Pergamon

PII: S0022-1910(96)00049-2

J. Insect Physiol. Vol. 42, No. 10, pp. 961–966, 1996

0022-1910/96 $15.00 + 0.00

A Role for Storage Proteins in Autogenous Reproduction in *Aedes atropalpus*

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Received 15 November 1995; revised and accepted 28 Feburary 1996

In the autogenous mosquito, *Aedes atropalpus*, storage proteins accumulated during the larval stage may serve as an amino acid reserve for oogenesis, in addition to metamorphosis. Hexameric storage proteins accumulate during larval development and include subunits of three different masses: 62.5, 66, 72.5 kDa. All three types of subunits are found in the female but only the larger two are in males. In females, storage proteins are only partially depleted by the time of eclosion. The remaining protein amounts to about 40% of the original store. Males, in contrast, exhaust their supply of stored protein during metamorphosis. In the female, the storage proteins disappear over the first days after eclosion, and are depleted before vitellogenin/vitellin levels reach their maximum. This suggests that the amino acids held in storage proteins are transferred to vitellogenesis, enabling autogenous egg development. The fact that these amino acids are not available for egg development until after eclosion, later than in many other insects, probably reflects a relatively recent evolution from blood-feeding ancestors. Copyright © 1996 Elsevier Science Ltd

Autogeny Arylphorin Oogenesis

INTRODUCTION

Two important stages in the lives of insects require large amino acid stores: metamorphosis and rapid embryogenesis. Holometabolous insects in particular accumulate large quantities of protein during the larval period as storage proteins, which are normally depleted during metamorphosis. In addition, female insects typically produce quantities of vitellogenin to provision eggs with sufficient amino acids to support embryonic development. Generally, nourishment for egg production is obtained during the adult stage, but in some species it can be accumulated during the larval stage. In insects that acquire all protein for egg production during the larval stage, the larval protein supply must be partitioned between metamorphosis and egg development.

Egg development in mosquitoes has been a major area of investigation for the past 25 years since, as blood-feeders, mosquitoes are an important vector of human and animal disease world-wide. A blood meal is generally the stimulus for egg development. Studies of autogenous mosquitoes, those that can produce at least some eggs without blood feeding, have provided comparative data enhancing our understanding of the nutritional ecology (O'Meara, 1985, 1987) and endocrine regulation of oogenesis (Masler *et al.*, 1980; Kelly *et al.*, 1981) of mosquitoes as a group.

The accumulation of larval reserves and their carry-over into the adult stage is generally believed to play a key role in autogeny. Fat, glycogen and protein are stored in the fat body and carried into the adult stage to a greater degree in autogenous mosquitoes than they are in anautogenous ones (Spielman, 1971; Clements, 1994). The specific biochemical basis of the protein reserve in autogenous mosquitoes, and how they are managed prior to vitellogenesis, has never been explored.

Given the increasing evidence for roles of storage proteins in adult insects (Wheeler and Martinez, 1995), we examined *Aedes atropalpus*, an autogenous mosquito species, to determine if storage proteins could serve as an amino acid reserve for oogenesis, as well as for metamorphosis.

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MATERIALS AND METHODS

Animals

Eggs of *Aedes atropalpus*, Bass Rocks strain, were hatched, then reared in pans containing 200 ml of water, at a density of about 100 larvae/pan. They were fed liver powder and maintained at 26°C on a 16:8 light:dark cycle. The Bass Rocks strain is 100% autogenous when larvae are well-fed (O'Meara and Krasnick, 1970).

Electrophoresis

Whole mosquitoes or their parts were ground in microcentrifuge tubes with plastic pestles in 200 μl bleeding buffer. Bleeding buffer consisted of Tris-buffered saline containing protease inhibitors, as described in Martinez and Wheeler (1994), except that 1 mM 4-(2-aminoethyl)benzenesulfonyl (AEBSF) substituted for phenylmethylsulfonyl fluoride (PMSF).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following the general method of Laemmli (1970), adapted to 6–15% gradient slab gels. Non-denaturing gradient 4–20% gels were run also using the Laemmli buffer system, but omitting SDS and beta-mercaptoethanol from the sample and running buffers. To establish a pore-limited state, the gel was run for 2500 Vh. Staining procedures are given in Wheeler and Buck (1995).

Molecular weight determinations

Molecular weights of proteins in SDS-PAGE and pore-limited native PAGE were estimated using commercial standards as described in Wheeler and Buck (1995). Determinations were made using samples from female pupae. Values given are averages for seven lanes of samples.

Densitometry

Protein bands on gels were quantified using an LKB Laser Densitometer, as described previously (Martinez and Wheeler, 1991). An internal standard of 1.0 or 0.5 μg of bovine serum albumin (BSA) was applied to each gel used for quantitative analysis. A standard curve was prepared using BSA; over the range of 0.3–20 μg, peak areas were linear with respect to protein quantity. Different gels could then be calibrated by referencing the internal standard to the BSA standard curve. To obtain an estimate of relative storage protein levels in late larvae, callow pupae and newly emerged adults, three samples of each stage of similar weight were run on one gel with a BSA standard.

To estimate protein levels in adult females after eclosion, individuals were collected as they emerged and frozen at various times from 0–72 hours later. Those frozen later were supplied with sugar water.

Western blots

Proteins from *A. atropalpus* were tested for cross-reactivity to antibodies against *Manduca sexta* arylphorin (from J. H. Law, Dept. of Biochemistry, Univ. of Arizona) and *Aedes aegypti* vitellin (from H. H. Hagedorn, Dept. of Entomology, Univ. of Arizona). Protein bands from SDS-PAGE gels of *A. atropalpus* extracts were transferred electrophoretically to nitrocellulose. Membranes were incubated with one of the two antisera. The arylphorin antibody was used as a 1:500 dilution and the vitellin antibody as a 1:25,000 dilution. Goat anti-rabbit IgG-conjugated alkaline phosphatase was used as the color agent.

RESULTS

Storage protein(s)

The native protein(s) migrates in a single sharp band with a relative mobility indicating a molecular mass of about 492,000 Da (Fig. 1). On SDS gels, extracts of females show three major polypeptides with molecular masses of 72,500, 66,000 and 62,500 Da (rounded to the nearest 500). Males have only the larger two of the three types of subunits found in females. None of the components in females were recognized by antibodies to *M. sexta* arylphorin. It is not clear from this study if the native proteins exist as homo- or hetero-hexamers.

In both sexes, the polypeptides are abundant by the end of the larval stage, and reduced in amount during pupal development (Fig. 2, lanes 1–5). In males, virtually no storage protein remains in males at the time of eclosion (Fig. 2, lane 5), but a substantial amount remains in females. There is also a color difference between male and female pupae. The fat body of females during late larval development and during the entire pupal stage appears blue–green.

Female larvae approaching metamorphosis and callow pupae contained similar amounts of storage protein. Quantification of three samples each for larvae, pupae and adults from the same gel are shown in Fig. 3. Of the

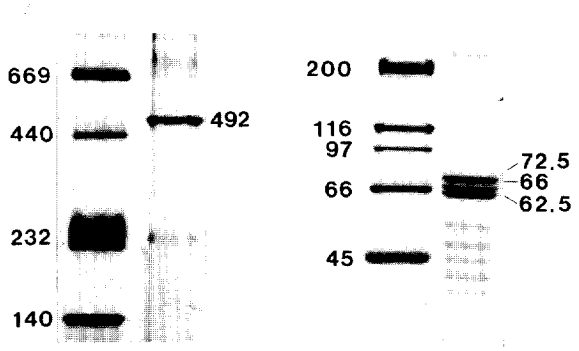

FIGURE 1. Storage proteins in female pupae of *A. atropalpus*, whole body extracts. Molecular masses of standards are given in kDa. (Right) SDS-gel electrophoresis reveals three subunits with different molecular weights. (Left) In non-denaturing, pore-limited electrophoresis, the storage protein appears as a single narrow band.

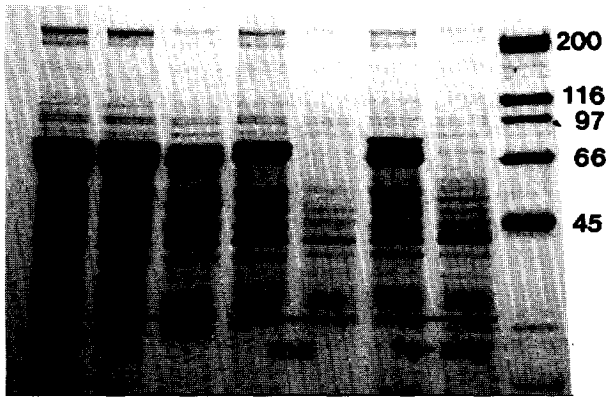

FIGURE 2. Storage proteins during development in *Aedes*. Lanes 1,2. Storage proteins accumulate to high levels in late larva and early pupal females (*A. atropalpus*). Lane 3. A substantial amount of storage protein still remains in newly emerged *A. atropalpus* females. Lanes 4,5. Male *A. atropalpus* also accumulate storage proteins by the beginning of the pupal stage, but virtually all is depleted by eclosion. Lanes 6,7. In *A. aegypti* females, virtually all of the storage proteins accumulated by the beginning of the pupal stage have been depleted at the time of eclosion to the adult. Lane 8. Protein standards. All mosquito samples represent 20% of the material extracted from one individual. ep = early (callow) pupa, fe = female, fe-aeg = female *A. aegypti*, la = late larva, ma = male, ne=newly emerged adult.

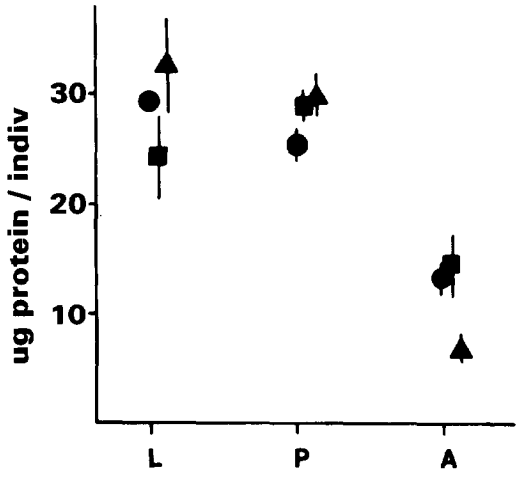

FIGURE 3. Depletion of the three types of storage protein monomers during development of females. Bars represent S.E. of the mean. Values were determined by quantitative scanning of SDS-polyacrylamide gels in which the three types of subunits were well-separated. Approximately 60% of the total store accumulated during the larval stage is used before adult eclosion. Circle—62.5 kDa, square—66 kDa, triangle—72.5 kDa.

total amount of storage protein present at the beginning of the pupal period, about 40% remained at eclosion. Only 23% of the original amount of the largest subunit (72.5 kDa) remained at eclosion, and about 50% of the two smaller storage protein subunits remained at the beginning of adulthood. In comparison, in females of the anautogenous mosquito *A. aegypti* females, the homologous proteins are depleted during pupal development, with virtually nothing remaining at the time of eclosion (Fig. 2, lanes 6 and 7).

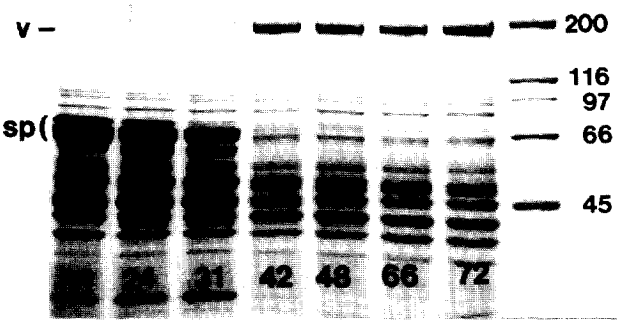

FIGURE 4. Storage protein depletion in females during the first 72h of adult life, illustrated with SDS-PAGE. Storage protein is depleted over the first 36h of adult life. Vitellogenin/vitellin is detectable by about 24h and is the dominant protein by about 48h. SP indicates the position of the storage protein monomers. V indicates the position of the large vitellogenin/vitellin subunit. The smaller unit is found in the same region as the storage proteins. Each lane represents 20% of the material extracted from an individual female.

In adult female *A. atropalpus*, storage proteins begin to disappear by 24h and are substantially gone by 36h (Figs 4 and 5). The largest subunit (72.5 kDa) was not detectable after 28h post-eclosion. Data are shown for the smaller two subunits (62.5 and 66 kDa) only to 32h. After that time, quantification of these proteins was confounded by the appearance of the small subunit of vitellogenin (66 kDa) (Dhadialla and Raikhel, 1990) (Figs 4 and 5). Vitellogenin, as quantified through the large subunit only, was found in some individuals by 24h. Vitellogenin/vitellin levels reached their maximum by 50h post-eclosion. The vitellogenin/vitellin band was recognized by antibodies to *A. aegypti* vitellin, and western blot confirmed that no vitellogenin was detectable at the time of eclosion (not shown). Dissection and analysis

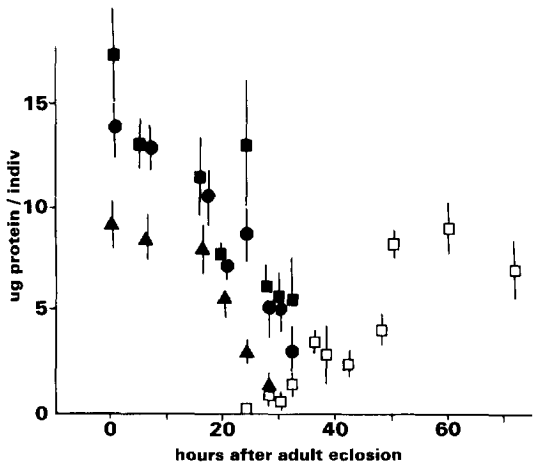

FIGURE 5. Quantification of storage protein depletion and the appearance of vitellogenin/vitellin. Bars represent S.E. of the means. Storage proteins cannot be quantified by scanning after about 30h because of the appearance of the small subunit of vitellogenin, which has a molecular mass of 66 kDa. Closed symbols—storage protein subunits: circle 62.5 kDa, square 66 kDa, triangle 72.5 kDa. Open square—vitellogenin/vitellin.

of females 50h post-eclosion showed that virtually all the egg protein was in the ovaries and none in the rest of the body (Fig. 6).

DISCUSSION

Mosquito storage proteins

The subunits of hexameric storage proteins in *Aedes atropalpus* include monomers of three different sizes. All three forms are found in females but only two in males. A recent survey of six other mosquito species in 2 subfamilies demonstrated the presence of two to three different storage proteins in larvae of each species. These proteins were recognized by antibodies to *Drosophila* and *Calliphora* storage proteins, confirming that they belong to the insect hexamerin family (Benes *et al.*, 1995; Korochkina *et al.*, in press).

Our data show that storage proteins decline sharply during the pupal period. Then, in the female, remaining storage protein begins to disappear immediately after eclosion and is substantially depleted by the middle of the second day (36h). After the first 24h, vitellogenin/vitellin levels begin to rise. These data are consistent with those of Van Handel (1976), who showed that the total protein content of *A. atropalpus* tissues remains constant for the first 3 days after eclosion, and that protein from the abdomen is transferred to the ovaries. These data are also consistent with the findings of Masler *et al.* (1980) who found that the growth of eggs increased dramatically after 48h. Although we noted that vitellogenin levels begin to rise earlier than 48h, uptake into developing oocytes should lag slightly behind synthesis.

In *Aedes aegypti*, the fat cells of newly emerged females contain protein granules, detectable by electron microscopy. The total volume of the granules begins to drop sharply immediately after eclosion and is virtually zero by 36h after emergence (Behan and Hagedorn, 1978; Raikhel and Lea, 1983). The electrophoretic data for *A. atropalpus* indicate a similar timetable of storage protein depletion, except that the amount remaining after eclosion is much greater. Therefore, regulation of breakdown may require no novel mechanisms in comparison to other mosquitoes; there is simply more residual protein to be degraded.

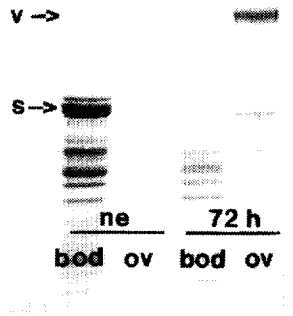

FIGURE 6. Transfer of protein from storage in the body to the ovaries. In newly emerged individuals (ne), virtually no protein is found in the undeveloped ovary (ov), and the dominant proteins are storage proteins. Seventy-two hours later, the dominant protein is found in the ovary, and the storage proteins are no longer found in the remainder of the body.

General relationships between storage proteins and egg production

In insects that do not feed at all as adults, or take only inconsequential amounts of protein after emergence, the protein accumulated during larval development must supply amino acids both for building the pupal and adult bodies and for providing eggs with yolk. In such insects, storage proteins can play a direct role in egg production by providing an amino acid source for vitellogenin synthesis. Wyatt (1991) has pointed out that temporal studies on the disappearance of storage proteins and the appearance of vitellogenin are particularly important as a basis for understanding the role of storage proteins in female reproduction.

Several possibilities exist for relationships between storage proteins and egg development in adult females. Males and females can accumulate the same types of hexamers but females accumulate more through enhanced synthesis as in *Bombyx mori* (SP2) (Tojo *et al.*, 1980) or through a longer larval period as in the gypsy moth (Karpells *et al.*, 1990). Females may also synthesize a different type of subunit than males. Female-specific subunits are reported from several species of Lepidoptera (Tojo *et al.*, 1980; Ryan *et al.* 1985, 1986; Webb and Riddiford, 1988; Bean and Silhacek, 1989).

In addition, storage proteins may serve directly as an egg protein and vice versa. This interchangeability underscores the fundamental role of both types of proteins as amino acid stores for building insect bodies—either an embryonic one or an adult one. For example, storage proteins are incorporated directly into developing eggs as yolk proteins in *Riptortus clavatus*. One of its hexameric storage proteins (CP-1) is synthesized by adult females and taken up directly by developing oocytes (Chinzei *et al.*, 1991, 1992). Pan (1971) noted that in *Hyalophora cecropia* some of the vitellogenin synthesized during the pupal stage disappears and suggested that it might be used as a source of protein for metamorphosing tissues. Vitellogenin may also have a role outside the egg in insect societies in which storage and reproductive functions have become dissociated and are carried out by different individuals. Martinez and Wheeler (1991, 1994) have described large accumulations of two storage proteins and vitellogenin in worker ants. Disappearance of all three proteins from nursing workers in the presence of larvae suggests that amino acids are transferred to larvae to support their growth.

Evolution of autogeny

A. atropalpus females contain a larger amount of the storage proteins found in both sexes, in part due simply to their larger size. In addition, females produce a female-specific storage protein. The *atropalpus* autogenetic strategy differs from that of Lepidoptera in the timing of the transfer of the amino acids from storage protein to vitellogenin. Lepidoptera accumulate essentially all of the protein used for egg production during the larval stage, but vitellogenin is synthesized in the larval or pupal stages (Ogawa and Tojo, 1981; Davis *et al.*, 1990; Lamison *et al.*, 1991). In mosquitoes, however, egg development is generally initiated by blood feeding in the adult stage. Mosquitoes appear to rely on evaluation of nutritional state after emergence to trigger egg development, even in many autogenous species (reviewed in Wheeler, 1996). In *A. atropalpus*, the initiation of vitellogenin synthesis after eclosion and the unusually large carry-over of storage protein from the pupal stage may reflect an evolution from obligately blood-feeding ancestors. If the trigger for vitellogenin synthesis could occur before eclosion, as it does in some autogenous mosquitoes (Watts and Smith, 1978; O'Meara and Lounibos, 1981), then the transfer of amino acids could begin earlier as well.

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Acknowledgements—Thanks to Tom Kelly and George Craig for supplying *Aedes atropalpus* eggs, and to TK for his detailed advice on rearing. H. Hagedorn supplied *Aedes aegypti* and vitellin antibody. The work was supported in part by NIAID 27403.