



Storage hexamer utilization in *Manduca sexta*

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Abstract

In preparing for metamorphosis insects store in their hemolymph and fat bodies a major nutrient reserve of 500-kDa hexamerins. At least three hexamerins serve this function in Lepidoptera, including arylphorin (ArH) and two high methionine proteins (M-MtH and V-MtH). Six day-old adults of *Manduca sexta* are shown here to have consumed over 99% of their pupal reserves of ArH and in the case of males, 99.8% of M- and V-MtH. In support of egg formation, however, females at this stage retain over 25% of their pupal reserves of the high methionine proteins. Demonstrated here are three factors contributing to the methionine protein reserves in day-6 adult females. (1) Pupal stores of the methionine proteins average 1.67 times larger in females than in males. (2) A fraction of this pupal store remains undiminished during pharate adult development: centrifugation of homogenates partitions the hexamerins into a fraction that is soluble in PBS and a smaller, particle-associated fraction that is not. Pharate adults consume most of the soluble fraction and relatively little of the particulate fraction, which then constitutes over half of the methionine protein reserves of post-eclosion females. (3) Both soluble and particle-associated reserves double in the week following eclosion and this suggests that adult females may resume the synthesis of V- and M-MtH. Though differing in amino acid sequence and antigenic properties, V-MtH and M-MtH showed no significant differences in their storage and utilization profiles.

Abbreviation:

ArH	arylphorin
M-MtH	moderately high methionine hexamerin
PBS	phosphate buffered saline
V-MtH	very high methionine hexamerin
Vg	vitellogenin

Introduction

Lepidopteran pupae store in their hemolymph and fat body high concentrations of three, and in some species four hexameric proteins with differing antigenic epitopes, developmental profiles and amino acid and conjugate compositions. All four are consumed primarily during somatic tissue metamorphosis, but sexual differences in the quantity of a methionine-rich hexamerin synthesized by larvae suggest that hexamerins can also be targeted for the support of egg production (Tojo et al., 1981; Ryan et al., 1985 B; Tojo et al., 1985; Bean and Silhacek, 1988). This possibility has been confirmed by differences in the timing of hexamerin consumption between one early and one late egg producing species (Pan and Telfer, 2001). The Cecropia moth (*Hyalophora cecropia*) produces essentially all of its eggs during the pupal-adult molt; by the time that the adult ecloses reserves of three hexamerins, including arylphorin (ArH) and two discrete methionine-rich hexamerins (V-

and M-MtH), were shown to have fallen to less than 1% of their respective pupal reserves. Monarch butterflies (*Danaus plexippus*) postpone egg production until exposed as adults to a long-day photoperiod; in this case four-week old, short-day adult females still contained 20% of their pupal store of M-MtH and 30% of V-MtH, while less than 2% of the pupal store of ArH remained in post-eclosion females.

A third category of species that are intermediate in their timing of egg formation (Wheeler, 1996) is exemplified by the tobacco hornworm (*Manduca sexta*). Yolk deposition begins in *M. sexta* late in pharate adult development (Nijhout and Riddiford, 1974), but the ovaries continue to produce eggs during the weeks following eclosion. The question raised here was whether, as in *D. plexippus*, the MtH's remain available in *M. sexta* to provide nutrients during post-eclosion egg formation.

M. sexta larvae produce, and their pupae store, three hexamerins. Phylogenetic trees based on deduced amino acid

sequences (Burmester et al., 1998), as well as the antigenic cross-reactions referred to below, confirm that these proteins are homologues of ArH, V-MtH, and M-MtH. Isolation, composition, amino acid sequences and developmental profiles of ArH and V-MtH have been studied in *M. sexta* larvae (Kramer et al., 1980; Riddiford and Hice, 1985; Ryan et al., 1985A and B; Webb and Riddiford, 1988A and B; Willott et al., 1989); the homologue of M-MtH is known primarily from analysis of a fat body cDNA clone (Corpuz et al., 1991). We show here that, as in *D. plexippus*, amounts of the two MtH's equivalent to approximately 25% of their pupal reserves are still present in females after the first six days of post-eclosion egg formation, while ArH content drops during this period to less than 1% of the pupal reserve.

Materials and Methods

Insects

Eggs and artificial diet for *M. sexta* were obtained from Carolina Biological Supply Company (www.carolina.com). Caterpillars and adults were kept on a long day regime (16L:8D) at 25° C. Under these conditions, 18 days elapsed between pupation and adult eclosion. Adult males and females were separately caged and manually fed to satiation once daily with 30% honey.

Antibodies

ArH, M-MtH, V-MtH and vitellogenin (Vg) were assayed using monospecific antisera in an immunodiffusion method. Preparation and reactions of the four antisera have already been described (Pan and Telfer, 2001). Immunizing antigens had been isolated from *H. cecropia* hemolymph, but *M. sexta* hemolymph and extracts produced with all four antisera a single zone of heterologous precipitation (e. g., Fig. 4 in Telfer et al. (1983) and Fig.1 in Pan and Telfer (2001)).

Immunodiffusion

The method used to measure changes in hexamerin stores that are soluble in phosphate-buffered saline (PBS) was initially introduced by Oudin (Oudin, 1948; Pan and Telfer, 2001). Extracts of pupae and adults were layered over appropriate antisera that had been diluted with saline containing 0.05% sodium azide and 0.3% agarose. Maximum dilutions consistent with clear visualization of the precipitates were 1:15 for Anti-ArH, 1:4 for Anti-M-MtH, 1:3 for Anti-V-MtH and 1:20 for Anti-Vg. After mixing at 45° C, the solution was allowed to gel at room temperature in 3 mm (id) glass tubes. Procedures for setting up the tubes, for measuring rates of advance of precipitation fronts and for constructing standard curves were previously described (Telfer et al., 1983; Telfer and Pan, 1988). The measurements were of relative concentrations and were expressed in each case as a percentage of the concentration in a standard solution. For the hexamerins, the standard was a hemolymph/soft tissue extract of female pupae; that for vitellogenin was an extract of day-6 adult female abdomens and their chorionated eggs.

Tissue preparation

The extraction medium was pH 7.2 PBS containing a

protease inhibitor cocktail from Boehringer Mannheim Biochemicals (www.roche.com) ("Complete"; one tablet per 25 ml), and 5 mM phenylthiourea (PTU). Hemolymph was drained through a dorsal abdominal slit into 0.2 ml of this medium. The midgut and, when present, the bursa copulatrix were discarded and the rest of the abdominal soft tissues, including primarily fat body and reproductive organs, were added to the collected hemolymph. When chorionated eggs were present, the ovaries were separately crushed with a mortar and pestle. Additional extraction medium was used to rinse residual hemolymph from the carcass and to bring the suspended tissues and hemolymph to a volume that yielded antigen concentrations convenient for measurement by immunodiffusion. The collected samples were stored frozen, thawed as needed and individually homogenized.

SDS-PAGE

Insoluble residues that would escape detection by immunodiffusion were visualized by SDS-PAGE in pre-cast, 4-15% gradient minigels. For this purpose, several homogenates from a chosen stage were vortexed and 0.1 ml samples of each were pooled. The combined sample was centrifuged for 4 min at 10,000g. Clear supernatants were decanted and mixed with five times their volumes of SDS-PAGE sample buffer. Pellets were separately dissolved in a volume of sample buffer equal to that of the diluted supernatants. Volumes added to the sample wells were standardized to contain a calculated three ten thousandths of a single insect homogenate. This dosage overloaded the lane containing pupal extract supernatants, but provided satisfactory resolution of polypeptides in adult extracts.

Results

Vitellogenin

Analysis of the developmental profile of Vg confirmed the utility of the methods employed and simultaneously confirmed, in terms of yolk protein accumulation, indications from the literature that *M. sexta* is intermediate in its timing of egg production. Vg is known to appear in the hemolymph of pharate adult *M. sexta* about five days before eclosion (Imboden and Law, 1983). In agreement with this, Oudin tests on soft tissue/hemolymph extracts detected four days before eclosion an amount of Vg equivalent to 0.01% of that in day-6 adult females (Table 1). At eclosion the amount had risen to 12%, in accord with the observation that the ovaries at this

Table 1. Relative concentration of vitellogenin in extracts of female *Manduca sexta*.

Stage * (n)	Relative Concentration (% ± SE)
Pupal day-1 (5)	0
Pupal day-12 (4)	0.01 ± 0.01
Pupal day-17 (4)	12 ± 3.5
Adult day-1 (6)	12.7 ± 3.0
Adult day-4 (6)	50.5 ± 4.1
Adult day 6 (6)	100 ± 17.1

*Adult eclosion occurs on pupal day-18.

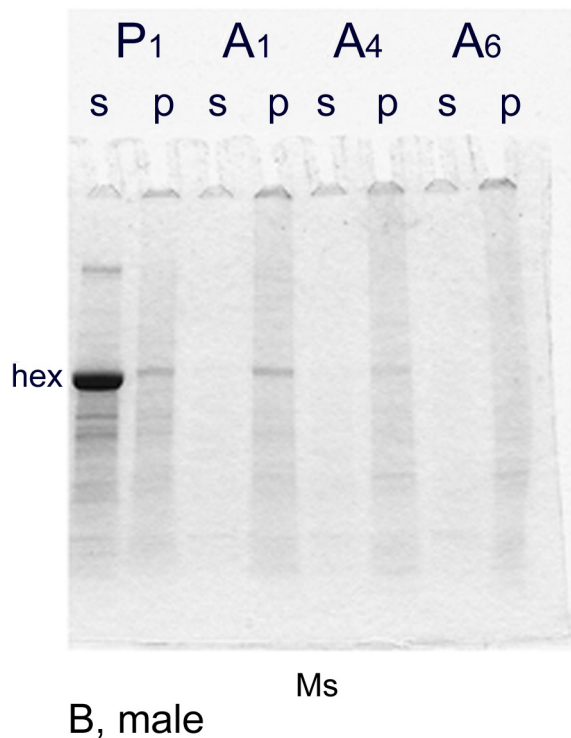
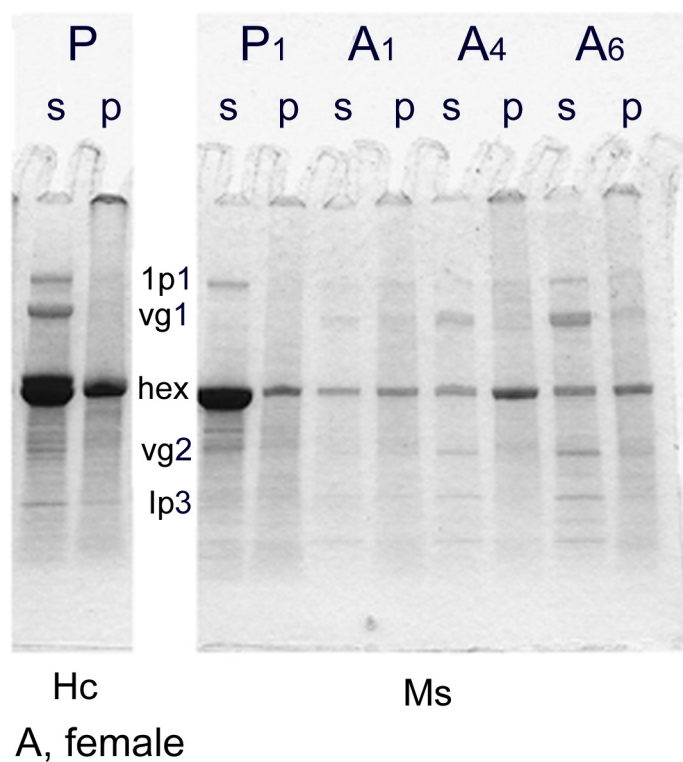


Figure 1. SDS-PAGE of centrifugal supernatants (s) and pellets (p) of soft tissue/hemolymph homogenates of *Manduca sexta* were run on 4-15%, precast minigels. Stages extracted include newly ecdysed pupae (P1) and adults one, four and six days after eclosion (A1, A4, A6). Serving as molecular weight standards were the major proteins in a hemolymph/soft tissue extract of diapausing *Hyalophora cecropia* pupae (left lane in A). The *H. cecropia* preparation includes 200-kDa apolipoprotein I (1p1), 180-kDa large subunit of Vg (vg1), unresolved 73-79-kDa subunits of ArH, V-MtH and M-MtH (hex), and 18-kDa apolipoprotein III (lp3). The heavy band lying above hex includes 85-kDa subunits of the riboflavin-binding hexamerin, a major *H. cecropia* hexamerin that is not detected in *M. sexta*.

stage already contain a small number of yolk-laden follicles (Nijhout and Riddiford, 1974). More follicles are added to this pool during subsequent days, and immunodiffusion reflected this by detecting a rise in vitellogenin content during the next six days (Table 1). SDS-PAGE of supernatants of adult extracts produced a female-specific doublet band (Vg1 in Fig. 1A, A-6, lane s) corresponding to the two circa 180 kDa forms of the large subunit of *M. sexta* Vg (Imboden and Law, 1983).

Pupal hexamerin stores

Females prepare for egg protein synthesis by storing larger MtH reserves than males. Male pupae contained only 50% as much soluble M-MtH as females ($n = 5$; $SE = 3.9$; $p > 0.02$ and < 0.01) and 70% as much V-MtH ($n = 5$; $SE = 12.5$; $p > 0.2$ and < 0.1). The latter difference was not significant statistically, but it is supported by the earlier report that this hexamerin reaches higher concentrations in the hemolymph of females than that of males (Ryan et al., 1985A). Contents of soluble ArH, by contrast, averaged 13% higher in males, and were not significantly different ($n = 5$; $SE = 7.1$; $p > 0.4$).

The sexual difference was also seen in SDS-PAGE (Fig. 1A and B; lanes s under P1). As in many other lepidopterans, ArH and the MtH's are the principle soluble proteins of *M. sexta* pupae (Kramer et al., 1980; Ryan et al., 1985A). At the 3×10^{-4} insect per lane dosage used here the hexamerins in pupal extracts were too concentrated to be resolved into their individual subunits (Fig. 1A and B, hex). The heavily stained overload band centered at around 75 kDa was consistently broader when produced by female supernatants than that produced by males.

Centrifugation of extracts separates insoluble, particle-associated proteins from a supernatant of PBS-soluble proteins (Pan and Telfer, 2001). In both male and female *M. sexta* the position of the principle component produced by centrifugal pellets corresponded to the upper edge of the PBS-soluble overload band but was much narrower and an order of magnitude less heavily stained than the latter (Fig. 1A and B; compare lanes s and p under P1). In both cases the pellet band resolved into a doublet; results described in the next section are consistent with the doublet's being formed by the two MtH's.

Developmental depletion of PBS-soluble hexamerins

During pharate adult development soluble forms of all three hexamerins decrease dramatically. In day-1 adult females ArH averaged only 2.3% of the soluble store in female pupae (Fig. 2 Female) while M-MtH and V-MtH averaged 6.3 and 3.4%, respectively. In males the declines were more extreme. Relative to their contents in pupal male extracts, ArH, M-MtH and V-MtH in day 1 adults averaged $0.02 \pm 0.01\%$, $0.28 \pm 0.21\%$ and $0.09 \pm 0.08\%$ respectively.

During six days following eclosion ArH continued to disappear in both sexes (Fig. 2); in day-6 females it had fallen to less than 1% of soluble pupal stores and in males it was no longer detectable in extracts of day-4 and -6 adults. MtH utilization, by contrast, exhibited a pronounced sexual difference. In day-6 females M-MtH and V-MtH levels had increased, respectively, to 12.9% and 11.6% of soluble female pupal stores, while levels in males had decreased, reaching values on day-6 of only 0.01% for M-MtH and

0.02% for V-MtH. Females at this stage thus contained 500 to 1000 as much soluble MtH's as males.

SDS-PAGE reflected these changes (Fig. 1A and B; lanes s under A1, A4 and A6). Female supernatants each produced a doublet with a staining intensity that increased in parallel with the rise in V- and M-MtH contents seen by immunodiffusion, while supernatants from males lacked detectable bands at the 75 kDa hexamerin subunit level.

Particle-associated hexamerins

Centrifugal pellets of adult female extracts produced doublet bands at the same position as those produced by supernatants (Fig. 1A; compare lanes p and s under A1, A4 and A6). Insolubility in PBS indicates that the particle-associated hexamerins are not hemolymph proteins but must instead be constituents of cells. This reinforces their identity, for the MtH's have been shown in several lepidopteran pupae to be stored primarily in the fat body (Tojo et al., 1978; Bean and Silhacek, 1988; Pan and Telfer, 1992), while ArH is the predominant storage protein in hemolymph (Kramer et al., 1980; Telfer et al., 1983; Pan and Telfer, 2001).

Particle-associated MtH's are largely spared from utilization during adult development, for pellet doublets from day-1 adults and pupae stained with similar intensities (Fig. 1A and B; compare lane p under P1 with that under A1). In males the pellet doublet grew weaker with time after eclosion, and was no longer detectable on day-6. By contrast, pellets from females produced doublets at all three adult stages examined. Rather than diminishing as in males, these stained more heavily at days-4 and 6 than at day-1.

At all three stages, doublets produced by pellets stained more intensely than those produced by the corresponding supernatants. Immunodiffusion, which measures only PBS-soluble antigens, therefore underestimated the adult hexamerin stores by more than 50%.

Discussion

The results describe the consumption schedules of the three *M. sexta* hexamerins and add another example to the earlier evidence from *D. plexippus* for the availability of MtH during post-eclosion egg formation. The soluble MtH's present in day-6 adults were 12-13% of the pupal stores of these two proteins, compared with less than 0.02% in males of the same age. Since SDS-PAGE showed that staining in adult female pellets was greater than that produced by corresponding supernatants, total content of the MtH's rises to a value that equals over 25% of their soluble pupal stores.

Immunodiffusion recorded a two to three-fold rise in PBS-soluble MtH content during six days following eclosion in *M. sexta* females, and this was confirmed in SDS-PAGE by increased staining of the doublet that is the principal component of centrifugal supernatants. In addition, doublets produced at this position by days-4 and -6 centrifugal pellets stained more heavily than that of the day-1 pellet. These increases suggest a resumption of MtH synthesis in adult females, though release from the thorax or from a tightly bound store that cannot be extracted by SDS-PAGE sample buffer is not ruled out.

A rise at eclosion in hexamerin concentrations in female hemolymph has been reported in another moth, *Plutella xylostella*.

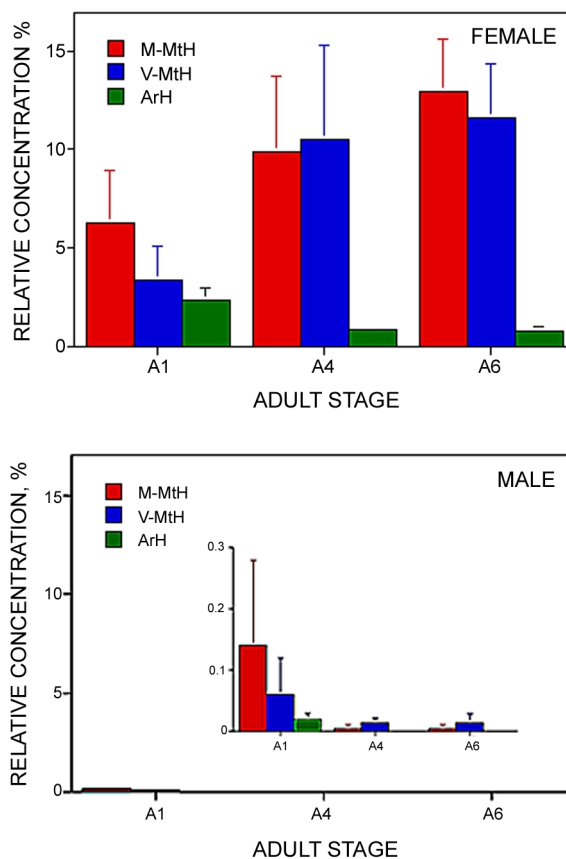


Figure 2. Soluble hexamerin concentrations measured by immunodiffusion in soft tissue/hemolymph extracts of abdomens of females (A) and males (B) of *Manduca sexta* at three times after eclosion. The concentrations of each antigen are expressed as percentages of those in extracts of female pupae. Values for males are plotted on an expanded scale in the inset. Error bars show standard errors; n = 6 in each case. Values for day-1 males relative to those of male pupae are indicated in the text.

In that case the value fell again rapidly within a day (Wheeler et al., 2000). Adult hexamerin synthesis has been described in several other orders of insects, differing from *M. sexta* in some cases by its occurrence in males as well as females and in its dependence on dietary protein intake (reviewed by Wyatt and Davey, 1996).

Aside from synthesis, two additional factors have the potential to enhance the MtH reserves of adult females. *M. sexta* is among the lepidopterans whose female pupae store larger quantities of the MtH's than males. Sexual differences were reported in the production of mRNA's for the two *M. sexta* MtH's by pharate pupae (Corpuz et al., 1991); and hemolymph concentrations of V-MtH reach much higher levels in female larvae and pharate pupae than in males (Ryan et al., 1985A); we confirm here the latter finding for soluble V-MtH in pupal tissue/hemolymph extracts, as well as showing that M-MtH is similarly enhanced in females.

In addition, centrifugation revealed that hexamerins stored in PBS-insoluble particles do not diminish during adult development. While only about a tenth of the total hexamerin store in pupae, this fraction becomes the major component of the MtH reserves of adult females. Of the three lepidopterans we have studied in this regard, *M. sexta* is unique. Adult *D. plexippus* store hexamerins primarily

in a PBS-soluble form (Fig. 4B in Pan and Telfer, 2001). And in *H. cecropia* the particulate store is exhausted along with soluble hexamerins as the eggs form in pharate adults.

ArH shows none of the attributes that favor the post-eclosion availability of the MtH's. Instead, this hexamerin disappears nearly entirely during somatic tissue metamorphosis.

Amino acids transferred from hexamerins to egg proteins can be assumed to pass through general pools and to be subjected to metabolic conversions. This has been made clear by experiments on another nectar-feeding sphingid, *Amphion floridensis*, (O'Brien et al., 2000; O'Brien et al., 2002). Differences in the stable isotope contents of larval and adult diets were exploited to show that carbon atoms in the amino acids of egg proteins can be derived from both sources. But amino acids that cannot be synthesized by the moth, as well as the amino groups of those that can, are derived only from the larval diet.

As noted above, ArH and V-MtH of *M. sexta* have been isolated from larval hemolymph and characterized with regard to composition and time of secretion. But M-MtH has eluded isolation in *M. sexta* (Ryan, personal communication). Its amino acid sequence and the timing of its mRNA production have been deduced instead from analyses of a larval fat body cDNA library (Corpuz et al., 1991). We show here that it precipitates antibodies against *Cecropia* M-MtH and is thus an antigenic homologue of the "protein 2" isolated by Tojo et al (1978) from the protein granules of *Cecropia* pupal fat body. Alignments of deduced amino acid sequences have since identified this protein in seven species of Lepidoptera (summarized by Zhu et al., 2002). Although M-MtH is still poorly known as an isolated protein, the antibody reactions described here reveal a utilization profile that is distinct from that of ArH but indistinguishable from that of V-MtH.

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