Differences in Development, Glycogen, and Lipid Content Associated With Cytochrome P450-Mediated Permethrin Resistance in *Culex pipiens quinquefasciatus* (Diptera: Culicidae)

M. C. HARDSTONE, X. HUANG, L. C. HARRINGTON, AND J. G. SCOTT[1]

Department of Entomology, Cornell University, Ithaca, NY 14853

J. Med. Entomol. 47(2): 188–198 (2010); DOI: 10.1603/ME09131

ABSTRACT Insecticide resistance in populations of mosquitoes is an escalating problem that can directly affect disease prevalence. Determining the fitness associated with an insecticide resistance mechanism (allele) will provide for greater understanding of the evolution of resistance, and help inform effective vector management programs. Previously, a population cage experiment in which the alleles of two highly related strains of *Culex pipiens quinquefasciatus* (Say) SLAB (susceptible) and ISOP450 (permethrin resistant because of cytochrome P450-mediated detoxification) were placed in direct competition in the absence of insecticide revealed that the P450 resistance allele frequency decreased over time. In the current study, SLAB and ISOP450 development, mortality and energetic resources derived from glycogen, other sugars and lipids were measured to identify biological parameters that might explain the previously observed fitness cost. SLAB exhibited shorter egg-to-adult female development time and larger body size when reared in groups when compared with ISOP450. ISOP450 female adults provided 20% sugar water lived longer than 20% sugar water fed females of the SLAB strain. No significant differences in larval development time, larval mortality, pupal stage duration time, pupal mortality, longevity of male and female adults provided with distilled water and males provided sugar water were found between the strains. The caloric content from glycogen and lipids were significantly higher in SLAB relative to ISOP450 in adults. The slower female emergence time and smaller body size when reared in groups combined with lower energy reserves (glycogen and lipids) associated with the resistance allele (in ISOP450) are likely fitness costs associated with the resistance allele of P450-mediated detoxification.

KEY WORDS Nutritional resources, calories, fitness cost, insecticide resistance, cytochrome P450 monooxygenases

The southern house mosquito, *Culex pipiens quinquefasciatus* (Say), is found throughout the southern hemisphere and is the most important urban vector of the nematode, *Wuchereria bancrofti*, which is the causative agent of lymphatic filariasis (Vinogradova 2000). *Cx. p. quinquefasciatus* is also a competent vector of West Nile virus, Japanese encephalitis virus, St. Louis encephalitis virus, Rift Valley fever virus, avian malaria parasites, and the nematode that causes dog heartworm (Turell et al. 2001, CDC 2002). Controlling adults of this species and other insect vectors of disease relies almost exclusively on the use of insecticides and especially the pyrethroid permethrin (Hougard et al. 2003, WHO 2006).

Pyrethroid insecticides are used to treat bed nets and for indoor residual spraying campaigns. Because of the extensive use of pyrethroids, resistant populations of *Culex* mosquitoes have been recorded throughout the world (Rodriguez et al. 1993, Ben Cheikh et al. 1998, Bisset and Soca 1998, Chandre et al. 1998, Kasai et al. 1998, Kolaczinski and Curtis 2004, Liu et al. 2004, Paul et al. 2004, Yebakima et al. 2004, Liu et al. 2005, Cui et al. 2006). Resistance to pyrethroids in *Culex* mosquitoes is conferred primarily by enhanced detoxification because of cytochrome P450 monooxygenases and target site insensitivity (L1014 F point mutation in *Vssc*, called *kdr*) (Kasai et al. 1998, Hardstone et al. 2007). In the JPAL strain of *Cx. p. quinquefasciatus*, cytochrome P450-mediated resistance metabolizes permethrin to 4′-OH permethrin (Kasai et al. 1998) and confers limited cross-resistance to other pyrethroid insecticides (Kasai et al. 1998, Hardstone et al. 2007).

Fitness studies in the context of metabolic-based insecticide resistance mechanisms in mosquitoes have focused on characteristics associated with esterase-based resistance (Chevillon et al. 1999, Lenormand et al. 1999, Raymond et al. 2001, Bourguet et al. 2004). In these studies, fitness costs have been implied because of lowered reproductive output (Berticat et al. 2002), decreased predator avoidance and parasite resistance

[1] Corresponding author, e-mail: jgs5@cornell.edu.

(Agnew et al. 2004, Berticat et al. 2004, Duron et al. 2006) and lower overwintering survival (Gazave et al. 2001). Thus far, a single study in mosquitoes has been conducted to examine the fitness specifically associated with cytochrome P450-mediated resistance (Hardstone et al. 2009). In that study, allele frequencies in a laboratory population cage experiment were measured when a resistance allele (from the ISOP450 strain) was placed in direct competition with the susceptible allele (from the SLAB strain). Under the standard laboratory rearing procedures (with no exposure to insecticides), the resistance allele frequency gradually decreased over time implying that a minor cost is associated with P450-mediated permethrin resistance (Hardstone et al. 2009).

The evolution of insecticide resistance in populations has been based on observations that mutations that confer resistance to insecticides are rare before insecticide application, increase in frequency only during insecticide application, and decrease in frequency in the absence of insecticide use (Crow 1956, Brown 1958). Therefore, these mutations are generally believed to be costly in the absence of insecticides. Fitness costs of resistance alleles in the absence of insecticides could be because of the reallocation of resources, changes in metabolic functions or alteration of developmental processes (Crow 1956, Roush and McKenzie 1987).

Because P450-mediated permethrin resistance in the resistant ISOP450 strain is known to be expressed in larvae (Hardstone et al. 2007), examination of larval fitness parameters was pursued. While a myriad of parameters are available to examine fitness in the adult stage, particularly those associated with reproductive capabilities, many of these factors are not possible to measure in the larval stage. Consequently, to determine the physical condition (fitness) of the larvae, energetic resources were measured along with mortality and development times.

Food is metabolized to provide necessary energy reserves in the form of nutrients such as carbohydrates (glucose, trehalose, and glycogen) and lipids for insects to complete development, perform basic metabolic processes, engage in flight and reproductive activities (Steele 1981) and provide substrates for the normal functioning of the nervous system (Strang 1981). Glucose is not readily used by insects for direct energy utilization; rather it is primarily used as a precursor in the synthesis of glycogen and trehalose (Friedman 1985) and therefore is usually found in low concentrations (Wyatt 1967). Glycogen can also be metabolized into trehalose, a sugar found in the hemolymph of insects, which provides immediate energy resources. Glycogen is used as an immediate source of energy in the early stages of flight (Steele 1981) because it is stored within the cell and there is no time needed to transport the molecule (Candy 1985). During insect development and particularly during feeding phases, ingested carbohydrates are converted into lipids (Van Handel and Lum 1961, Candy 1985, Downer 1985). Lipids are used through catabolism to yield trehalose and glycogen that can then directly be used during molting, prolonged flight, oogenesis, and normal body functions (Beenakkers et al. 1981). Therefore, energetic levels of larvae and adults can alter survival and reserves used in flight possibly altering mating, host seeking, and oviposition behaviors.

The aim of this study was to measure biological parameters associated with development and energy resources between highly related (>99.9%) resistant (ISOP450) and susceptible (SLAB) strains. If ISOP450 larvae and adults were slower to develop, had increased mortality or accumulated lower energy reserves as compared with the susceptible SLAB strain, these patterns could explain the previously observed fitness cost present in the ISOP450 strain (Hardstone et al. 2009). Differences in the biological parameters whereby SLAB out-performs ISOP450 would be suggestive of a fitness cost being associated with the resistance allele of P450-detoxification.

Materials and Methods

Mosquito Strains and Experimental Setup. SLAB is a susceptible laboratory strain of *Cx. p. quinquefasciatus* that has been in colony for ≈40 yr without exposure to insecticides (Georghiou et al. 1966). JPAL is a permethrin resistant strain originally collected in 1984 from Saudi Arabia and subsequently was selected with permethrin for 20 generations. It contains P450-mediated detoxification and *kdr* (Kasai et al. 1998). ISOP450 is a strain of *Cx. p. quinquefasciatus* that has been in colony since 2005 and is highly related (>99.9%) to SLAB. ISOP450 was isolated through repeated backcrosses of JPAL to SLAB with permethrin selection of backcross progeny such that the final strain was 1,300-fold resistant to permethrin conferred by cytochrome P450 monooxygenase detoxification (Hardstone et al. 2007). Permethrin resistance in ISOP450 is incompletely dominant, autosomally linked, monofactorially inherited, and expressed in the larvae, but not in adults (Hardstone et al. 2007).

Strains were maintained in a chamber with a regime of programmed natural daily temperatures cycling from 22 to 30°C, 80% RH and a photoperiod cycle of 14L:10D h. The light phase started and finished with a 2 h twilight period. Adult mosquitoes in colony cages were provided a 20% sugar solution ad libitum and an anesthetized mouse for 30 min twice per week (Cornell University Approved Animal Use Protocol #2001–0056).

Mosquito body size determinations were made using an Olympus DF PLAPO dissecting microscope with an Olympus DP25 camera and DP2-B5W (Olympus) computer software. Fourth instar larvae were immobilized by placing them on a piece of filter paper and the width of the thorax was measured as an indication of body size (Timmermann and Briegel 1998). Adult size was determined by measuring the length of the right wing, removed and mounted on a glass microscope slide with clear tape, from the axillary incision to the apical margin, not including fringe (Nasci 1990).

Development and Mortality Parameters. Measurements of developmental parameters for individual mosquitoes were made by placing 24 newly hatched first instar larvae individually into a 60 ml plastic cup (Fabri-Kal, Kalamazoo, MI) with 50 ml of distilled water and 18 mg of powdered food consisting of a 1:2:1 mixture of ground TetraFin goldfish flakes (Spectrum, Atlanta, GA), ground rabbit pellets (Hartz Mountain Corporation, Secaucus, NJ) and liver powder (MP Biomedicals, Solon, OH) for a total of 12 replicates. First instars were obtained randomly from a minimum of 14 egg rafts. Mortality or pupation of larvae was recorded at 24 h intervals. Pupae were transferred to individual plastic test tubes containing 3 ml of distilled water. Tubes were monitored every 24 h for mortality or adult emergence. Newly eclosed adults were kept individually in 177 ml paper hot cups (International Paper, Memphis, TN) covered with mesh. Each sex was divided into two cohorts and exposed to environments with differing levels of calories available from food (sugar water and distilled water). One cohort was continuously provided cotton saturated with 20% sugar water (an environment rich in calories) and the other received distilled water (an environment absent of calories). Adults were monitored at 24 h intervals for mortality. Within 24 h of death, wings were removed and measured to estimate body size as described above. All life stages were maintained at 27 ± 1°C.

Nutritional Energetic Measurements. Egg rafts were collected en masse from colony cages. To obtain mosquitoes for experimentation within the range of large sizes collected in the field (Day et al. 1990), 75 first instar larvae were placed into a plastic rearing tray (26.7 cm × 20.3 cm × 7.6 cm, Lock & Lock, New South Wales, Australia) containing 2 liters of distilled water. Larvae were provided 200 mg of powdered food (described above) daily. Pupae were placed individually into plastic tubes with 3 ml of distilled water to allow for adult eclosion. Emerged adults were sorted and maintained in same-sex cages. All life stages were maintained at 27 ± 1°C. Glycogen, other sugars (primarily glucose and trehalose) and lipid measurements were obtained for fourth instar larvae, teneral females (within 24 h of emergence), virgin females with access to 20% sugar water at 2 and 4 d old and virgin females with access to distilled water at 2 and 4 d old. The adult life stages were chosen because 100% of adults provided distilled water died after 4 and 2 d old adults provided a data point between emergence and death. Seventy-five individuals were included in each age group.

To determine nutrient content, mosquitoes were fixed in an oven for 30 min at 100°C and prepared following the methods of Van Handel and Day (1988) as modified by Harrington et al. (2001). Briefly, fixed mosquitoes were individually homogenized for 30 s in 200 μl of 2% sodium sulfate solution followed by the addition of 1.5 ml chloroform:methanol (1:2) solution. Tubes were centrifuged for 1 min at 450 × *g*. Precipitate (containing glycogen) was used for glycogen measurements. Supernatant (containing other sugars and lipids) was transferred into two new glass test tubes. One of the tubes containing supernatant was evaporated under a fume hood at 25°C for 24 h (until 200 μl remained) and used to measure sugars other than glycogen. The second tube containing supernatant was evaporated under a fume hood at 25°C for 72 h (dried completely) and used for lipid analysis. Serial dilutions of *D*-glucose (Fisher, Fair Lawn, NJ) were prepared in duplicate to obtain standard curves for glycogen and other sugars each day samples were measured. Measurements of samples for glycogen and other sugars as well as the *D*-glucose standards were determined using the hot anthrone-based assay (Van Handel 1985b). New standard curves to measure lipids were analyzed in duplicate using serial dilutions of peanut oil (nSpired Foods, San Leandro, CA) each day samples were measured. Lipid levels of samples and standards were determined using the vanillin reagent assay (Van Handel 1985a). Optical densities were measured using a Beckman DU 640 Spectrophotometer (Beckman-Coulter, Fullerton, CA) at 625 nm for glycogen and other sugars and 525 nm for lipids.

Nutritional measurements of micrograms per mosquito were corrected for body size (by dividing micrograms per mosquito by millimeter thorax length of larvae or millimeter wing length of adults) and converted to calories (by multiplying micrograms glycogen and other sugars by 0.004 and microgram lipids by 0.009 [Briegel 1990, Clements 1992]). These conversion rates account for the total amount of energy provided by the nutrient based on the Atwater system (Merrill and Watt 1973) and allowed for the direct comparison of energetic content provided by each nutrient between the strains.

Statistical Analyses. Development parameters were compared between SLAB and ISOP450 strains with one-way ANOVA (JMP 7.0, SAS Institute, Cary, NC). Larval mortality and pupal mortality were arc-sin transformed and statistically compared using one-way ANOVA. Additionally, Kaplan-Meier analysis was conducted on the survival curves created for adults of both sexes maintained on the diet treatments. Tukey-Kramer honestly significant difference (HSD) test was used to compare adult body size (millimeter wing length) from individually reared mosquitoes between the diet treatments.

Comparisons of body size measurements (millimeter thorax width or millimeter wing length) between SLAB and ISOP450 when reared in groups were made using one-way ANOVA. Levels of each nutrient at each age group between the strains were compared using one-way ANOVA. Comparisons of the nutrient content corrected for body size per age group between SLAB and ISOP450 strains were made using an ANOVA random block design test with date of analysis as the random variable.

Results

Development and Mortality Parameters. The resistant strain (ISOP450) had a significantly longer egg-to-adult female development time than the suscepti-

Table 1. Comparisons of development and mortality parameters between susceptible (SLAB) and resistant (ISOP450) strains (24 individuals/replicate, 12 replicates) of *Cx. p. quinquefasciatus* held at 27 ± 1°C

Life stage	Parameter	SLAB (mean ± SEM)	ISOP450 (mean ± SEM)
Larval	Development (d)	8.24 ± 0.05	8.35 ± 0.05
	Mortality (%)	7.39 ± 0.67	7.90 ± 0.77
Pupal	Duration (d)	1.85 ± 0.03	1.83 ± 0.03
	Mortality (%)	9.84 ± 0.67	8.29 ± 0.41
Adult	Egg-to-adult male (d)	9.85 ± 0.05	9.82 ± 0.07
	Egg-to-adult female (d)	10.22 ± 0.05	10.51 ± 0.08*

* Statistically greater than SLAB ($P \leq 0.05$) using one-way ANOVA.

Table 2. Wing sizes of surviving SLAB and ISOP450 adults maintained individually and provided 20% sugar water or distilled water for the duration of their lives

Treatment	Sex	Wing size in mm (n)	
		SLAB	ISOP450
20% sugar water	Male	2.62 ± 0.01 (58)a	2.64 ± 0.01 (58)a
20% sugar water	Female	3.11 ± 0.02 (53)b	3.08 ± 0.02 (55)b
Distilled water	Male	2.62 ± 0.01 (59)a	2.63 ± 0.01 (58)a
Distilled water	Female	3.15 ± 0.02 (54)b	3.11 ± 0.02 (57)b

Means ± SEM followed by different letters indicate statistical differences ($P \leq 0.01$) using Tukey-Kramer HSD test. Values are mean ± SEM (n in parenthesis).

ble strain (SLAB) ($F = 10.43$, df = 1, $P = 0.002$) when the strains were held at the same temperature. All other development parameters measured (larval development time, larval mortality, pupal duration, pupal mortality, and egg-to-adult male development time) were not statistically different between the two strains (Table 1).

Survivorship curves of adult males and females of ISOP450 and SLAB under the two diet treatments are shown in Fig. 1. As expected, the food limited adults (distilled water treatment) had significantly lower survivorship than those provided sugar water ($\chi^2 = 462$, df = 1, $P < 0.0001$). Within the distilled water treatment, no differences in male survival (SLAB median longevity = 4 d, ISOP450 median = 4 d; $\chi^2 = 0.519$, df = 1, $P = 0.471$) or female survival (SLAB median = 4 d, ISOP450 median = 3 d; $\chi^2 = 0.356$, df = 1, $P = 0.551$) were detected. Within the sugar water treatment, between SLAB and ISOP450 there were no significant differences in survival between males (SLAB median = 26 d, ISOP450 median = 28 d; $\chi^2 = 1.41$, df = 1, $P = 0.235$). Survival of females provided 20% sugar water were significantly different between strains ($\chi^2 = 8.17$, df = 1, $P = 0.004$), with median longevity of SLAB being 26 d while that of ISOP450 was 30 d. As expected females were larger than males, but size of males or females did not differ between strains or diet treatments (Table 2).

Nutritional Energetic Measurements. Table 3 lists the values (in micrograms per mosquito) of glycogen, other sugars and lipids of SLAB and ISOP450 fourth instar larvae, teneral females, 2 and 4 d old females provided 20% sugar water and 2 and 4 d old females provided distilled water. There were no significant

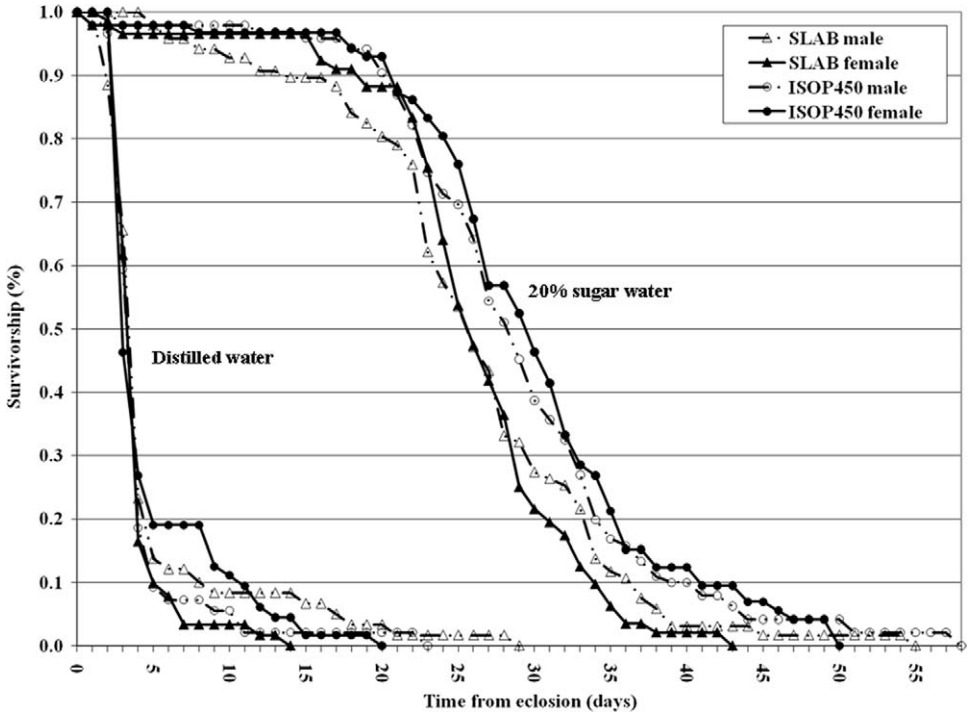

Fig. 1. Survival curves of adult male and female susceptible (SLAB) and resistant (ISOP450) *Cx. p. quinquefasciatus* maintained on 20% sugar water or distilled water (n listed in Table 2). Day 0 indicates day of eclosion.

Table 3. Glycogen, other sugars, and lipid content of different age groups of SLAB and ISOP450 strains of *Cx. p. quinquefasciatus*. Values are mean ± SEM (n = 75 per age group)

Age group	Glycogen (ug/mosquito)		Other sugars (ug/mosquito)		Lipid (ug/mosquito)	
	SLAB	ISOP450	SLAB	ISOP450	SLAB	ISOP450
Fourth instar larvae (male and female)	42.13 ± 1.92	45.18 ± 2.74	9.07 ± 1.59	9.86 ± 1.66	65.25 ± 4.01	58.14 ± 3.81
Teneral female adults	24.15 ± 0.94**	20.67 ± 0.92	4.46 ± 0.63	3.98 ± 0.49	83.77 ± 3.21	79.76 ± 2.59
2 d old female adults provided sugar water	53.51 ± 3.29	46.18 ± 5.03	30.64 ± 2.32	33.34 ± 4.08	120.26 ± 4.77**	95.33 ± 5.35
2 d old female adults provided distilled water	11.55 ± 0.61**	8.85 ± 0.64	1.46 ± 0.23	2.03 ± 0.35	39.75 ± 1.45*	35.75 ± 1.22
4 d old female adults provided sugar water	70.36 ± 3.05**	58.85 ± 2.90	23.02 ± 1.93	22.27 ± 1.80	163.46 ± 9.13**	110.18 ± 5.51
4 d old female adults provided distilled water	8.93 ± 0.44	8.23 ± 0.39	3.92 ± 0.54	2.70 ± 0.34	13.95 ± 0.97	12.39 ± 0.99

Asterisks indicate significantly greater value within a nutrient class per age group between strains using one-way ANOVA (*, $P < 0.05$; **, $P < 0.01$).

differences between strains at the larval stage (Table 3). However, SLAB generally had higher glycogen and lipid content compared with ISOP450 and this was significant ($P \leq 0.01$) in the majority of adult age groups (Table 3).

When reared in groups, SLAB adult mosquitoes were significantly larger ($P < 0.05$) than ISOP450 in four of five adult age groups (Table 4). Given that female body size correlates with fecundity (Clements 1992, Lima et al. 2003), this observation suggests that a fitness disadvantage is associated with the resistance allele in ISOP450. Given the larger size of the SLAB females reared from groups of larvae, we examined whether the higher glycogen and lipid levels were simply a function of their body size. Therefore, micrograms per mosquito measurements were body size (millimeters) corrected for all age groups and then converted to calories (to obtain energy measurements). This allowed for the direct comparison of energy obtained from each nutrient between the two strains.

Glycogen caloric content per millimeter body size was statistically higher in SLAB compared with ISOP450 teneral females ($F = 6.85$, df = 1, 138, $P = 0.010$), 2 d old females provided distilled water ($F = 10.7$, df = 1, 134, $P = 0.001$) and 4 d old females provided sugar water ($F = 6.51$, df = 1, 143, $P = 0.012$). SLAB levels of glycogen (glycogen caloric content/millimeter body size) were greater for 2 d old females provided sugar water and 4 d old females provided distilled water as well, but these differences were not significant (Fig. 2). Fourth instar larvae contained more glycogen than did any adult age group for both strains. Among the adult age groups, glycogen calorie levels tended to increase with age when adult mosquitoes had access to sugar water. From tenerals to 4 d old females provided sugar water, calories from glycogen increased by 300% in SLAB and 284% in ISOP450. The converse occurred to adults deprived of sugar; calories from glycogen tended to decrease with age (Fig. 2) where 64.3 and 60.0% of the glycogen measured in teneral adults had disappeared in SLAB and ISOP450 4 d old females provided distilled water, respectively.

Table 4. Body sizes of the SLAB and ISOP450 strains of *Cx. p. quinquefasciatus* reared in groups at different age groups

Age group	Body size (mm)	
	SLAB	ISOP450
Fourth instar larvae (male and female)	1.06 ± 0.01	1.07 ± 0.01
Teneral female adults	3.41 ± 0.01*	3.37 ± 0.01
2 d old female adults provided sugar water	3.40 ± 0.01	3.43 ± 0.01
2 d old female adults provided distilled water	3.42 ± 0.01*	3.38 ± 0.01
4 d old female adults provided sugar water	3.36 ± 0.01*	3.29 ± 0.01
4 d old female adults provided distilled water	3.43 ± 0.01*	3.35 ± 0.01

* Statistically greater than ISOP450 ($P \leq 0.05$) using one-way ANOVA. Values are mean ± SEM (n = 75 per age group).

The caloric content per millimeter body size of other sugars was not statistically different between SLAB and ISOP450 larvae or adults (Fig. 3). Neither SLAB nor ISOP450 consistently had higher levels of calories from other sugars throughout the age groups. Calorie levels from other sugars ranged from 1.6- to 4.6-fold lower than glycogen calorie levels.

The greatest differences between SLAB and ISOP450 strains were measured in calories obtained from lipids. Energetic resources from lipids (calories/millimeters) were higher in SLAB than ISOP450 fourth instar larvae ($F = 5.88$, df = 1, 134, $P = 0.017$), 2 d old females provided sugar water ($F = 12.0$, df = 1, 125, $P = 0.001$), 4 d old females provided sugar water ($F = 56.3$, df = 1, 142, $P < 0.0001$) and 4 d old females provided distilled water ($F = 5.82$, df = 1, 144, $P = 0.017$). Similar to the pattern observed with glycogen, calories/millimeter from lipids within the adult age groups increased with age with access to sugar water and decreased with age when only provided distilled water (Fig. 4). Between the teneral female and 4 d old female life stages, when sugar water was provided, lipid levels increased by different amounts for SLAB and ISOP450, 200 and 143%, respectively. When females were placed under caloric deprivation (distilled water treatment) the calories from lipids decreased by similar amounts (84.0% for SLAB and 84.4% for ISOP450) when measured between the teneral female and 4 d old female life stages.

Discussion

The P450 resistance locus present in ISOP450 confers a slightly lower fitness in the absence of insecticide compared with the isogenic susceptible strain (SLAB), which agrees with a previous report (Hard-

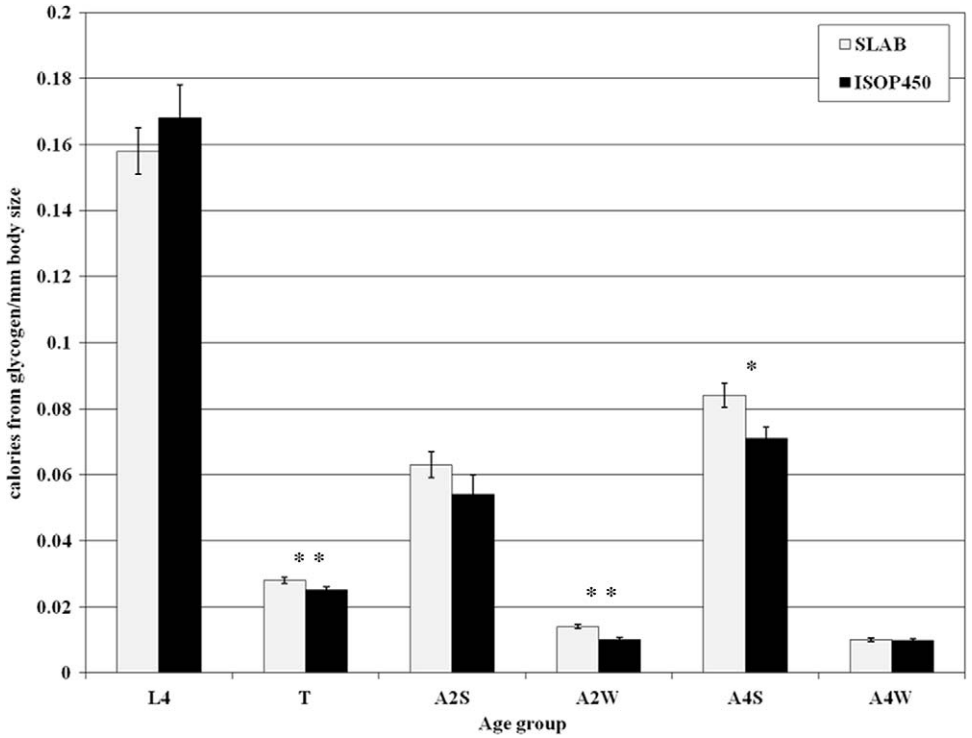

Fig. 2. Energetic resources from glycogen corrected for body size of susceptible (SLAB) and resistant (ISOP450) strains of *Cx. p. quinquefasciatus* at different life stage age groups (L4, fourth instar larvae; T, teneral females; A2S, 2 d old females provided 20% sugar water; A2W, 2 d old females provided distilled water; A4S, 4 d old females provided 20% sugar water; A4W, 4 d old females provided distilled water). Values are mean ± SEM ($n = 75$ mosquitoes/age group/strain). Asterisks indicate significant differences per age group between strains (*, $P < 0.05$; **, $P < 0.01$).

stone et al. 2009). Given that the P450-mediated resistance in ISOP450 was larval-specific (Hardstone et al. 2007), it was expected that biological differences might be most evident at this life stage, although no larval or pupal developmental differences were found between SLAB and ISOP450. However, adult parameters differed between the strains where females of SLAB developed faster and were larger when reared in a group than ISOP450 female adults suggesting a developmental deficiency associated with ISOP450 when placed in competition for food. For both strains, when larvae were reared in groups the size of adult females were larger than when larvae were reared individually, this indicates that developing alone presents a less optimal environment to the mosquitoes. Energy resources from glycogen were higher in SLAB adult females of the teneral, 2 d old provided distilled water and 4 d old provided sugar water life stages compared with ISOP450. In SLAB, energy provided by lipids was higher in fourth instar larvae, 2 d old females provided distilled water and 4 d old females provided sugar water, compared with ISOP450. The energy levels measured in this study are consistent with previous reports (Van Handel 1965, Timmermann and Briegel 1999). Thus, the estimates of fitness measured in this study show potential costs associated with P450-mediated resistance in ISOP450 in a smaller female body size when reared in groups as larvae, a longer egg-to-adult female eclosion time and lower relative energetic resources provided from glycogen and lipid nutrients relative to SLAB.

Body size of adult females in SLAB and ISOP450 strains were dependent on the number of other larvae present during development. A stronger negative developmental effect of group living was present in the ISOP450 strain such that SLAB females reared in groups were larger in size than the equivalently treated ISOP450 females. Size differences within a sex were only observed between the two strains when larvae were reared in groups suggesting a possible decreased ability for ISOP450 larvae to obtain food when competing with conspecifics. It is well known in mosquitoes that a positive correlation exists between body size as measured by adult wing length and egg production (Clements 1992, Lima et al. 2003). Particularly because *Culex spp.* lay rafts of eggs, larvae will develop in clusters rather than alone. Therefore, it is likely that SLAB females have higher reproductive potential than ISOP450 females based on the body size differences that result from developing in groups.

When provided sugar water, ISOP450 adult females survived ≈4 d longer than SLAB adult females. In multiple mosquito species it has been observed that female fecundity is inversely correlated to age (Jalil 1974, Akoh et al. 1992), though positive correlations of egg laying with age have also been reported (Styer et

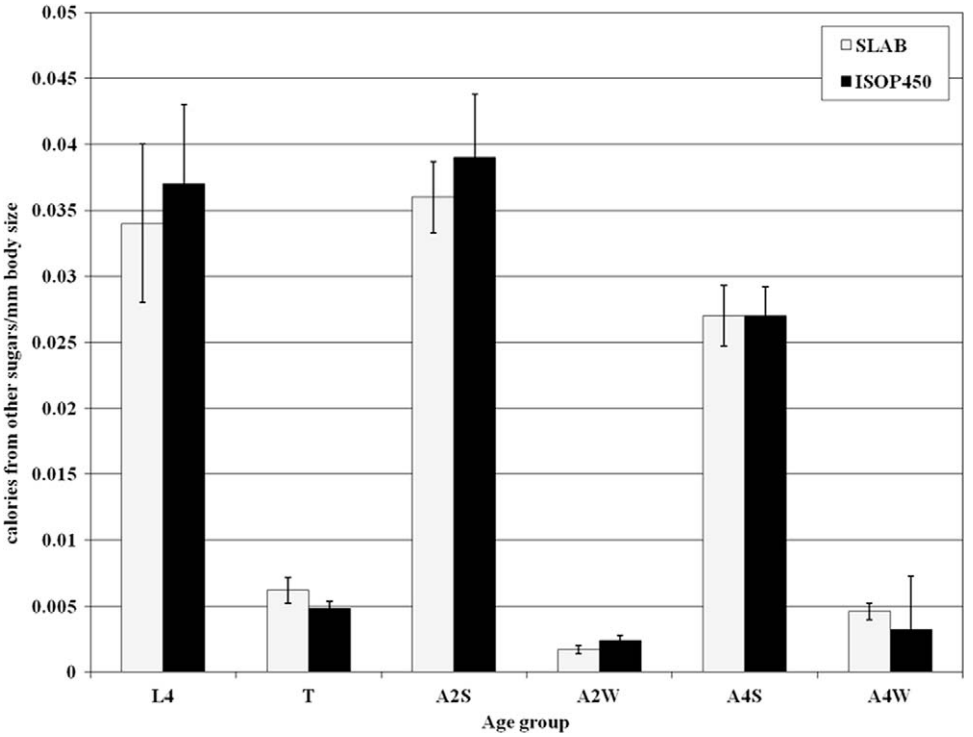

Fig. 3. Energetic resources from sugars other than glycogen corrected for body size of susceptible (SLAB) and resistant (ISOP450) strains of *Cx. p. quinquefasciatus* at different life stage age groups (L4, fourth instar larvae; T, teneral females; A2S, 2 d old females provided 20% sugar water; A2W, 2 d old females provided distilled water; A4S, 4 d old females provided 20% sugar water; A4W, 4 d old females provided distilled water). Values are mean ± SEM ($n = 75$ mosquitoes/age group/strain). Asterisks indicate significant differences per age group between strains (*, $P < 0.05$; **, $P < 0.01$).

al. 2007). Thus, it is unclear if the longevity of ISOP450 females is a reproductive benefit especially because wild females are not likely to survive as long as a female kept under laboratory conditions.

Emergence time is an important factor to consider for fitness. SLAB females emerged earlier than females of the ISOP450 strain by almost 7 h. Males of both strains did not significantly differ in emergence time such that females of SLAB emerge 9 and 9.5 h and ISOP450 females emerge 16 and 16.5 h after SLAB and ISOP450 males, respectively. In small laboratory mating cage experiments, Williams and Patterson (1969) observed that at 82 ± 1°F (28 ± 1°C) male *Cx. p. quinquefasciatus* did not actively seek females to mate with until males were 72 h old and no females were found to be inseminated by males <24 h old (Williams and Patterson 1969) because inversion of the male genitalia occurs in a temperature-dependent manner during this period (ranging from 6 h to >72 h postemergence to complete depending on temperature) (Clements 1992). Observations from this study provide a scenario whereby males of both strains have an equal opportunity to mate with the earlier emerging SLAB females, allowing the susceptible P450 allele to be increased in the population given that female *Culex* mosquitoes are thought to be monogamous (Kitzmiller and Laven 1958, Craig 1967, Bullini et al. 1976). Because field populations of mosquitoes have overlapping generations, it is unclear whether this pattern would drive the P450 resistance allele out of a resistant wild population. This observation could be what provided the susceptible allele an advantage over the resistant allele in the insecticide-free population cage experiment since the experimental design included discrete generations (Hardstone et al. 2009).

No differences in glycogen energy stores were found at the fourth instar larval stage indicative of the ability for both strains to store equal amounts of glycogen from the larval diet provided. Calories obtained from glycogen in teneral female adults, 2 d old females provided distilled water and 4 d old females provided sugar water differed between ISOP450 and SLAB strains. Intriguingly, a significant difference was observed at the teneral female adult stage where ISOP450 levels of calories obtained from glycogen were lower than SLAB levels (Fig. 2). This difference may be because of ISOP450 using more of the glycogen resources for metamorphosis or the inability of ISOP450 to store energy as readily as SLAB. ISOP450 does not have as much energy from glycogen for various biological processes as compared with SLAB. The biological significance of the differences observed between the 2 d old females provided distilled water and 4 d old females provided sugar water of ISOP450 and SLAB are unknown because no differences in glycogen levels were measured between the 2 d old

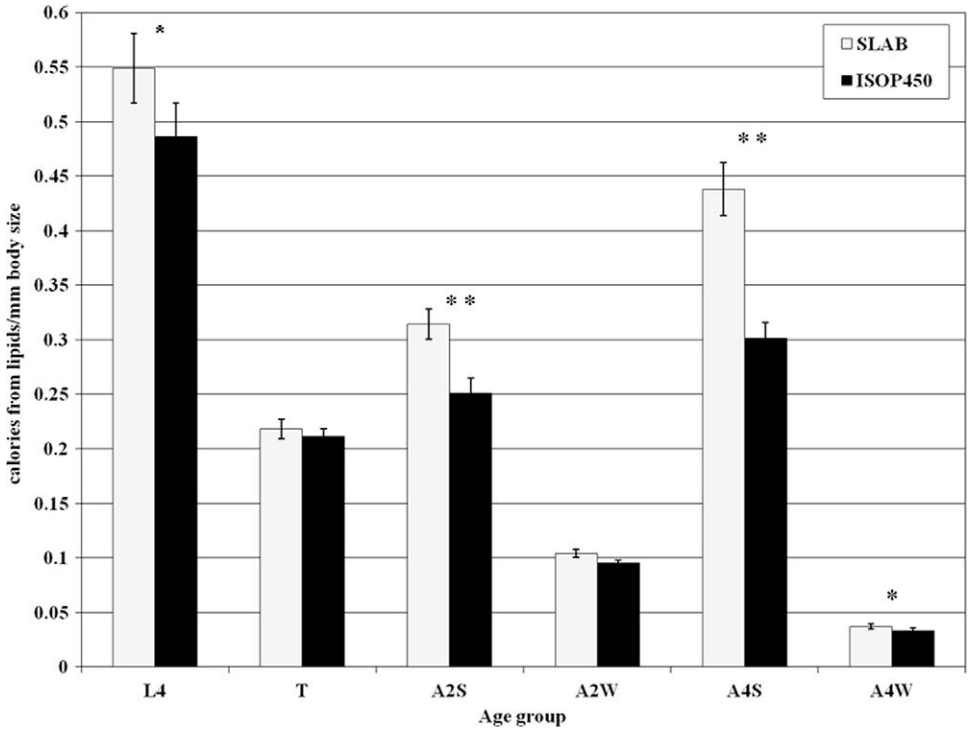

Fig. 4. Energetic resources from lipids corrected for body size of susceptible (SLAB) and resistant (ISOP450) strains of *Cx. p. quinquefasciatus* at different life stage age groups (L4, fourth instar larvae; T, teneral females; A2S, 2 d old females provided 20% sugar water; A2W, 2 d old females provided distilled water; A4S, 4 d old females provided 20% sugar water; A4W, 4 d old females provided distilled water). Values are mean ± SEM (n = 75 mosquitoes/age group/strain). Asterisks indicate significant differences per age group between strains (*, $P < 0.05$; **, $P < 0.01$).

females provided sugar water and 4 d old females provided distilled water. Both strains were able to equally gain glycogen resources when provided sugar water as adults, they also both equally deplete glycogen resources when starved (Ivanovic 1991).

No significant differences in calories from other sugars were found between SLAB and ISOP450. Both strains were equally able to store other sugars over time when provided a food source (larval food or sugar water) and other sugars accounted for the least amount of energy of the three nutrient types measured.

Lipid reserves differed significantly with SLAB larvae containing greater levels of reserves than ISOP450. For a number of insect species, the lipid content corrected for dry weight is higher in larvae compared with adults (Fast 1964) as was observed for both SLAB and ISOP450. At the teneral adult stage, the levels become equal between the strains and when adults were provided sugar water, ISOP450 were less efficient at storing lipids or used more lipids for survival. Under conditions of adult starvation, both strains exhausted the long-term lipid stores to survive (Van Handel and Lum 1961, Fast 1964). Previous reports on insecticide resistance (mostly resistance to DDT) and lipid content have shown that they are not correlated and that increased lipids are not a mechanism of resistance (Reiser et al. 1953, Bridges and Cox 1959, Matsubara 1960, Ascher and Neri 1961, Fast 1964, Kalra et al. 1967). Therefore, it appears that lipid levels are not associated with the resistance status of a population (in which case ISOP450 would be expected to have higher levels), but are developmentally important suggesting that ISOP450 has inferior development compared with SLAB. Additionally, because mosquito oocytes and egg yolks are primarily composed of lipids (Clements 1992), the higher level of lipids measured in SLAB adult females (particularly those provided sugar water) may result in greater fecundity (Briegel et al. 2002) when compared with ISOP450 females.

Laboratory analysis of fitness associated with insecticide resistance using highly related strains, such as SLAB and ISOP450, can provide valuable information because the genetic background is more uniform than using unrelated strains. While developmental and energetic indices suggest that a minor fitness cost under laboratory conditions is associated with P450-mediated resistance in ISOP450, other fitness parameters (mating competition, sperm competition, predator avoidance, egg viability, fitness/metabolic levels under field conditions, etc.) not studied here could also be informative. Therefore, further investigations into reproductive output or life table construction of SLAB and ISOP450 are necessary. Development of allele specific markers to differentiate individuals that are homozygous or heterozygous for P450 resistance will

open the door for studies of the population genetics (and fitness) of this allele under both laboratory and field conditions.

ISOP450 has lower estimates of fitness (using measurements of development and energetic resources) when compared alongside SLAB in the laboratory. Most notably, smaller body size when reared in groups, slower female preimaginal development time and lower calories (energy) from nutrients (Mostowy and Foster 2004) give ISOP450 inferior measurements of fitness indices when compared with SLAB. Determining whether the laboratory results observed in this study are relevant for resistant populations in the field will require future experimentation such as determining fitness of SLAB and ISOP450 provided carbohydrates from various natural sources.

Acknowledgments

The authors thank C. J. M. Koendraadt and A. E. Douglas for valuable discussion about the nutrition assays, B. P. Lazzaro for reviewing early versions of this manuscript and F. Vermeylen for statistical assistance. This work was supported by Cornell University CALS Mellon Grant (MCH), by the United States Environmental Protection Agency (EPA) under the Science to Achieve Results Graduate Fellowship Program (MCH), the Grace Griswold fund, and the Sarkaria Institute of Insect Physiology and Toxicology. The EPA has not officially endorsed this publication and the views expressed herein may not reflect the views of the EPA.

References Cited

Agnew, P., C. Berticat, S. Bedhomme, C. Sidobre, and Y. Michalakis. 2004. Parasitism increases and decreases the costs of insecticide resistance in mosquitoes. Evolution 58: 579–586.

Akoh, J. I., F. I. Aigbodion, and D. Kumbak. 1992. Studies on the effect of larval diet, adult body weight, size of bloodmean and age on the fecundity of *Culex quinquefasciatus* (Diptera: Culicidae). Insect Sci. Applic. 13: 177–181.

Ascher, K.R.S., and I. Neri. 1961. Lipoid content and resistance in the housefly, *Musca domestica* L. Entomol. Exp. & Appl. 4: 7–19.

Beenakkers, A.M.T., D.J.V.D. Horst, and W.J.A.V. Marrewijk. 1981. Role of lipids in energy metabolism, pp. 53–100. *In* R.G.H. Downer [ed.], Energy Metabolism in Insects. Plenum, New York.

Ben Cheikh, H., Z. Ben Ali-Haouas, M. Marquine, and N. Pasteur. 1998. Resistance to organophosphorus and pyrethroid insecticides in *Culex pipiens* (Diptera: Culicidae) from Tunisia. J. Med. Entomol. 35: 251–260.

Berticat, C., G. Boquien, M. Raymond, and C. Chevillon. 2002. Insecticide resistance genes induce a mating competition cost in *Culex pipiens* mosquitoes. Genet. Res. Camb. 79: 41–47.

Berticat, C., O. Duron, D. Heyse, and M. Raymond. 2004. Insecticide resistance genes confer a predation cost on mosquitoes, *Culex pipiens.* Genetical-Research 83: 189–196.

Bisset, J. A., and A. Soca. 1998. Cross-resistance to malathion in Cuban *Culex quinquefasciatus* induced by larval selection with deltamethrin. Med. Vet. Entomol. 12: 109–112.

Bourguet, D., T. Guillemaud, C. Chevillon, and M. Raymond. 2004. Fitness costs of insecticide resistance in natural breeding sites of the mosquito *Culex pipiens.* Evolution 58: 128–135.

Bridges, R. G., and J. T. Cox. 1959. Resistance to houseflies to γ-benzene hexachloride and dieldrin. Nature 184: 1740–1741.

Briegel, H. 1990. Metabolic relationship between female body size, reserves, and fecundity of *Aedes aegypti.* J. Insect Physiol. 36: 165–172.

Briegel, H., M. Hefti, and E. DiMarco. 2002. Lipid metabolism during sequential gonotrophic cycles in large and small female *Aedes aegypti.* J. Insect Physiol. 48: 547–554.

Brown, A.W.A. 1958. Insecticide resistance in arthropods. World Health Organization, Geneva, Switzerland.

Bullini, B., M. Coluzzi, and A.P.B. Bullini. 1976. Biochemical variants in the study of multiple insemination in *Culex pipiens* (Diptera, Culicidae). Bull. Entomol. Res. 65: 683–685.

Candy, D. J. 1985. Intermediary metabolism, pp. 1–43. *In* G. A. Kerkut and L. I. Gilbert [eds.], Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon Ltd., Oxford, United Kingdom.

[CDC] Centers for Disease Control and Prevention. 2002. Provisional surveillance summary of the West Nile Virus epidemic–United States, January–November 2002 Morb. Mort. Wkly. Rep. 51: 1129–1133.

Chandre, F., F. Darriet, M. Darder, A. Cuany, J.M.C. Doannio, N. Pasteur, and P. Guillet. 1998. Pyrethroid resistance in *Culex quinquefasciatus* from West Africa. Med. Vet. Entomol. 12: 359–366.

Chevillon, C., M. Raymond, T. Guillemaud, T. Lenormand, and N. Pasteur. 1999. Population genetics of insecticide resistance in the mosquito *Culex pipiens.* Biol. J. Linnean Soc. 68: 147–157.

Clements, A. N. 1992. The biology of mosquitoes: development, nutrition and reproduction. Chapman & Hall, London, United Kingdom.

Craig, G. B. 1967. Mosquitoes: female monogamy induced by male accessory gland substance. Science 156: 1499–1501.

Crow, J. F. 1956. Genetics of insect resistance to chemicals. Annu. Rev. Entomol. 1: 227–246.

Cui, F., M. Raymond, and C. Qiao. 2006. Insecticide resistance in vector mosquitoes in China. Pest Manag. Sci. 62: 1013–1022.

Day, J. F., A. M. Ramsey, and J.-T. Zhang. 1990. Environmentally mediated seasonal variation in mosquito body size. Environ. Entomol. 19: 469–473.

Downer, R.G.H. 1985. Lipid metabolism, pp. 77–114. *In* G. A. Kerkut and L. I. Gilbert [eds.], Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon Ltd., Oxford, United Kingdom.

Duron, O., P. Labbe, C. Berticat, F. Rousset, S. Guillot, M. Raymond, and M. Weill. 2006. High *Wolbachia* density correlates with cost of infection for insecticide resistant *Culex pipiens* mosquitoes. Evolution 60: 303–314.

Fast, P. G. 1964. Insect lipids: a review. Mem. Entomol. Soc. Can. 37: 1–50.

Friedman, S. 1985. Carbohydrate metabolism, pp. 44–76. *In* G. A. Kerkut and L. I. Gilbert [eds.], Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon Ltd., Oxford, United Kingdom.

Gazave, E., C. Chevillon, T. Lenormand, M. Marquine, and M. Raymond. 2001. Dissecting the cost of insecticide resistance genes during the overwintering period of the mosquito *Culex pipiens.* Heredity 87: 441–448.

Georghiou, G. P., R. L. Metcalf, and F. E. Gidden. 1966. Carbamate resistance in mosquitoes. Selection of *Culex*

pipiens fatigans Weidemann for resistance to Baygon. Bull. World Health Org. 35: 691–708.

Hardstone, M. C., B. P. Lazzaro, and J. G. Scott. 2009. The effect of three environmental conditions on the fitness of cytochrome P450 monooxygenase-mediated permethrin resistance in *Culex pipiens quinquefasciatus.* BMC Evol. Biol. 9: 42.

Hardstone, M. C., C. A. Leichter, L. C. Harrington, S. Kasai, T. Tomita, and J. G. Scott. 2007. Cytochrome P450 monooxygenase-mediated permethrin resistance confers limited and larval specific cross-resistance in the southern house mosquito, *Culex pipiens quinquefasciatus.* Pestic. Biochem. Physiol. 89: 175–184.

Harrington, L. C., J. D. Edman, and T. W. Scott. 2001. Why do female *Aedes aegypti* (Diptera: Culicidae) feed preferentially and frequently on human blood? J. Med. Entomol. 38: 411–422.

Hougard, J. M., V. Corbel, R. N'Guessan, F. Darriet, F. Chandre, M. Akogbeto, T. Baldet, P. Guillet, P. Carnevale, and M. Traore-Laminzana. 2003. Efficacy of mosquito nets treated with insecticide mixtures or mosaics against insecticide resistant *Anopheles gambiae* and *Culex quinquefasciatus* (Diptera: Culicidae) in Cote d'Ivoire. Bull. Entomol. Res. 93: 491–498.

Ivanovic, J. P. 1991. Metabolic response to stressors, pp. 28–67. *In* J. Ivanovic and M. Jankovic-Hladni [eds.], Hormones and Metabolism in Insect Stress. CRC, Inc., Boca Raton, FL.

Jalil, M. 1974. Observation on the fecudity of *Aedes triseriatus* (Diptera: Culicidae). Entomol. Exp. & Appl. 17: 223–233.

Kalra, R. L., A. S. Perry, and J. W. Miles. 1967. Studies on the Mechanism of DDT-Resistance in Culex pipiens fatigans. Bull. World Health Org. 37: 651–656.

Kasai, S., I. S. Weerashinghe, and T. Shono. 1998. P450 monooxygenases are an important mechanism of permethrin resistance in *Culex quinquefasciatus* Say larvae. Arch. Insect Biochem. Physiol. 37: 47–56.

Kitzmiller, J. B., and H. Laven. 1958. Tests for multiple fertilization in *Culex* mosquitoes by the use of genetic markers. Am. J. Hyg. 67: 207–213.

Kolaczinski, J. H., and C. F. Curtis. 2004. Investigation of negative cross-resistance as a resistance-management tool for insecticide-treated nets. J. Med. Entomol. 41: 930–934.

Lenormand, T., D. Bourguet, T. Guillemaud, and M. Raymond. 1999. Tracking the evolution of insecticide resistance in the mosquito *Culex pipiens.* Nature 400: 861–864.

Lima, C. A., W. R. Almeida, H. Hurd, and C.M.R. Albuquerque. 2003. Reproductive aspects of the mosquito *Culex quinquefasciatus* (Diptera: Culicidae) infected with *Wuchereria bancrofti* (Spirurida: Onchocercidae). Mem. Inst. Oswaldo Cruz. 98: 217–222.

Liu, H., Q. Xu, L. Zhang, and H. Liu. 2005. Chlorpyrifos resistance in mosquito *Culex quinquefasciatus.* J. Med. Entomol. 42: 815–820.

Liu, H., E. W. Cupp, K. M. Micher, A. Guo, and N. Liu. 2004. Insecticide resistance and cross-resistance in Alabama and Florida strains of *Culex quinquefaciatus (sic).* J. Med. Entomol. 41: 408–413.

Matsubara, H. 1960. Studies on the sexual difference of susceptibility of the insect against insecticides. I. On the sexual difference of characteristics of houseflies' lipids. Botyu-Kagaku 25: 138–143.

Merrill, A. L., and B. K. Watt. 1973. Energy value of foods basis and derivation. ARS–U.S. Department of Agriculture, Handbook no. 74.

Mostowy, W. M., and W. A. Foster. 2004. Antagonistic effects of energy status on meal size and egg-batch size of *Aedes aegypti* (Diptera: Culicidae). J. Vect. Ecol. 29: 84–93.

Nasci, R. S. 1990. Relationship of wing length to adult dry weight in several mosquito species (Diptera: Culicidae). J. Med. Entomol. 27: 716–719.

Paul, A., L. C. Harrington, L. Zhang, and J. G. Scott. 2004. Insecticide resistance in *Culex pipiens* from New York. J. Am. Mosq. Cont. Assoc. 21: 305–309.

Raymond, M., C. Berticat, M. Weill, N. Pasteur, and C. Chevillon. 2001. Insecticide resistance in the mosquito *Culex pipiens:* what have we learned about adaptation? Genetica 112–113: 287–296.

Reiser, R., D. S. Chadbourne, K. A. Kuiken, C. F. Rainwater, and E. E. Ivy. 1953. Variations in lipid content of the boll weevil and seasonal variation in its resistance to insecticides. J. Econ. Entomol. 46: 337–340.

Rodriguez, M., E. Ortiz, J. A. Bisset, J. Hemingway, and E. Saledo. 1993. Changes in malathion and pyrethroid resistance after cypermethrin selection of *Culex quinquefasciatus* field populations of Cuba. Med. Vet. Entomol. 7: 117–121.

Roush, R. T., and J. A. McKenzie. 1987. Ecological genetics of insecticide and acaricide resistance. Annu. Rev. Entomol. 32: 361–380.

Steele, J. E. 1981. The role of carbohydrate metabolism in physiological function, pp. 101–133. *In* R.G.H. Downer [ed.], Energy Metabolism in Insects. Plenum, New York.

Strang, R.H.C. 1981. Energy metabolism in the insect nervous system, pp. 169–206. *In* R.G.H. Downer [ed.], Energy Metabolism in Insects. Plenum, New York.

Styer, L. M., S. L. Minnick, A. K. Sun, and T. W. Scott. 2007. Mortality and reproductive dynamics of *Aedes aegypti* (Diptera: Culicidae) fed human blood. Vector-Borne Zoo. Dis. 7: 86–98.

Timmermann, S. E., and H. Briegel. 1998. Molting and metamorphosis in mosquito larvae: a morphometric analysis. Mitteilungen der Schweizerischen Entomologischen Gesellschaft 71: 373–387.

Timmermann, S. E., and H. Briegel. 1999. Larval growth and biosynthesis of reserves in mosquitoes. J. Insect Physiol. 45: 461–470.

Turell, M. J., M. L. O'Guinn, D. J. Dohm, and J. W. Jones. 2001. Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile Virus. J. Med. Entomol. 38: 130–134.

Van Handel, E. 1965. The obese mosquito. J. Physiol. 181: 478–486.

Van Handel, E. 1985a. Rapid determination of total lipids in mosquitoes. J. Am. Mosq. Control Assoc. 1: 302–304.

Van Handel, E. 1985b. Rapid determination of glycogen and sugars in mosquitoes. J. Am. Mosq. Control Assoc. 1: 299–301.

Van Handel, E., and P.T.M. Lum. 1961. Sex as a regulator of triglyceride metabolism in the mosquito. Science 134: 1979–1980.

Van Handel, E., and J. F. Day. 1988. Assay of lipids, glycogen and sugars in individual mosquitoes: correlations with wing length in field-collected *Aedes vexans.* J. Am. Mosq. Control Assoc. 4: 549–550.

Vinogradova, E. B. 2000. *Culex pipiens pipiens* mosquitoes: taxonomy, distribution, ecology, physiology, genetics, applied importance and control. Pensoft Publishers, Sofia, Bulgaria.

Williams, F. M., and R. S. Patterson. 1969. Effect of age on mating of *Culex pipiens quinquefasciatus.* Florida Entomol. 52: 259–261.

[WHO] World Health Organization. 2006. Pesticides and their application for the control of vectors and pests of public health importance. WHO/CDS/NTD/WHOPES/GCDPP/2006. 1: 125.

Wyatt, G. R. 1967. The biochemistry of sugars and polysaccharides in insects, pp. 287–360. *In* J.W.L. Beament [ed.], Advances in Insect Physiology. Academic Inc. Ltd., London, United Kingdom.

Yebakima, A., M. Marquine, J. Rosine, M.-M. Yp-Tcha, and N. Pasteur. 2004. Evolution of resistance under insecticide selection pressure in *Culex pipiens quinquefasciatus* (Diptera: Culicidae) from Martinique. J. Med. Entomol. 41: 718–725.

Received 19 May 2009; accepted 28 October 2009.