also during

non-feeding periods in adult life. They also provide amino acids for vitellogenesis during egg development, for cuticle maturation, and serve as transport proteins for carrying riboflavin and ecdysteroids (reviewed in Peter & Scheller, 1991).

Typically hexamerins are synthesized by fat bodies during larval stages and secreted into the hemolymph. Through a receptor mediated uptake mechanism, the hexamerins are stored in pupal fat bodies as specific protein granules (reviewed in Haunerland, 1996; Levenbook, 1985). The proteins accumulated in the fat bodies during larval stages are mobilized and utilized during metamorphosis and vitellogenesis for egg production. Hexamerins account for nearly 60% of total proteins in the larval hemolymph just before pupation.

In autogenous female mosquitoes, about 60% of the hexamerin reserves are utilized for metamorphosis and the remaining 40% reserves are taken up during vitellogenesis. The mobilization of these hexamerin reserves from fat body and their incorporation in eggs during vitellogenesis is reflected by the depletion of hexamerins in the fat body about 36hrs after ecdysis (Wheeler and Buck, 1996) and appearance of vitellin proteins thereafter.

Zakharkin and co-workers (2001) showed that the fourth-instar larvae of an autogenous mosquito, *Ochlerotatus (Aedes) atropalpus* synthesizes one protein, Hexamerin- 1.2 (Aat Hex-1.2) that is found only in female larvae and pupae. This implies female-specific expression of a hexamerin gene in *A. atropalpus* larvae. Accumulation of a methionine-rich subunit of hexamerin-1 in *Aedes aegypti* (Aa Hex-1 γ) is responsible for higher levels of Hex-1 γ protein in early female pupae

as shown by Gordadze et al (1999). Male mosquitoes tend to utilize all the accumulated hexamerins by the end of ecdysis.

Vitellogenin or vitellin is the principal proteinaceous component of insect egg yolk. Mosquitoes acquire this vitellin protein from two different sources: firstly, from the stored hexamerin proteins that are accumulated during larval stages; and secondly, from exogenous sources, like a blood meal during adult life. Only proteins derived after ingestion of blood can be utilized for egg production in anautogenous mosquitoes (Smith & Brust, 1971; Lang, 1978). Both hexamerin and vitellogenin proteins are similar in regards to high aromatic amino acid content (O' Meara, 1972; Chen, 1994; Romans, 1995).

There is an association between autogenous behavior and geographic location of the species or populations and season. Species at higher latitudes are known to exhibit autogeny to a greater extent. Sota (1994) noted autogenous behavior of the mosquito species *Aedes togoi* in Northern Kyushu, Japan only during spring and autumn but not in summer. Adult females that emerged during spring and autumn (shorter day cycles and lower temperatures) were larger in size and laid more eggs.

Transition from autogeny to anautogeny in the same species is exemplified by *Wyeomyia smithii*, and is correlated with the geographic origin of populations. A blood meal is not required by *W. smithii* for vitellogenesis at latitudes higher than 40°N and for multiple clutches of egg laying (O' Meara, 1981) *i.e.*, they are obligatorily autogenous. An external source of protein in the form of a blood meal (Bradshaw, 1980) is required by *W. smithii* at latitudes south of 36°N for ovarian

development. This transition from autogeny to anautogeny likely is due to the fact that the carrying capacity of pitcher plants in the southern region is almost always saturated conferring developmental constraints on the growth of larvae (Bradshaw & Holzapfel, 1982). This occurs because reproduction continues almost year round, with a brief diapause during the summer. As a result, the adult females emerge with underdeveloped ovaries. Follicles are immature and are in the previtellogenic phase in the newly emerged females of southern population (O' Meara & Lounibos, 1981). In contrast, vitellogenesis starts earlier for adult females of *W. smithii* from the northern region that emerge with follicles at stage III of development. At optimum conditions, the follicles mature within 48 hrs (Smith & Brust, 1971). In northern populations, a lengthy winter diapause results in a cessation of reproduction, and overall, population sizes are reduced relative to those in southern populations.

Heat tolerance of organisms like *W. smithii* with complex life cycles (i.e. those organisms with two or more distinctive phases), depend on habitat thermal stability and individual mobility. Heat tolerance is greater in life stages that are exposed to thermally variant than thermally stable environments. Sessile life stages have more heat tolerance than mobile life stages (reviewed in Zani et al, 2005).

Larval density and larval nutrition influence genetic expression of autogeny. Larval density affects time to pupation in *W. smithii* (Bradshaw, 1982; Istock, 1975). The survival of larvae of *Wyeomyia* species is also dependent on duration of environmental stressors. Shorter periods (≤ 2 week) of exposure to high larval

density did not effect survival in *W. felicia* (Seifert, 1980). The survival was affected in *W. vanduzeei* when the duration was more than three months (Frank & Curtis, 1977). Istock (1975) showed that *W. smithii* from Northern regions were not influenced by larval density for at least 25 days.

The geographic subpopulations of the purple-pitcher plant mosquito *W. smithii* in North Carolina, Georgia and Florida have previously been observed to exhibit variation in feeding behavior (autogeny), genetic structure (isozymes), reproduction (autogenous egg production) and production/utilization of hexamerin proteins. The geographically intermediate population from Georgia has generally been intermediate in all factors assessed (Irby, personal communication). *W. smithii* are autogenous in North Carolina, anautogenous in Florida and their feeding behavior intermediate in Georgia (i.e. autogenous and anautogenous), with anautogeny apparently increasing in frequency during the past 10 years (Irby, personal communication). In 1998, only 1 out of 100 mosquitoes from the Georgia population was observed probing during a cage feeding trial. In these trials, mated mosquitoes seven or more days after emergence were offered the opportunity to blood feed on a human arm inserted into the cage for 10 minutes. In more recent trials (2005, 2007 and 2008), using mosquitoes collected as larvae during each year and reared to adults in the laboratory, feeding trials have shown that from 10-25% of mated females attempted to blood feed, with feeding success (probing resulting in imbibing of blood) also increasing during that time.

Following the temporal sequence of hexamerin protein levels in the larval, pupal and newly emerged male and female pitcher plant mosquitoes can provide insight into this unique female-specific feature of storage and utilization of hexamerin proteins. It also sheds some light on understanding egg production (autogenous) behavior of mosquitoes, thereby helping in designing methods for altering/halting egg laying and preventing mosquito-borne infections. Finally, it provides an excellent model system for investigating the evolutionary processes leading to blood-feeding behavior in a population that had stopped expressing this significant phenotype, but appears to be expressing it again, possibly as a result of climate change.

The purpose of this study was to compare the amount of hexamerin protein accumulation in the three geographic subpopulations of *W. smithii* during larval, pupal and adult stages in both sexes. The study also focused on determining the time from ecdysis until hexamerins almost completely disappear from the newly emerged male and female mosquitoes of *W. smithii*.

CHAPTER 2

MATERIALS AND METHODS

a) Collection of larvae, pupae and adults:

Larvae of *W. smithii* were collected from populations of the purple-pitcher plant, *Sarraccenia purpurea*, from the Highlands Biological Research Station, Highlands North Carolina (35.054688°N, 83.187956 °W); the Apalachicola National Forest, near Wilma, Florida (30.126329 °N, 84.989237 °W); and near Manassas in Tattnall County, Georgia (32.167911 °N, 82.010772 °W) These larvae were fed fish food (Tetramin “Tropical Flakes”) and were maintained at 26°C with 16:8 daily light: dark cycles until their emergence into the pupal stage. Larvae from the three geographically distinct areas were followed through their emergence into the pupal stage and up to three days of adult life. Towards this end, based on the morphology of genital tubercles, pupae were sexed and frozen within 18 hours of their emergence. Adult samples of both sexes were collected and frozen every 6 hours for 3 days after their emergence from the pupal stage.

b) Sexing pupae:

Pupae were sexed based on the morphology of genital tubercles, by looking for consistent differences in these pupal structures, and correlating them with sex after emergence to the adult stage. Pupae were laid flat on their dorsum over a glass slide and examined under a light microscope. Males were identified by the presence of forked terminal abdominal segment; females were identified by the presence of a rectangular distal abdominal segment as shown in the Fig 1.

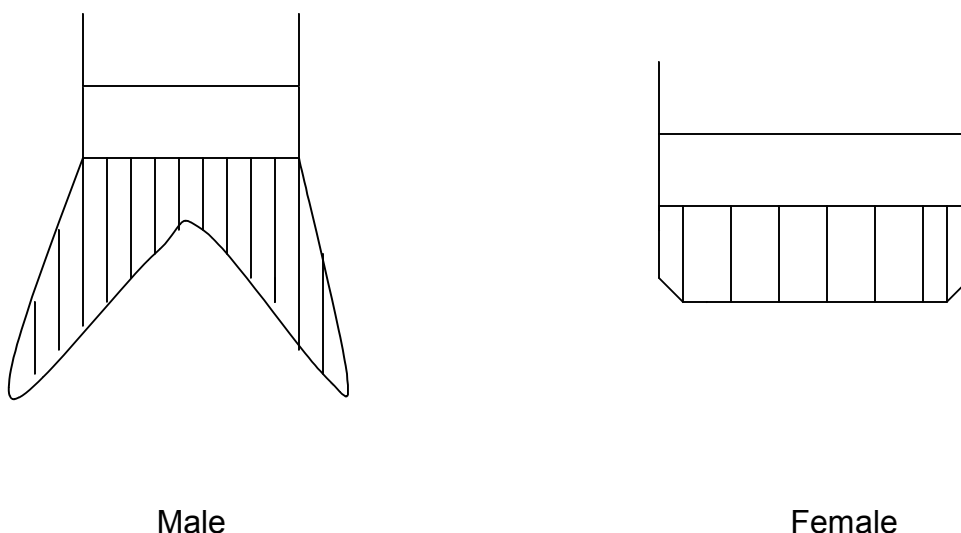


Figure 1: Sexing pupae of *Wyeomyia smithii*. Examination of ventral aspect of last abdominal segment reveals the consistent sex related morphological difference.

c) Measurement of adult size:

Wing lengths of mosquitoes are proportionate to their body sizes, varying allometrically. For standardization relative to hexamerin concentration, left wing lengths of representative sub-samples of adult mosquitoes from each of the three populations were measured in this study using an ocular micrometer on a compound microscope (APPENDIX A). Longer wing length, implies a greater abundance of somatic proteins that were accrued during larval stages and which will be directed towards oogenesis.

d) Preparation of specimens for electrophoresis:

Whole specimens (pupae or adults) were placed in 1.5 mL micro centrifuge tubes and were ground with a plastic pestle in 50 μ L PBS (pH 7.2). Fifty μ L of sample buffer (100Mm Tris, pH 6.8, 2% SDS, 5% β -mercaptoethanol, 15%

glycerol, 0.02% bromophenol blue) were added and the specimens were heated for 10 min in a boiling water bath.

e) Gel electrophoresis and immunoblotting:

Discontinuous SDS-PAGE (Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis) was carried out after loading the wells with 40 μ L of specimen. Stacking gels were 2% acrylamide, pH 6.9, and separating gels were 7.5 % acrylamide, pH 8.9. Ten μ L/ well of molecular weight markers (Sigma Chem. Co.) were used for molecular weight estimation. Two gels were run simultaneously: one was used only for protein electrophoresis and the other was used for immunoblotting. Both the gels were identical in all aspects except that wide molecular weight range Sigma markers (M.W. 6-205 KDa) were used for regular electrophoresis gels and wide range Sigma color markers (M.W. 6,500-205,000) were used for immunoblotting gels. Electrophoresis was carried out at an initial current of 20 mA/ gel which was increased to 40 mA/ gel once the bands entered the separating gel. The gel that was used only for electrophoresis was stained with Coomassie blue (0.125% Coomassie blue R-250, 50% methanol, 10% acetic acid), and destained with destaining solution I (50% methanol, 10% acetic acid). Finally the gel was fixed in destaining solution II (7% acetic acid, 5% methanol). The gel electrophoresis was repeated three times with each set of specimens.

The other gel was used for immunoblotting. Immunoblotting is ten times more sensitive than Coomassie blue staining and potentially provides a clearer resolution of the proteins of interest with less background. Protein bands from

SDS-PAGE gels of *W. smithii* were transferred electrophoretically to nitrocellulose paper and used for immunoblot assays. Assembled in a sandwich with nitrocellulose paper, the gels were washed in three changes of 25 mM Tris, 192 mM glycine, and 20% methanol (transfer buffer) over 45 min, and blotted at 150V for 2h in transfer buffer cooled to 4⁰C in a Trans Blot Cell (Bio-Rad) electroblotting device. After disassembling the sandwich, the nitrocellulose blot (replica) was used in an immunoblot assay.

f) Preparation of anti-hexamerin antibodies:

Twenty early female pupae (<18 hrs after emergence from larval stage) of *W. smithii* from Apalachicola, Florida were used as the source of hexamerins for use as antigen. All pupae were ground in a single micro centrifuge tube in 100 μ L PBS (pH7.2) and 100 μ L electrophoresis sample buffer was added. The sample was then heated in a boiling water bath for 10 minutes. Fifty μ L of sample was added to each of two 10 cm wide wells, and electrophoresis was carried out as described above. Hexamerin protein bands, identified by abundance and molecular weight, were cut from the gel, emulsified (made up to 1 mL with normal saline (0.9%), and drawn back and forth between two 5 mL syringes connected by a short length of tubing until mixed as well as possible), and then further mixed with adjuvant by drawing the above preparation and 1 mL of Freund's complete adjuvant (Sigma Chem. Co.) in and out of a 5 mL syringe with a 23 g needle attached until the material obtained a "whipped cream" consistency. This preparation was used as antigen for the first set of injections into New Zealand white rabbits (*Oryctolagus cuniculi*). For this initial exposure to antigen, two

subcutaneous injections of 0.5 mL each and two intramuscular injections of 0.5 mL each were administered into the rabbit. Three booster doses (similarly prepared, but using Freund's incomplete adjuvant, Sigma Chem. Co.) were administered on days 7, 19, and 49. Test trials of the immunoblot assay, using blots of gels prepared as described above for preparation of antigen, were done to determine adequacy of anti-hexamerin antibody development using serum drawn two weeks after the 19 day injection, and two weeks after 49 day injection.

g) Immunoblot assays:

The immunoblot assays were carried out at room temperature in individual styrene acrylonitrile containers (Nalgene). Incubations and washes were done on a shaker table. Blots were first incubated for 1 h in 100 ml of PBS with 0.05% Tween-20 blocking buffer (BB), 4 liters of PBS-Tween 20 was prepared by dissolving 9.08g sodium phosphate, 2.16 g potassium phosphate, 24.28 g NaCl and 2 mL Tween-20. The BB was removed and blots were incubated for 2 h in 100 mL of BB containing hexamerin specific antibodies. The hexamerin antibodies were used at a 1:800 dilution. The diluted antibodies were removed and blots were washed three times (15 min each) with 100ml each of BB. After the third wash, blots were incubated for 1hr in 100ml of BB containing horse radish peroxidase – conjugated goat anti-rabbit IgG (Sigma Chem. Co.). The solution was decanted and blots were washed again three times (15 min each) with BB. After the last wash, blots were incubated for 5-7 min in 100 mL of a substrate buffer containing 30 mg diaminobenzidine and 166 μ l of 3% hydrogen peroxide dissolved in 50 mM Tris-HCl, pH 7.4. After decanting the substrate

buffer and rinsing the blots with several changes of distilled water, the enzyme-substrate reactions were stopped. Blots were dried with filter paper and stored under pressure in the dark until photographed.

CHAPTER 3

RESULTS

a) Detection of putative hexamerins in polyacrylamide gels.

SDS- PAGE gel electrophoresis of *W. smithii* pupae revealed the sub units of putative hexamerin bands in the molecular weight range 62 to 85 KDa (Fig 2). This determination was based on the relative abundance of these prominent proteins, their molecular weight, and their disappearance during the time course of adult development. It was not possible to resolve six polypeptides, possibly because of co-migration of similar size molecules.

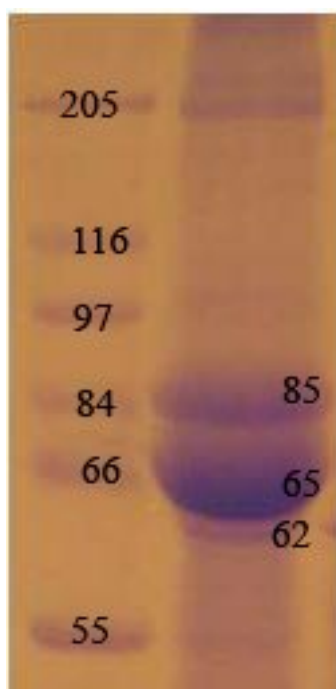


Figure 2: Putative hexamerin proteins from whole body extracts of female *W. smithii* pupae from Highlands, North Carolina. Standard molecular weights are represented towards left in KDa. SDS-PAGE electrophoresis reveals putative hexamerin sub units with their molecular weights in KDa on the right.

b) Comparison of putative hexamerins in male and female pupae and adults from three geographically separate populations

There is a significantly greater amount of stored proteins in pupae compared to newly emerged adults in all three geographic subpopulations. Newly emerged adult females accumulate more storage proteins compared to males (Fig 3). The gel electrophoresis was repeated three times with each set of specimens in this study and the results were consistent.

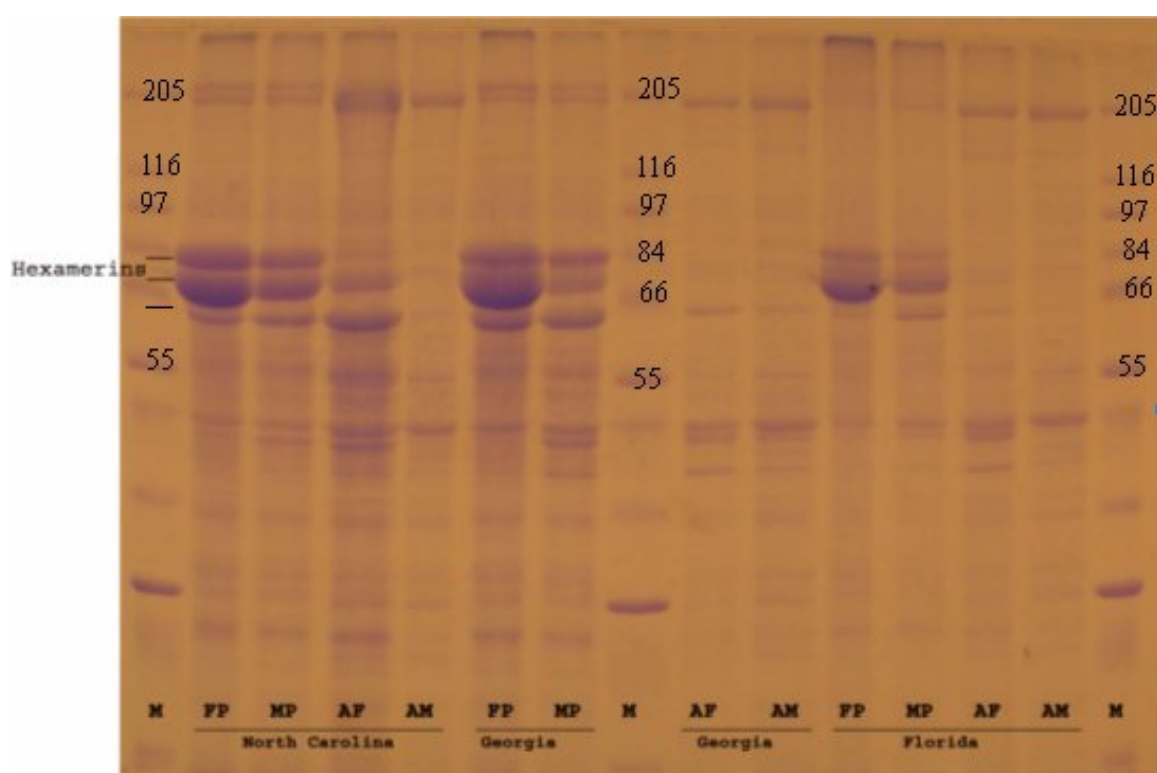


Figure 3: SDS gel electrophoresis showing the differences in the amounts of putative hexamerins in the three populations of *W. smithii* mosquitoes. All the samples were whole specimens and were collected within 3 hours after emergence. Female pupae in general, accumulated greater amounts of storage proteins compared to males in all the three populations. The higher level of putative hexamerins in adult females compared to adult males is also evident. (M

– Molecular weight markers, FP – Female Pupa, MP – Male Pupa, AF – Adult Female, AM – Adult Male).

The 65 KDa subunit of hexamerin is the most abundant polypeptide in all the pupae and adults of both sexes across the three subpopulations of *Wyeomyia smithii*. This 65 KDa subunit has been quantified (Table 1) by measuring the size and internal density of the bands on the SDS-PAGE gels using Imagej software (<http://rsbweb.nih.gov/ij>) (see APPENDIX B for details).

Table 1: Quantification of 65 KDa polypeptide.

	Area	IntDen
Female pupa		
North Carolina	422	39143
Georgia	530	49222
Florida	189	17780
Adult female		
North Carolina	204	22743
Georgia	52	6449
Florida	93	13667
Male pupa		
North Carolina	258	26614
Georgia	157	17451
Florida	142	15215
Adult Male		
North Carolina	46	5720
Georgia	35	4338
Florida	66	8052

The internal density of the bands is directly proportional to the area of the polypeptide band. Female Pupa from Georgia have the highest amount of hexamerin 65KDa polypeptide (area 530 & Int Den 49222) and pupa from Florida have the least (area 189 & IntDen 17780). It is interesting to note that newly

emerged adult females of Georgia have lowest amounts of polypeptide content (area 52 & IntDen 6449) among all the three subpopulations of adult females. This implies that most of the stored hexamerins are utilized for ecdysis in Georgia subpopulation.

Male pupa from North Carolina accrue more proteins compared to those from Georgia, followed by Florida ones. There is no significant difference in the amount of stored putative hexamerin 65KDa subunit in newly emerged males.

c) Time course of disappearance of putative hexamerins in male mosquitoes after adult emergence from three geographically separate populations

Newly emerged adult male mosquitoes utilize majority of the hexamerin proteins during ecdysis. Moreover, adult male mosquitoes are non-hematophagous. In a newly emerged adult male, almost all the stored proteins are utilized in less than 6 hours. This feature is common to all the three geographic sub populations (Fig 4a, 4b, 4c).

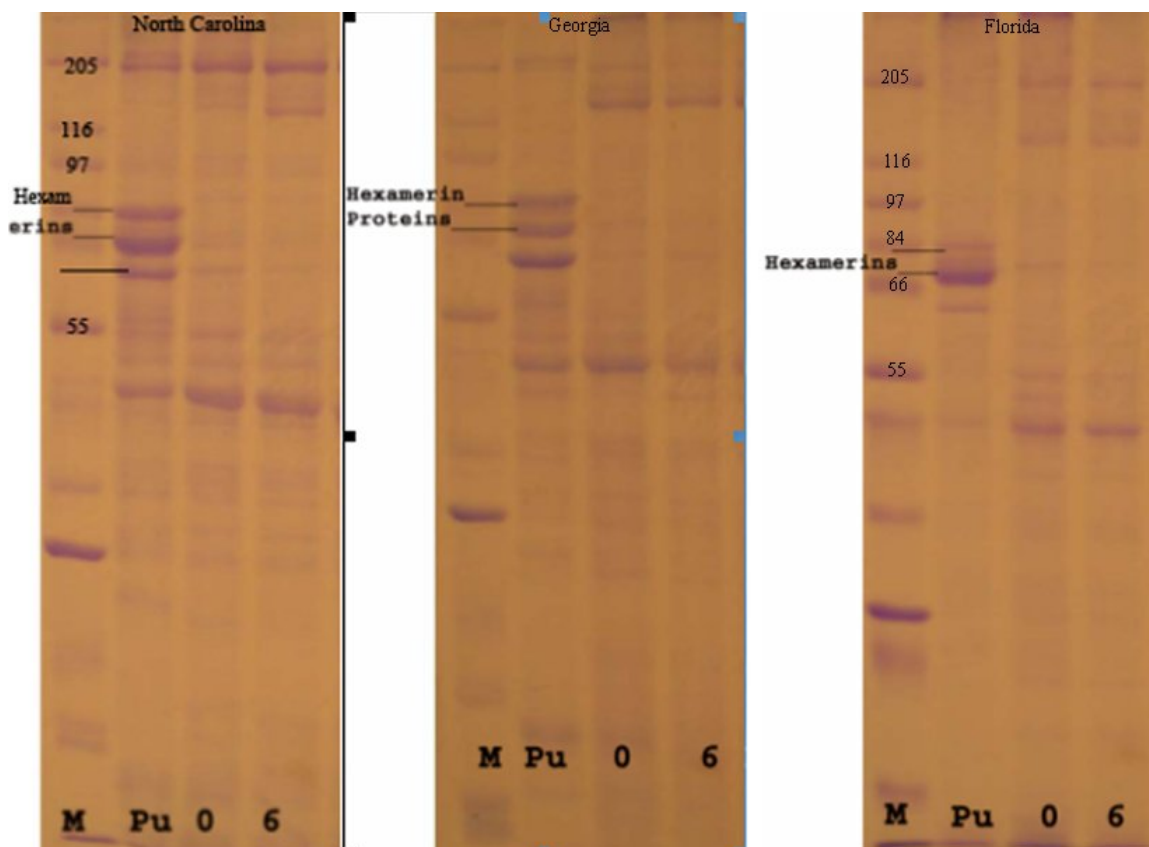


Fig 4a

Fig 4b

Fig 4c

Figure 4: SDS electrophoresis showing the temporal sequence (6 hourly) in the accumulation of putative hexamerins in adult male *W. smithii* mosquitoes.

Highlands, North Carolina; Tattnall County, Georgia; and Apalachicola, Florida are represented in Fig 4a, 4b & 4c respectively. Almost all the stored proteins are depleted within 6 hours of adult life. (M – Molecular weight markers in KDa, Pu - Pupa)

d) Time course of disappearance of putative hexamerins in female mosquitoes after adult emergence.

i) Highlands, North Carolina

The temporal disappearance of the three putative hexamerin sub units is distinct. Although most of the stored putative hexamerin subunits are utilized

within 30 hours after emergence, the smaller 62 KDa subunits last for ≥ 66 hours and the larger 85 KDa subunits disappear immediately after adult emergence (Fig 5).

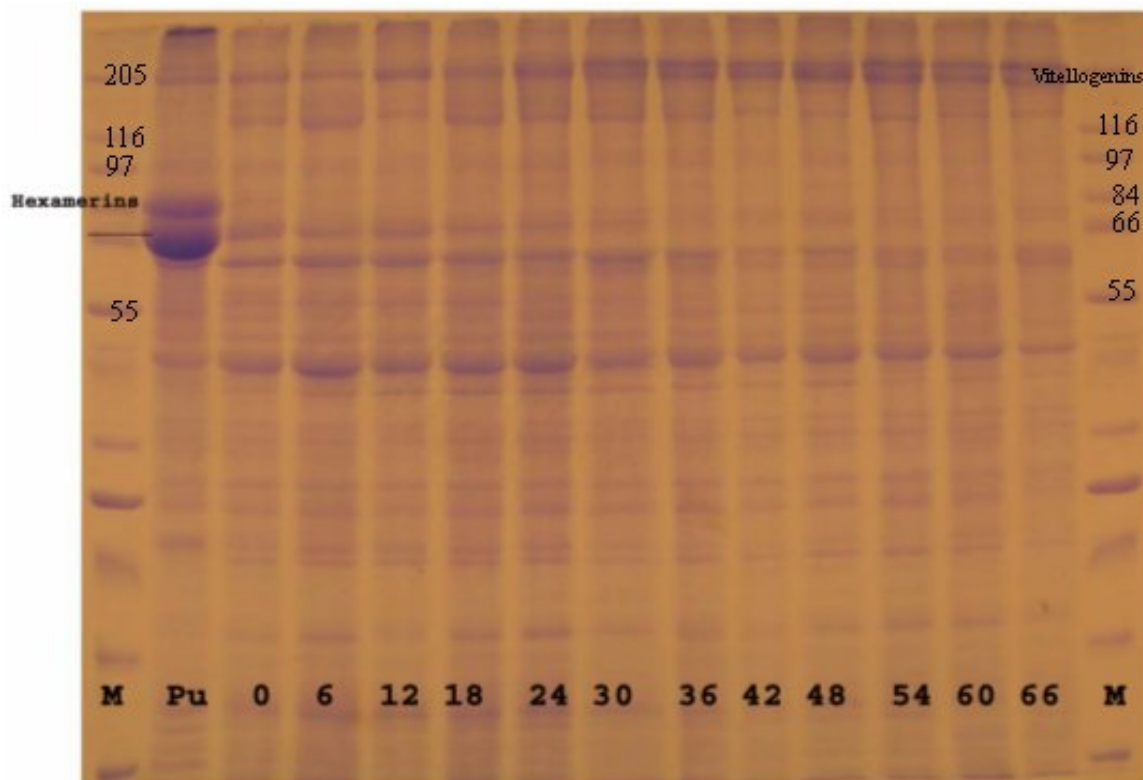


Figure 5: SDS electrophoresis of adult female *W. smithii* from Highlands, North Carolina. The first and last lanes represent standard molecular weight markers. The second lane from left represents pupae that were < 3 hrs after emergence from larval stage. The subsequent lanes from left represent whole body extracts of adult females that were collected every 6 hours starting from 0 hour through 66 hours (M – Molecular weight markers, Pu - Pupa).

ii) Tattnall County, Georgia

All putative hexamerin protein reserves are utilized within 42 hours after adult female emergence. The larger 65 KDa subunits disappears in 18 hours and the

smaller 62 KDa subunit lasts for 42 hours. There is no evidence of 85 KDa subunits in newly emerged adults. Vitellogenin protein subunits begin to increase after 36 hours of adult life (Fig: 6).

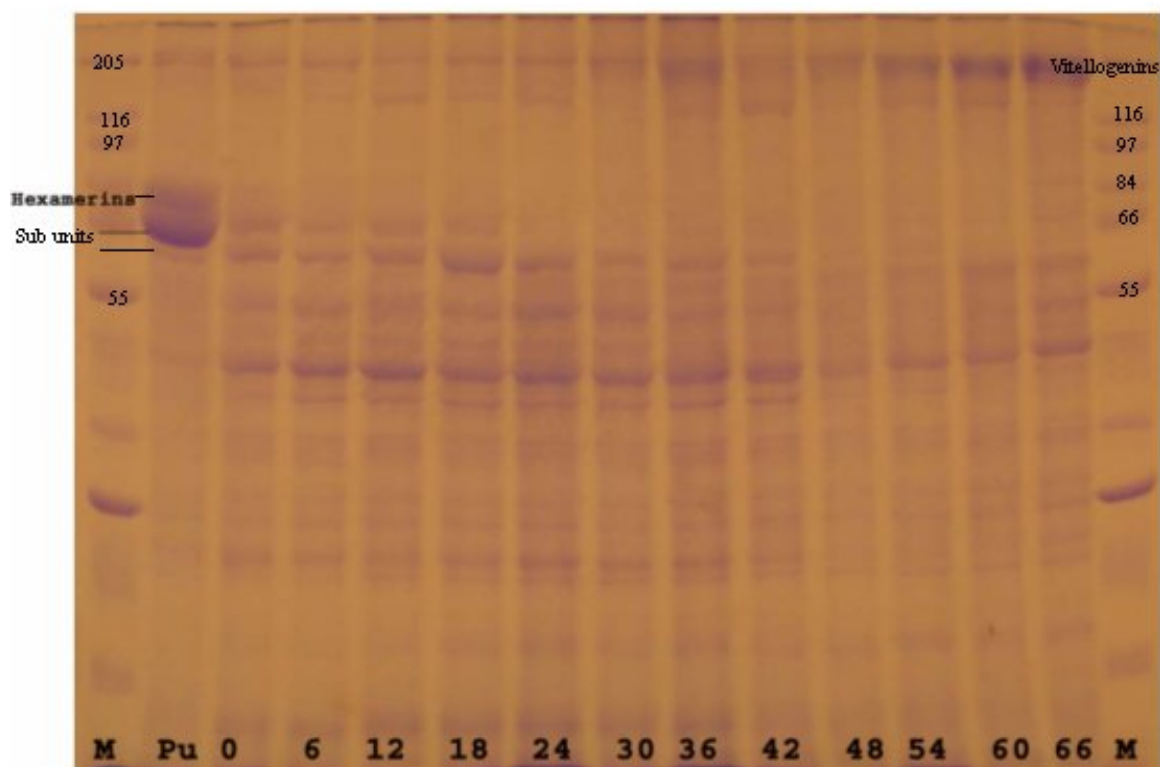


Figure 6: Gel electrophoresis of adult female *Wyeomyia smithii* mosquitoes from Tattnall County, Georgia. Standard molecular weight markers are represented on either side and newly emerged female pupae (<3 hrs) in second column from left (Pu). Starting from the third lane towards the left, represent a single whole adult female collected every 6 hourly from 0 hour through 66 hours (M – Molecular weight markers, Pu - Pupa).

iii) Apalachicola, Florida

Again noted is the different time course of disappearance of the hexamerin polypeptides. The larger 85 KDa polypeptide is not apparent in a newly emerged

adult. The 72.5 KDa subunit disappears in 36 hours while the smaller 68KDa subunit is evident even at 66 hours. The delayed (60 hours and later) increase in vitellogenin proteins is also evident (Fig 7).

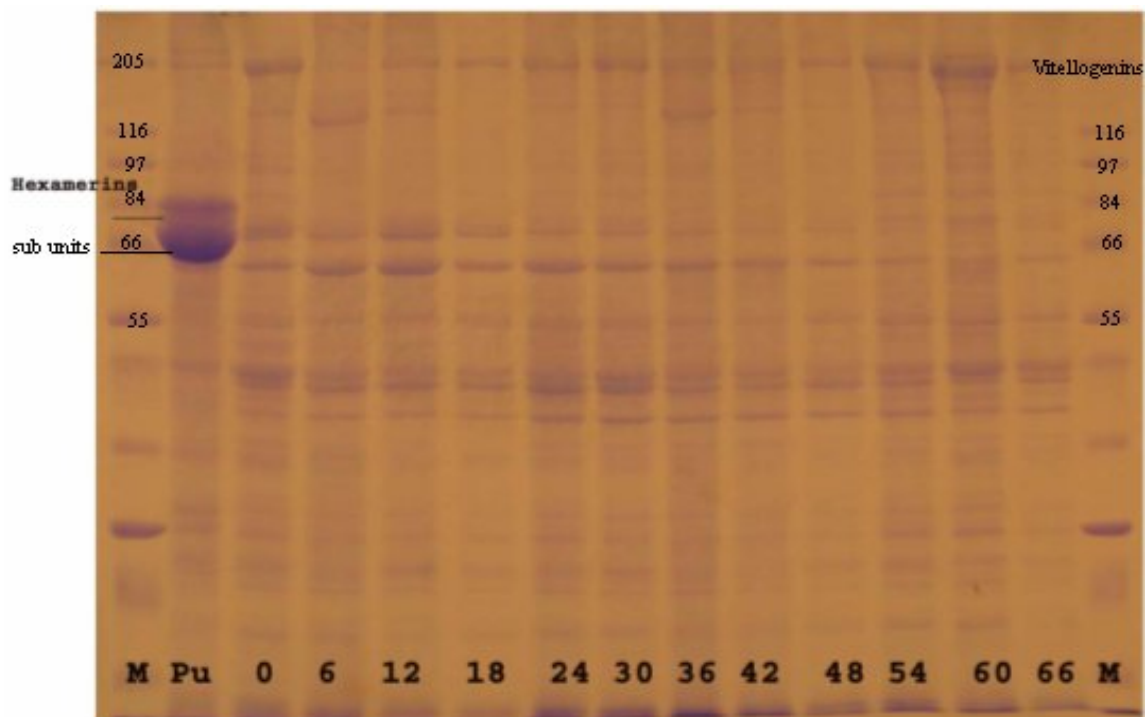


Figure 7: Gel electrophoresis of female *W. smithii* from Apalachicola, Florida. Gel shows disappearance of hexamerin proteins with time (hrs) in newly emerged adult females. The first and last lanes represent standard molecular weight markers. The second lane (Pu) represents whole female pupa. Subsequent lanes represent newly emerged adult females from 0 hours through 66 hours collected every 6 hours.

e) Comparison of wing lengths of adult females from three geographically separate populations

The average adult mosquito size (based on left wing length) in both sexes from North Carolina, Georgia, and Florida are shown in Table 2. As would be

expected, autogenous mosquitoes have large body size (indicated by large wing length) and vice versa. The larger adult body size of *Wyeomyia smithii* in North Carolina is a reflection of this autogenous behavior. Anautogenous *W. smithii* from Florida are small in size and that of *W. smithii* from Georgia have intermediate body size.

Table 2: Average wing length of adult *Wyeomyia smithii* mosquitoes.

	Geographic region					
	<u>Highlands, NC</u>		<u>Tattnall County, GA</u>		<u>Apalachicola, FL</u>	
	M	F	M	F	M	F
Average left wing length (mm)	2.12	2.48	1.98	2.31	1.91	2.29
Number (n)	11	11	11	11	12	12
Std Dev	0.06	0.10	0.06	0.14	0.10	0.16

Analysis of the results was done using 2 way ANOVA for sex ($F= 6.34$, $df = 1$, $p < 0.014$) and geographic location ($F= 0.49$, $df = 2$, $p < 0.615$).

f) Results of immunoblot assays of putative mosquito hexamerin depletion

Although antiserum prepared in rabbits to hexamerins were reactive in preliminary assays, when the antiserum was used with blots containing the entire array of mosquito proteins, significant non-specific binding occurred with non-hexamerin proteins. As a result, immunoblots (not shown) provided no additional information about the relative concentration and time course of depletion of hexamerins from the three populations of *W. smithii*. Additionally, antisera did not

significantly increase detection of hexamerins as compared to Coomassie blue staining, so the anticipated ability to more sensitively detect the proteins did not occur.

CHAPTER 4

DISCUSSION

Mosquitoes are a holometabolous insect, *i.e.*, they undergo multiple stages of development (egg – larva – pupa – adult). The pupal stage in the life cycle of a mosquito is a non-feeding stage. Only proteins that are stored during feeding larval stages are carried on to early adult lives. The hexamerins serve as nutritional reserves and are converted to vitellogenins (egg proteins) during oogenesis.

Spielman (1971) established that autogenous behavior in mosquitoes is influenced by larval factors. There was no effect on autogeny or fertility in *Wyeomyia smithii* mosquitoes when females were not given sugar feeds after their emergence from a well-fed larval stage.

In each of the three geographic subpopulations examined in this study, the hexamerin protein content of pupae or adults of *Wyeomyia smithii* is abundant among females as compared to males favoring the production and utilization of these proteins in egg laying.

Regardless of the geographic distribution or regardless of the sex, the total hexamerin protein content of pupae is always more as compared to adults. Pupae tend to accumulate more hexamerin proteins either by an efficient protein synthesis mechanisms as in *Bombyx mori* (Tojo et al., 1980), through increased gene expression (Zakharin et al., 2001), or even through prolonged larval stages (Karpells et al., 1990). Mahmood (1999) noted that the fourth instar larval stage of female *Wyeomyia smithii* is prolonged by 2.1 days when compared to their

male counterparts. *W. smithii* have longer 3rd and 4th instar larval stages during hibernation and aestivation (Istock, 1975). These adaptive mechanisms offer a survival advantage by giving a chance for more storage protein accumulation.

Though significantly less, male pupae did carry hexamerins to some extent as compared to their female counterparts. Adult males did not contain any hexamerins beyond 6 hours after emergence from pupal stage. These proteins were consumed during ecdysis or shortly after emergence into the adult stage. It also indicates the lack of further necessity of these proteins in adult males.

Adult male *W. smithii* emerge two days earlier than adult females from their pupal stages (Mahmood, 1999), *i.e.*, have a shorter larval developmental time. The longer pre-adult life stages in females provides them more time to gather protein reserves and utilize them efficiently for egg production.

The current study is in accord with that of Wheeler and Buck (1996) which demonstrates the disappearance of most hexamerin proteins within 36 hours after ecdysis in adult female mosquitoes of *Wyeomyia smithii*. The transition from autogenous to anautogenous behavior of *W. smithii* is reflected in the relative decrease in hexamerin content of adult mosquitoes as we move from North to South in this study, with greater expression of hexamerin accumulation, in mosquitoes from North Carolina than in those from Georgia and Florida, but more rapid depletion of hexamerins in mosquitoes from Florida and Georgia than those from North Carolina. Overall, as in other phenotypic measures, the *W. smithii* from Georgia are intermediate with regard to hexamerin utilization. This variation is evident based on the observable thickness, size and density of the

bands in gels performed for this study. However, spectral photographic analysis of these bands needs to be conducted to further quantify the differences between the amounts of hexamerins produced and used by these populations of mosquitoes.

The combination of prolonged diapause because of a more adverse climate in the Northern regions associated with lower larval density, less competition for acquiring nutritional resources, and lower vertebrate host densities results in emergence of larger sized adult females of *W. smithii* (based on wing length). Autogeny is favored in this geographic subpopulation.

A blood meal is not required by *W. smithii* from Northern regions for multiple clutches of egg laying (O' Meara, 1981). The autogenous behavior in *W. smithii* from Northern regions is a recent change in the evolutionary process as evident by the presence of blood sucking mouth parts (Hudson, 1970). The larval stages are prolonged to facilitate more hexamerin protein accumulation and the larval densities are low in the pitcher plants. As a result, newly emerged pupae have abundant reserves of hexamerin proteins. These hexamerins are partially utilized during non-feeding pupal stages and the rest of the storage proteins are mobilized from storage sites to form egg protein, vitellogenin, during early adult life.

Wyeomyia smithii from Tattnall County, Georgia are both autogenous and anautogenous. Over the past decade, anautogeny has been noted to be increasing in this subpopulation (Irby, personal communication). Environmental stressors like locally increased average temperature, greater competition for

larval nutrition, and greater larval density might have been contributing to the increasing host seeking/blood-feeding abilities of this geographic subgroup.

Wyeomyia smithii in Florida reproduce perennially except during summers. The larvae are exposed to high competition for obtaining nutritional resources. The larval density is high in the pitcher plants in this region. They are dependent on exogenous protein from hematophagy during early adult life for egg laying. These factors probably dictate the largely anautogenous behavior of adult *W. smithii* in this region.

There is statistically significant difference in the body size of adult male and adult female *W. smithii* across the three subpopulations. The average wing length of adult female mosquitoes of *W. smithii* were 2.48 mm, 2.31 mm, and 2.29 mm for Highlands, North Carolina; Tattall County, Georgia; and Apalachicola, Florida, respectively. Though statistically insignificant, the geographic variation in body size of *W. smithii* noted in our study correlates with their relative differences in their autogenous behavior. On an average, larger wing lengths were noted in adult female *W. smithii* from North Carolina which are autogenous compared to their counterparts in Florida which have smaller wings and are anautogenous. The clear decrease in adult female body size with decreased hexamerin storage protein content and anautogenous behavior likely reflects the more limited availability of nutritional resources in the southern region because of increased competition for larval nutrients that occurs in warmer climates. This is similar to the relation between autogeny and body size in *Aedes togoi*, which depends on geographic location (Mogi, 1995), with

increased autogeny among subarctic populations and decreased autogeny among tropical populations.

A positive relation between wing length and frequency of autogeny is seen in *Ochlerotatus vigilax* (Hugo, 2003). As noted by Armbuster and Hutchinson (2002), larger wing length in adult female *Aedes albopictus* and *A. geniculatus* correspond to their higher fecundity. The geographic variation in body size of *Wyeomyia smithii* noted in this study correlate with their relative differences in their anautogenous behavior. Wing lengths are larger in adult female *W. smithii* from North Carolina which are autogenous than their counterparts in Florida which have smaller wings and are largely anautogenous.

The pitcher-plant mosquito, *Wyeomyia smithii* exhibits variation in autogeny based on the environmental influences. Adult female *W. smithii* from Highlands, North Carolina, is autogenous, with large body size (based on wing length) and accumulate more hexamerin proteins during larval stages. The stored hexamerins are converted to egg proteins, vitellogenins, within 30 hours to lay the first and subsequent batches of eggs, without an exogenous protein source like a blood meal. This autogenous behavior in *W. smithii* from Northern regions is an adaptation to overcome the long winters associated with difficulty in finding a vertebrate host.

The subpopulation of *Wyeomyia smithii* from Georgia is intermediate in size and exhibits both autogenous and anautogenous behavior. Though the larval forms gather a large reserve of hexamerin storage proteins, most of them are utilized during ecdysis, more than the other two subpopulations. As a result,

newly emerged adult females carry relatively less protein and have to depend on hematophagy for protein acquisition. Larval accrued hexamerins last for about 42 hours after emergence and are utilized for oogenesis. There has been a recent increase in anautogenous behavior in this subpopulation, probably as a result of climate change.

Subpopulation of *Wyeomyia smithii* from Apalachicola, Florida are mostly anautogenous, and are smaller in size. Due to the high larval carrying capacity of Pitcher plants in this region, there is a significant competition for obtaining larval nutrition. Newly emergent adult females have scant hexamerin reserves and depend on blood sucking for vitellogenesis. There is delayed utilization of these hexamerin reserves (hexamerins are seen even after 66 hours on SDS-PAGE gels) for vitellogenin synthesis.

The storage and utilization of hexamerin proteins in *Wyeomyia smithii* is genetically regulated. Molecular cloning and expression of hexamerin cDNA from the three geographic subpopulations of *W. smithii* during their larval, pupal, and adult stages can be done to better understand the genetic regulation of autogenous behavior.

Environmental factors like temperature influence hexamerin gene expression. It can be speculated that there would exist a critical temperature above which hexamerin gene expression would increase. The recent observation of increasing anautogenous behavior in *W. smithii* from Georgia may be a result of global warming leading to triggering the thermal threshold for hexamerin gene expression in this subpopulation.

Also, isolation and analysis of the hexamerin subunits and vitellogenin polypeptides, and their precise quantification is essential to clearly understand the process of oogenesis.

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APPENDICES

Appendix A:

WING LENGTHS (LEFT WING) OF ADULT *Wyeomyia smithii* MOSQUITOES OF
BOTH SEXES FROM THREE GEOGRAPHIC SUBPOPULATIONS

Hours after Emergence	<u>Left wing length in mm</u>					
	Highlands, North Carolina		Tattnall County, Georgia		Apalachicola, Florida	
	M	F	M	F	M	F
0	2.21	----	2.09	2.10	1.97	2.42
6	----	2.58	1.97	2.23	1.90	2.51
12	2.15	2.38	1.97	2.61	1.94	2.21
18	2.11	2.61	1.97	2.45	1.82	2.02
24	2.12	2.59	----	2.35	1.86	2.31
30	2.16	2.52	1.92	2.28	1.86	2.31
36	2.06	2.48	2.00	2.24	1.96	2.46
42	2.20	2.46	2.06	----	1.87	2.48
48	2.01	2.34	1.98	2.2	1.68	2.09
54	2.11	2.39	1.96	2.24	2.08	2.24
60	2.11	2.57	2.03	2.40	2.02	2.28
66	2.11	2.38	1.88	2.33	1.94	2.19

APPENDIX B:

 QUANTIFICATION OF 65 KDa POLYPEPTIDE – MEASUREMENT OF
 BAND SIZE AND INTERNAL DENSITY (USING imagej SOFTWARE –
<http://rsbweb.nih.gov/ij>)

Label	Area	Mean	StdDev	Min	Max	Width	Height	IntDen
NC FP	422	92.756	7.664	80	118	36	14	39143
NC MP	258	103.155	4.202	96	113	31	11	26614
NC AF	204	111.485	2.275	107	120	31	8	22743
NC AM	46	124.348	1.037	123	127	20	3	5720
GA FP	530	92.872	8.196	76	114	39	16	49222
GA MP	157	111.153	1.733	107	116	27	8	17451
GA AF	52	124.019	0.874	122	127	24	3	6449
GA AM	35	123.943	0.802	122	125	23	3	4338
FL FP	189	94.074	6.483	67	108	27	9	17780
FL MP	142	107.148	1.822	104	114	26	7	15215
FL AF	93	122.627	1.135	120	125	25	9	13667
FL AM	66	122.0	1.203	120	125	25	4	8052

NC FP (North Carolina Female Pupa); NC MP (North Carolina Male Pupa); NC AF (North Carolina Adult Female); NC AM (North Carolina Adult Male); GA FP (Georgia Female Pupa); GA MP (Georgia Male Pupa); GA AF (Georgia Adult Female); GA AM (Georgia Adult Male); FL FP (Florida Female Pupa); FL MP (Florida Male Pupa); FL AF (Florida Adult Female); FL AM (Florida Adult Male).