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INFLUENCE OF METHOPRENE AND DIETARY PROTEIN ON MALE *ANASTREPHA SUSPENSA* (DIPTERA: TEPHRITIDAE) LIPID AND PROTEIN CONTENT

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ABSTRACT

Because both the application of a juvenile hormone analog, methoprene, and the addition of protein to the adult diet increased the sexual success of male Caribbean fruit fly, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae), it was hypothesized that both might also impact male nutritional status. Total content of lipid and of protein in *A. suspensa* males were measured to discover if there was an effect of these treatments alone or in combination on the content of each of these subtstances. In the first 24 hours following adult emergence, 6 different treatments were applied (all possible combinations of methoprene in acetone solution or acetone alone, and protein-diet enrichment). Adult weight was determined for all treatments at 5, 10, 15, 20, 25, 30 and 35 d post-emergence. Dietary protein had a positive effect on the weight and total lipid and protein contents during the first 35 d of adult male life. There were minimal negative impacts from methoprene applications. Even though males were more active sexually, there was no significant change in weight or protein content during the study period. However, total lipid content decreased with age. The usefulness of methoprene to enhance the sexual performance of mass-reared tephritids destined for sterile release appears to outweigh any physiological costs/limitations that such treatment might confer.

Key Words: adult age, adult weight, Caribbean fruit fly, hydrolyzed yeast, juvenile hormone, sexual maturation

RESUMEN

Debido a que tanto la aplicación de un análogo de la hormona juvenil, metopreno, y la adición de proteínas a la dieta del adulto aumento el éxito sexual del macho de la mosca de la fruta de Caribe, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae), se planteó la hipótesis de que ambos también podrían afectar el estatus nutricional masculino. Se midió el contenido total de lípidos y de proteínas en los machos de *A. suspensa* para descubrir si había un efecto de estos tratamientos solos o en combinación sobre el contenido de cada una de estas sustancias. En las primeras 24 horas después de la emergencia de adultos, se aplicaron 6 diferentes tratamientos (todas las combinaciones posibles de metopreno en solución de acetona en solución o solo acetona y con el enriquecimiento de proteínas en la dieta). Se determinó el peso adulto para todos los tratamientos a los 5, 10, 15, 20, 25, 30 y 35 días después de la emergencia. Las proteínas dietéticas tuvo un efecto positivo sobre el peso y el total de lípidos y proteínas durante los primeros 35 días de vida de los machos adultos. Hubo un mínimo de impactos negativos de las aplicaciones de metopreno. A pesar de que los machos eran más activos sexualmente, no hubo ningún cambio significativo en el peso o el contenido de proteína durante el período de estudio. Sin embargo, el contenido de lípidos totales disminuyeron con la edad. La utilidad de metopreno para mejorar el desempeño sexual de moscas tefrítidas criadas en masa destinadas para programas que liberan los machos estériles parece superar los costos fisiológicos y las limitaciones que dicho tratamiento puede conferir.

Topical application of the juvenile hormone analog, methoprene, on the dorsal surface of adult male Caribbean fruit flies, *Anastrepha suspensa* (Loew) (Diptera: Tephritidae), increases male sexual success (Pereira et al. 2009), apparently because it increases the production of male sex pheromone. In addition it accelerates sexual maturation by several days (Teal & Gomez-Simuta 2002). The addition of protein to the adult diet has a similar effect on sexual performance, but the underlying cause(s) has yet to be investigated experimentally. When methoprene and protein are combined there is an additive increase in male sexual performance, and males are ~ 4 times more likely to mate than males not exposed to methoprene nor given access to protein (Pereira et al. 2010).

Presumably, increased pheromone production occurring at an earlier age, as well as accelerated sexual activity, is energetically demanding and

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may affect the balance of the metabolic compounds (Teal et al. 2000). One hypothesis frequently mentioned for the relatively long, sometimes more than 2 weeks, pre-reproductive period found in many adult frugivorous Tephritidae is that the time is used to acquire resources needed for reproduction (Sivinski et al. 2000). Decreases in resource foraging time due to accelerated sexual maturation and increases in body nutrients resource expenditure might be expected to result in substantive changes in a fruit fly's nutritional status that could affect longevity and long-term sexual performance. We further supposed that these expenses could be particularly difficult to incur in the absence of a protein enriched adult diet.

As a result, we hypothesized that the nutritional effects of methoprene and diet, both alone and in combination, might have an important effect on sexual performance of male flies reared for sterile insect technique (SIT) programs. It is from this perspective that we address the following questions: (1) What influence does methoprene treatment have on male *A. suspensa* weight and lipid/protein nutrient stores over a period of 35 d; ~ 33% of males survive to this age under laboratory conditions (Sivinski 1993); and (2) do protein enriched and protein deprived diets affect male weight and lipid/protein content, and is there an interaction between diet and methoprene treatment?

Considering the potential importance of adult diet to SIT, relatively little nutritional work has been done with *A. suspensa*. The rate and the temporal patterns of consumption of carbohydrates, proteins, and amino acids by adults (Sharp & Chambers 1984; Landolt & Davis-Hernandez 1993), as well as the role of food availability and quality on male pheromone production have been studied to some extent (Epsky & Heath 1993; Teal et al. 2000; Teal & Gomez-Simuta 2002). A positive influence of sucrose on male pheromone calling (Landolt & Sivinski 1992) and survival (Teal et al. 2004) has been reported. However, no work has been done specifically on the nutritional impact of protein incorporation into adult diets. This is the first study of male tephritid nutritional balance challenged by artificially elevated "juvenile hormone" titers to improve sexual performance.

Material and Methods

Insects

The Caribbean fruit flies used in this study were obtained from laboratory colony at the Center for Medical, Agricultural and Veterinary Entomology (CMAVE) USDA-ARS, at Gainesville, FL. At the time of the study, the colony was 3 years old and had been produced according to the conventional mass rearing protocols (FDACS 1995). Pupae were collected from the colony and sorted by size in a pupal sorting machine (FAO/IAEA/USDA 2003). Pupal size was homogenized to reduce male size and weight variability; large males have been shown to have a sexual advantage over smaller males (Burk & Webb 1983; Burk 1984; Webb et al. 1984; Sivinski & Dodson 1992; Sivinski 1993). Males used for this experiment were from pupal size class of 10.9 0.71 mg (n = 30) in weight. This is considered a mid-size pupal weight for field collected *A. suspensa* males in infested guava fruits (Hendrichs 1986). Throughout the experiment flies were maintained in a laboratory room with a photoperiod of 13L:11D (light from 0700 to 2000 h), a light intensity of 550 50 lux, a temperature of 25 1°C and a relative humidity of 55 5%.

Diet and Hormonal Treatments

Following emergence, males were subjected to 1 of the following 6 diet and hormonal treatments:

- M^+P^+: topical methoprene in acetone; access to sugar and hydrolyzed yeast
- M^+P^-: topical methoprene in acetone; access to sugar
- M^-P^+: topical acetone; access to sugar and hydrolyzed yeast
- M^-P^-: topical acetone; access to sugar
- P^+: no topical application; access to sugar and hydrolyzed yeast
- P^-: no topical application; access to sugar.

Methoprene (5 µg in 1 µL acetone) was applied topically within the first 24h after emergence. Controls consisted of application of 1 µL acetone only (M^-) or no topical application (P^+ and P^-). In order to conduct the topical application, males were immobilized in a net bag, and the solution was applied through the mesh on the dorsal surface of the thorax from a micro-pipette. No anaesthesia was used to immobilize the flies. Precautions were taken to avoid cross contaminations among experimental subjects. Male flies exposed to the different treatments were maintained in screen cages (30 cm by 30 cm by 30 cm), with a maximum male density of 200 flies/cage. Flies were allowed free access to food (according to above treatments) and water. In protein-deprived treatments (P^-) flies were only provided with sugar. Protein was provided to the flies in the form of hydrolyzed yeast mixed with sugar (1:3 parts, respectively). This mixture is considered a high quality diet for *Anastrepha* species (Jácome et al. 1995; Aluja et al. 2001).

Experimental cages were maintained for up to 35 d. For weight and chemical analysis, male flies

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were sampled at the following ages: 5, 10, 15, 20, 25, 30, and 35 d of adult age. For each age and treatment, 5 flies were randomly sampled and stored at -84ºC until used for analysis. In order to obtain the base line information after emergence, 5 newly emerged (without access to any food or water) and untreated flies were collected as well. Because lipid content in *Ceratitis capitata* (Wied.) has been found to vary according to the time of the day, due to the different activities in which males were engaged (Warburg & Yuval 1997), we sampled males at the same time each day (16:30 h, immediately before the beginning of the calling period). Flies were weighed individually prior to homogenization for lipid and protein determination.

Quantification of Lipids and Proteins

Individual male flies were homogenized in a solution of PBS buffer at pH 7.25 (8.77 g of 0.15 M NaCl and 7.1 g of 50 mM Na_2HPO_4 in 1 L of water). The homogenate was then brought up to 4.0 mL with PBS. Lipids were extracted from the homogenate by adding 40 mg of Na_2SO_4 to half of the initial volume, and 3.75 mL of chloroform: methanol (1:2) (Bligh & Dyer 1959) was used to separate polar and non-polar constituents of the homogenate. An additional 1.25 mL of chloroform was added to the homogenate and vortexed for 4 min at 4,000 rpm. The non-polar chloroform phase was collected. The remaining solution was re-extracted with chloroform (1.875 mL), vortexed and collected. Chloroform was evaporated in a Speed Vac device (Thermo Savant, San Jose, CA).

Lipid contents were determined by the vanillin reagent method (Van Handel 1985; Warburg & Yuval 1996), with triolein being used as a standard. Quantification of lipids was done by reacting 10 µL of sample with 190 µL of vanillin reagent. Lipid content was determined colorimetrically at 530 nm in a spectrophotometer (Bio-tek Instruments, Winooski, VT).

Protein determination was done according the Pierce BCA protein assay (Pierce, Rockford, IL). One mL of the polar fraction of the homogenate was centrifuged for 1 min at 14,000 rpm. Half the volume was mixed with 100 µL of sodium deoxychoate reagent (0.15 w/v) and 100 µL of 72% (w/v) tricloroacetic acid (TCA) to precipitate the proteins. After incubation at room temperature for 10 min and centrifugation for 10 min at 14,000 rpm the supernatant was discarded. The precipitate was dissolved and reacted with 50 µL of 5% (w/v) sodium dodecyl sulfate (SDS) and 1 mL of Pierce micro BCA™ protein assay reagent (Pierce, 1999). After incubation in a water bath at 37ºC for 30 min, proteins in samples and standards were determined colorimetrically at 562 nm in a spectrophotometer (Bio-Tek Instruments, Winooski, VT).

Statistical Analyses

Data were analyzed by two-way analysis of variance (ANOVA) to detect the interactions between age and treatment for the parameters studied, independently (weight, lipid content, and protein content). These analyses were followed by an ANOVA to detect differences between means in the treatments. Tukey's test was used to separate means (Ott & Longnecker 2001). Statistical analyses were performed with R software (version 2.1.0, www.r-project.org).

Results

Male weight

Average adult weight varied between 5.8 mg and 11.6 mg (Fig. 1). There was no interaction between treatment and adult age ($F_{35,192} = 1.16$, $P = 0.256$), and no effect of age ($F_{7,192} = 1.59$, $P = 0.140$; Table 1) on adult weight. There was, however, a significant effect of treatment ($F_{5,192} = 24.46$, $P < 0.05$). Protein-fed males generally had significantly higher fresh weights than sugar fed males (Fig. 1, Table 2).

Lipid content

Significant effects of treatment ($F_{5,192} = 131.37$, $P < 0.001$), adult age ($F_{7,192} = 83.14$, $P < 0.001$), and the interaction of adult age and treatment ($F_{35,192} = 6.34$, $P < 0.001$) were found. Male lipid content per treatment per age (Fig. 2) differed both among ages and for different treatments (Table 1) and among treatments for different ages (Table 2). In protein-deprived males, lipid contents dropped at 5 d after emergence, while protein-fed males maintained stable lipid levels during the first 10 d of adult life (Fig. 2). Afterwards, lipids dropped to lower levels. Methoprene treatment did not affect lipid levels in either protein-fed or protein-deprived male flies.

Protein Content

Significant effects of treatment ($F_{5,192} = 44.63$, $P < 0.001$), adult age ($F_{7,192} = 15.00$, $P < 0.05$), and the interaction of adult age and treatment ($F_{35,192} = 4.77$, $P < 0.001$) were found. Significant differences in protein content among the different ages were found within each treatment except for treatment M^-P^+ (Fig. 3, Table 1), and among treatments at all ages (Table 2). Protein-fed males maintained higher protein levels than protein-deprived males (Fig. 3). In protein-fed males, protein content steadily increased through time, while in protein-deprived males, protein levels declined. Methoprene did not affect the level of protein in either protein-fed or protein-deprived males.

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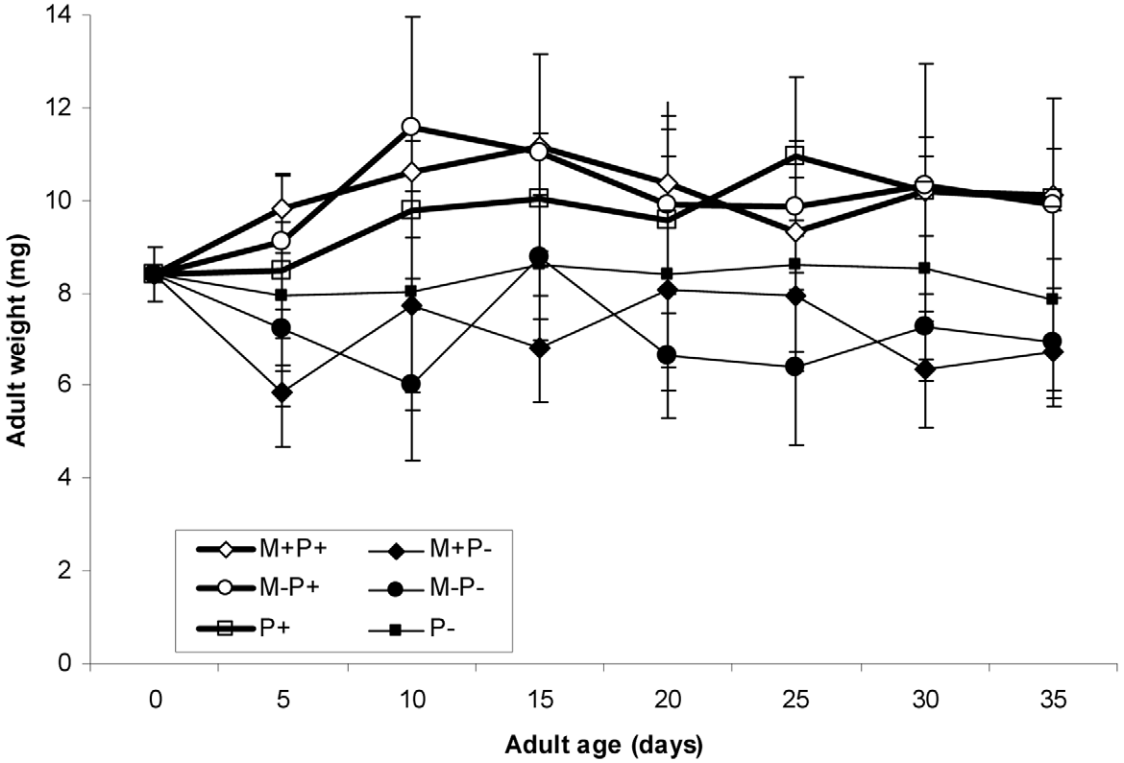


Fig 1. Mean (±SD) adult weight (n = 5) of male Caribbean fruit flies at different adult ages among the 6 treatments featuring methoprene (M) and protein (P) and their various combinations.

Discussion

In male *A. suspensa* there was a clear effect of a protein-enriched diet on weight, total lipid, and total protein content over the first 35 d of adult life. In contrast, there was no effect of methoprene or acetone application on the studied parameters. Regardless of diet type, weight and total protein content were relatively stable during adult life. In contrast, total lipid content steadily decreased with age. This decline began later, however, in flies fed a protein-enriched diet (10 d after emergence).

In all the treatments, consumption of protein resulted in insects able to regulate their weight, protein levels, and lipid content at higher level than insects without a protein food source. This is broadly consistent with what has been observed in Tephritidae in general and other *Anastrepha* spp. in particular (Aluja et al. 2001). In nature, adult tephritids feed on a variety of carbohydrates and proteins derived from fruit juices, honeydew, and bird feces (Hendrichs et al. 1991; Warburg & Yuval 1997; Yuval & Hendrichs 2000). Protein enhances reproductive performance in *C.*

Table 1. Analysis of variance (ANOVA) for male Caribbean fruit fly weight, total lipids, and total proteins among different ages in 6 different treatments featuring methoprene (M) and protein (P) and their various combinations (NS, non significant differences, $P > 0.05$; * $0.01 < P < 0.05$; *** $P < 0.001$).

Treatments	Male weight	Total lipids	Total proteins
M+P+	$F_{7,32} = 1.1559$ (ns)	$F_{7,32} = 7.5932$ ***	$F_{7,32} = 3.2711$ *
M+P-	$F_{7,32} = 1.9367$ (ns)	$F_{7,32} = 32.76$ ***	$F_{7,32} = 8.6007$ ***
M-P+	$F_{7,32} = 1.8041$ (ns)	$F_{7,32} = 11.124$ ***	$F_{7,32} = 2.0186$ (ns)
M-P-	$F_{7,32} = 2.0277$ (ns)	$F_{7,32} = 35.906$ ***	$F_{7,32} = 7.7853$ ***
P+	$F_{7,32} = 1.0071$ (ns)	$F_{7,32} = 12.504$ ***	$F_{7,32} = 2.9685$ *
P-	$F_{7,32} = 0.1291$ (ns)	$F_{7,32} = 20.805$ ***	$F_{7,32} = 4.8732$ ***

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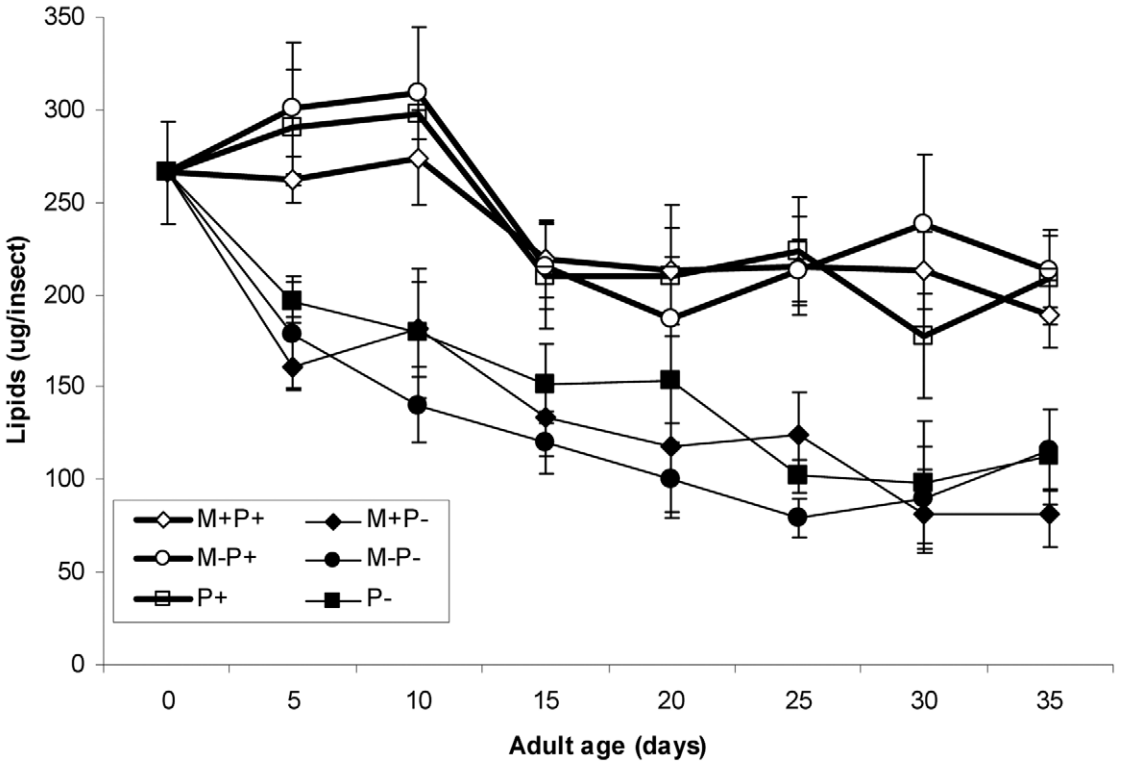


Fig. 2. Mean (±SD) total lipid content (n = 5) of male Caribbean fruit flies at different adult ages among the 6 treatments featuring methoprene (M) and protein (P) and their various combinations.

capitata (Warburg & Yuval 1997; Kaspi et al. 2000; Shelly & Kennelly 2002; Shelly et al. 2002; Yuval et al. 2002), and protein-fed males start to call earlier in life (Papadopoulos et al. 1998). Protein-fed males are more competitive in terms of post copulatory sexual selection as well (Taylor & Yuval 1999). In *Bactrocera dorsalis* (Hendel), incorporation of protein into adult diet significantly increases survival and mating success (Shelly el al. 2005). Among *Anastrepha* species, Aluja et al. (2001) evaluated the effects of different adult nutrients, including protein and sugar, on male sexual performance in adults of 4 species, (*A. ludens* (Loew), *A. obliqua* (Macquart), *A. serpentina* (Wied.), *A. striata* Schiner). Overall, protein-fed males were more sexually successful than protein-deprived, except for *A. ludens* where no differences were found. Neither did male diet influence *A. ludens* female reproductive potential following trophalaxis (Mangan 2003). However, in a more recent study protein did improve *A. ludens* sexual performance (Aluja et al. 2008).

Thus, perhaps not surprisingly, protein-enhanced diets typically, but not always, enhance

TABLE 2. ANALYSIS OF VARIANCE (ANOVA) FOR MALE CARIBBEAN FRUIT FLY WEIGHT, TOTAL LIPIDS, AND TOTAL PROTEINS AMONG TREATMENTS FOR DIFFERENT AGES FEATURING JUVENILE HORMONE (JH) AND PROTEIN (P) AND THEIR VARIOUS COMBINATIONS (* $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$).

Adult age (days)	Male weight	Total lipids	Total proteins
5	$F_{5,24} = 4.546$ **	$F_{5,24} = 26.285$ ***	$F_{5,24} = 3.5202$ *
10	$F_{5,24} = 4.566$ **	$F_{5,24} = 28.881$ ***	$F_{5,24} = 26.629$ ***
15	$F_{5,24} = 5.521$ **	$F_{5,24} = 12.548$ ***	$F_{5,24} = 10.277$ ***
20	$F_{5,24} = 2.624$ *	$F_{5,24} = 13.873$ ***	$F_{5,24} = 8.9948$ ***
25	$F_{5,24} = 4.370$ **	$F_{5,24} = 50.275$ ***	$F_{5,24} = 4.2223$ **
30	$F_{5,24} = 4.429$ **	$F_{5,24} = 21.339$ ***	$F_{5,24} = 8.9493$ ***
35	$F_{5,24} = 5.120$ **	$F_{5,24} = 36.197$ ***	$F_{5,24} = 9.5048$ ***

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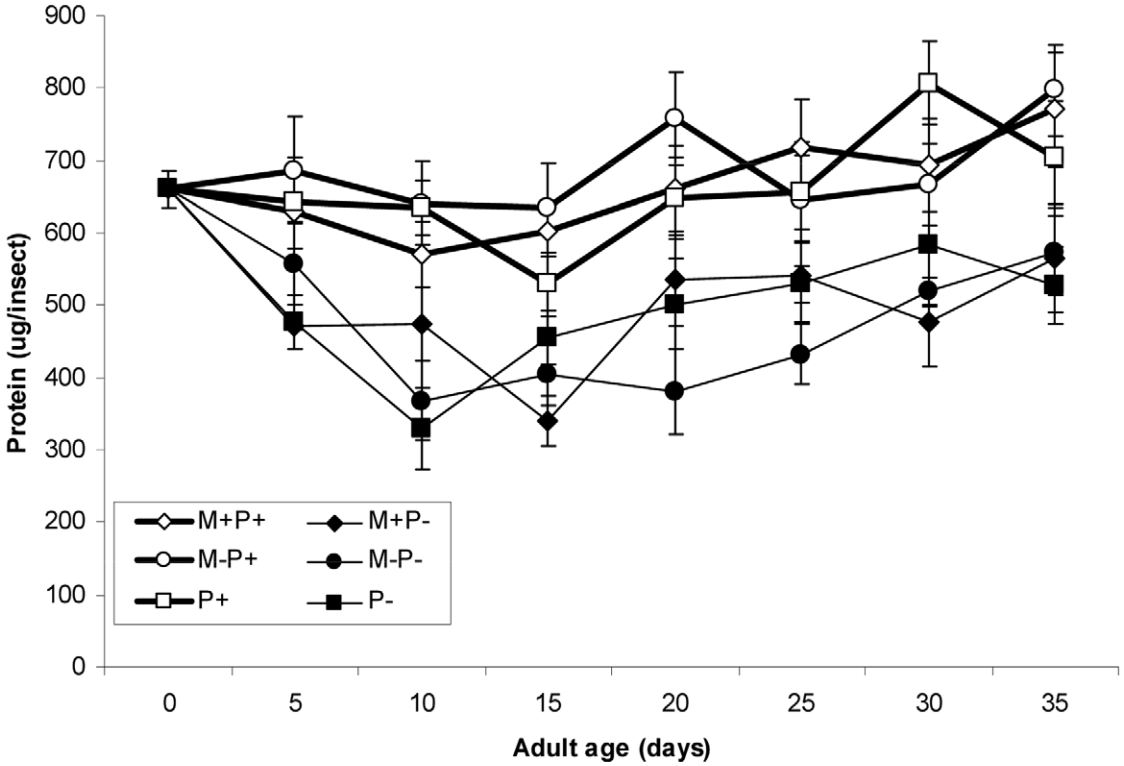


Fig. 3. Mean (±SD) total protein content (n = 5) of male Caribbean fruit flies at different adult ages among the 6 treatments featuring methoprene (M) and protein (P) and their various combinations.

sexual success. However, there are perhaps revealing differences in physiology and foraging tactics for food and mates among males of different species with different diets and activities. For instance, unlike *A. suspensa*, protein-fed *C. capitata* males have lower lipid content than those that are protein-deprived (Kaspi et al. 2000). In addition, while lekking males are heavier and contain significantly more protein and sugar than resting males, they do not contain more lipids (Yuval et al. 1998). Lipids (fatty acids, phospholipids, and sterols) have a nutritional role distinct from carbohydrates and proteins. Yuval et al. (1994) described lipids metaphorically as an energetic trust fund, whereas carbohydrates are comparable to a readily accessible cash account. Perhaps the difference between *A. suspensa* and *C. capitata* in their use of protein for lipogenesis reflects a difference in energy use patterns, with *A. suspensa* putting more reserves in "long-term" accounts for future use. This in turn might reflect more predictably encountered food sources for *C. capitata* or a lower daily chance of mortality for *A. suspensa* that leads in turn to "planning" for the future.

Many kinds of fatty acids and phospholipids are synthesized by insects, but all insects require sterols in their diet (Chapman 1998). Reduction of total lipid content with age in *A. suspensa* can be the result of somatic activities, since lipids represent stored energy, even if some restoration of lipid reserves occurs by lipogenesis (Warburg & Yuval 1996). In male *A. suspensa*, at least in the first 10 days of adult life of protein-fed males, there is a slight increase in lipid content. The same phenomenon occurs in *C. capitata* (Warburg & Yuval 1996) and *A. serpentina* (Jácome et al. 1995). Total lipid content declined following male *A. suspensa* sexual maturation. Sharp decreases indicate that males started to utilize their metabolic reserves, and this seems likely to correspond to the energetic requirements of any number of sexual and agonistic behaviors and processes (e.g., Sivinski et al. 2000). One of these potential expenditures that can be indirectly examined with the present data is pheromone production.

Nestel et al. (1986) suggested that lipid reserves in male *C. capitata* may play an important role in the regulation and production of sex pheromone. Nestel et al. (2005) found a decrease in lipid body content after sexual maturation (as the present data reveal for *A. suspensa*), but later on the content displayed a harmonic pattern where total lipid content increased and decreased at a periodicity of 10 days. In *A. suspensa*, male pheromone production increases when methoprene is

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applied (Teal et al. 2000). However, we found that application of methoprene did not affect lipid content of flies maintained on different diet treatments.

The differences in total protein content between protein-fed and protein-deprived males may be influenced by ingested protein in the gut. However, the gradual increase in total protein content over time in both protein-deprived (after 10 d as adult) and protein-fed males is both difficult to explain and inconsistent with artificial-diet protein alone accounting for the difference. Tephritids are known to feed on animal excrement (Prokopy et al. 1993; Epsky et al. 1997), and perhaps the consumption of bacteria from their own feces or bacteria growing on dead flies or on food sources, inadvertently provided them with a protein source. Regardless of the origin of this additional protein, the difference in protein contents on the different diets suggests it was not sufficient to completely satisfy nutritional requirements.

The findings of this study have implications for SIT programs. Among the most important is that while the addition of methoprene has male sexual advantages it appears to have no immediate nutritional detriments. Thus it is a relatively "cost-free" means of improving the performance of mass-reared and released flies. The incorporation of dietary protein has a positive effect on adult weight and lipid and protein content all of which are plausibly related to performance as well (Yuval et al. 1998). Due to these effects, the incorporation of protein in adult diet for SIT programs is also recommended.

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