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QUARTERLY

Vitellogenesis in virgin and mated females of the mealworm beetle, *Tenebrio molitor**

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A comparison between vitellogenesis in virgin and mated females of *Tenebrio molitor* showed significant differences at each investigated developmental stage. Yolk protein deposition in oocytes, measured as an increase in their size parameters (length, width, and volume), proceeded much faster and was more efficient in mated females as compared to virgins. In fertilized females the gonadotropic cycle showed a cyclicity with an eight-day period while virgin females finish their vitellogenic stage after the first cycle. These differences were reflected in changes in the rate of protein synthesis in the fat body of females completing vitellogenesis or entering the next oogenetic cycle. In the haemolymph, in addition to a large (158 kDa) and two small (56 kDa and 45 kDa) subunits of vitellogenin, there was an abundance of proteins of 80 kDa and 60 kDa.

Vitellogenesis, a complex process of yolk protein synthesis, transport *via* the haemolymph, and deposition in growing oocytes, has been studied in several insect orders including Coleoptera (for review see [1, 2]). Generally, yolk proteins are synthesized in the female fat body as high molecular mass precursors — vitellogenins. Slight post-translational modifications, mainly lipo- and/or glycosylation, are processes typical of this family of proteins and do not influence significantly electrophoretic mobility of the final product, vitellins, when analysed in polyacrylamide gel [3, 4].

Insect species differ in the mode of endocrine regulation of oogenesis. In some species juvenile hormone (JH) is known to play a pivotal role [5, 6]. JH stimulates protein synthesis in the fat body, including vitellogenin synthesis [7] and its release into haemolymph [8]. Vitellogenesis was also shown to be influenced by ecdysone as in the case of *Aedes aegypti* [9] and *Drosophila melanogaster* [10]. The influence of both neurohormones and juvenile hormone in the fat body protein synthesis associated with ovarian maturation was observed in *Locusta migratoria*. In male locust an adipokinetic hormone (*Lom*AKH), and an extract of glandular lobes of *corpora cardiaca* (CC) inhibits the protein synthesis in the fat body *in vitro* [11]. In contrast, extracts of brain or storage lobes of CC

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Abbreviations: cc, corpora cardiaca; JH, juvenile hormone; *Lom*AKH, *Locusca* migratoria adipokinetic hormone; LTP, lipid transfer particle.

stimulate *in vitro* the fat body protein synthesis [12].

In *Tenebrio molitor*, neurosecretory factors act synergistically with *corpus allatum* in the regulation of oocyte development [13, 14]. It is known that mating stimulates both oocyte maturation and oviposition in young *Tenebrio molitor* females [15].

In this study we followed the yolk protein synthesis in the fat body, as well as changes in the levels of these proteins in the haemolymph of both virgin and mated females of *Tenebrio molitor*. The deposition of vitellins in their oocytes during adult development was also investigated.

MATERIALS AND METHODS

Experimental animals. The mealworm (*Tenebrio molitor*) were maintained in the laboratory and reared as described by Rosiński *et al.* [16]. The insects were collected as pupae and sexed according to [17]. Every day newly emerged adults were removed from the basic culture and placed in individual boxes.

Oocyte measurements. Oocytes taken from females of different age were placed in Ringer solution, pH 6.8, and measured under a microscope by means of a specially designed drawing device. It allowed precise measurement of both length and width of the oocytes, and calculating their volume in mm^3. Follicles were then categorized according to their length. Oocytes 0.01–1.3 mm long were designated "small", and those 1.31–3.00 mm long were classified as "big".

Haemolymph collection. The insects were anaesthetized for 5 min with carbon dioxide. Haemolymph (5–6 µl) was collected on ice from injured prothoracic limb and stored at –20°C.

In vivo assay for protein synthesis. Adult females (2–8 days after adult emergence) were injected with 2 µCi of [^{14}C]leucine (spec. act. 238 mCi/mmol, from Institute for Research and Application of Radioisotopes, Czech Republic). Six hours later the radioactivity incorporated into proteins of the fat body, haemolymph, and oocytes was determined. Proteins present in the haemolymph were precipitated with 10% trichloroacetic acid on filter paper according to [18]. Radioactivity was expressed as c.p.m./µl of haemolymph.

Assay for RNA and protein synthesis in vitro. Fat body was dissected from 2–20 day-old females, rinsed with Ringer solution, and incubated at 30°C in 200 µl of Grace medium in the presence of 5 µCi of [5,6-^{3}H]uridine (spec. act. 47 Ci/mmol, Amersham) for RNA labelling, or with 0.25 µCi of [^{14}C]leucine (spec. act. 25 Ci/mmol, Amersham) for protein labelling. Incubation time was 3 h for uridine incorporation into RNA, and 6 h for leucine incorporation into proteins.

RNA and protein extraction and measurements. Fat body or oocytes were either dissected from experimental females after *in vivo* labelling, or the fat body was labelled *in vitro* during 3 h incubation. The tissues were then homogenised in 0.3 M $HClO_4$ and the sediment thoroughly washed with 0.3 M $HClO_4$ at 4°C. RNA was extracted with 0.5 M $HClO_4$ for 20 min at 70°C. The residual pellet was washed several times with cold 0.3 M $HClO_4$ and treated with 1 M KOH at 70°C for 1 h [19]. Radioactivity was measured in aliquots of these extracts by scintillation counting in dioxane.

Protein content was determined according to [20], using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis. This was carried out in 7.5% or 10% polyacrylamide gel according to Laemmli [21]. After electrophoresis, proteins were stained using Coomassie Brilliant Blue R-250. Mobility of the protein was compared to that of a set of globular proteins relative molecular mass markers (Sigma Chemical Co.).

RESULTS

Females of *Tenebrio molitor* initiate vitellogenesis about 2 days after adult emergence. The changes in length, width and volume of oocytes in virgin and mated females are presented in Fig. 1A and B, respectively. The volume of oocytes was the greatest on day 7 after adult emergence in mated and on day 9 in virgin females. It should be pointed out that in unfertilized females the oocytes present in the oviduct were twice as large as those of mated females. The differences in the number of large oocytes on consecutive days of adult development (Fig. 2) clearly illustrate that mated beet-

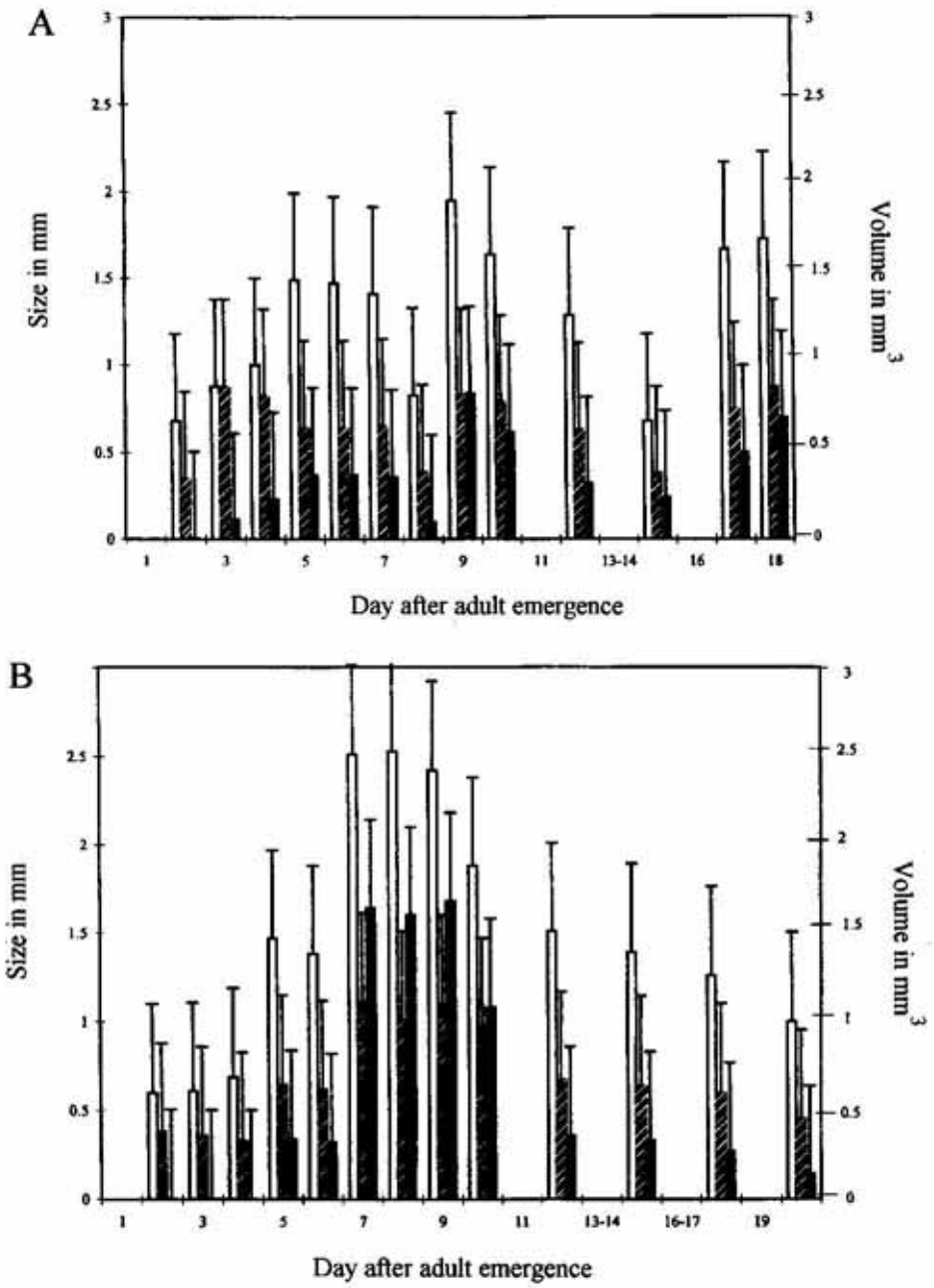

Fig. 1. Developmental changes in size of oocytes of virgin (A) and mated (B) females of Tenebrio molitor. Means (±SD) were based on measurements of oocytes from 2–10 females. White bars, oocyte length; hatched bars, oocyte width; black bars represent oocyte volume.

les started their second gonadotropic cycle on day 8, whereas virgin females maintained their large oocytes until day 18 with practically no changes in their size.

Now, the question arises whether the yield of vitellogenin deposition in the oocytes of virgin and mated females at different stages of adult development can be measured. The intensity of synthesis of proteins which are later deposited as yolk proteins in oocytes is shown in Fig. 3. On the 2nd day the rate of protein synthesis measured by [^{3}H]leucine incorporation per mg of oocyte protein during 6 h incubation *in vivo* showed no statistically significant differences between the two groups of investigated females. On day 6, and especially on day 8 when the intensity of oocyte growth differed depending on mating, there were also differences in the level of newly synthesized yolk proteins (compare Fig. 1 and 2). In the oocytes of mated females the yolk protein synthesis was about twice as high as in virgins.

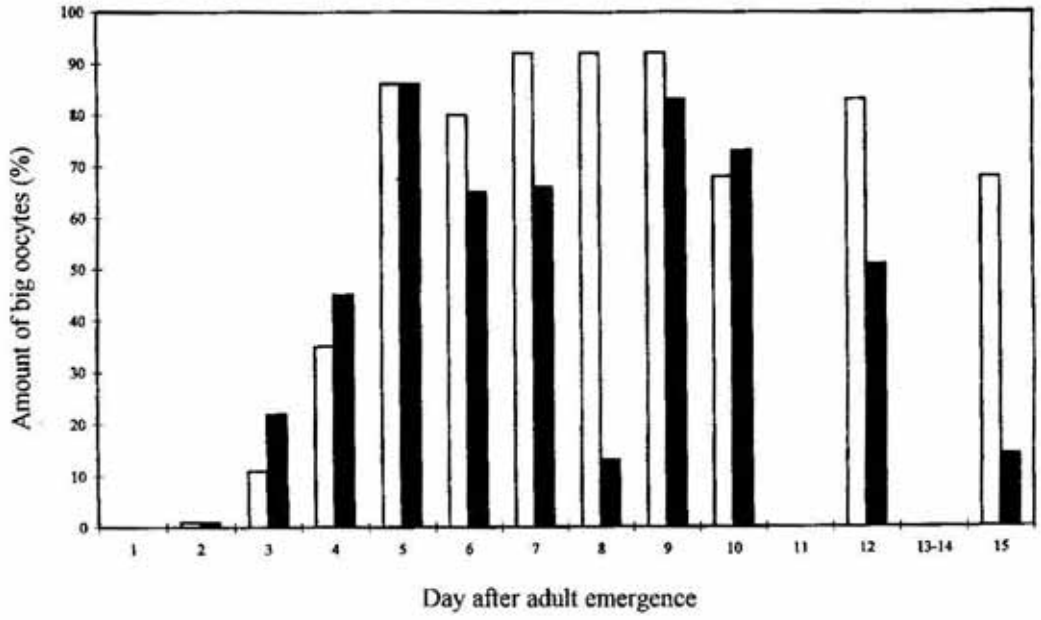

Fig. 2. Content of big oocytes in virgin (white bars) and mated (black bars) females of Tenebrio molitor during consecutive days of adult development.

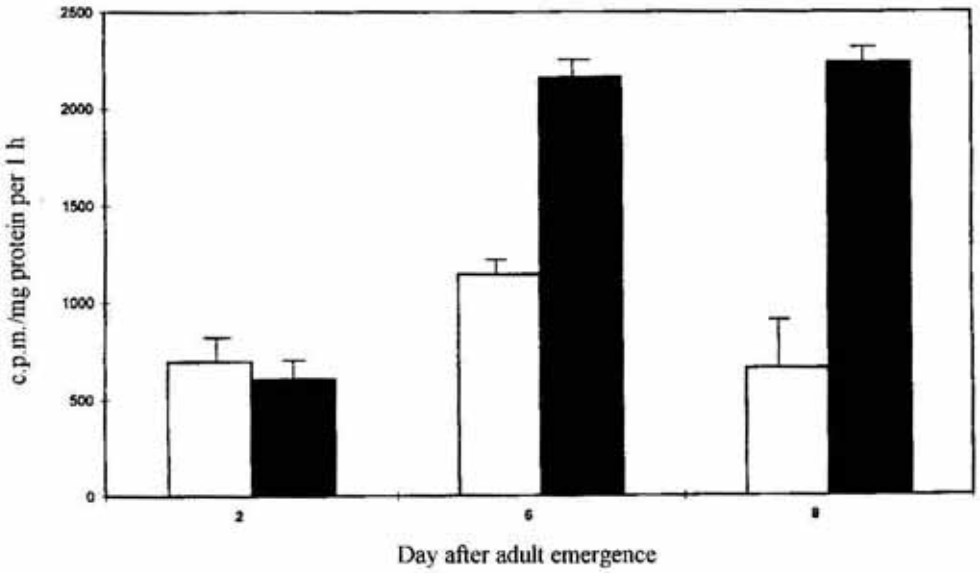

Fig. 3. Deposition of newly syntesized proteins measured as the rate of protein synthesis in oocytes of virgin (white bars) and mated (black bars) females during the first gonadotropic cycle.

It can be expected that differences in yolk protein deposition in growing oocytes of virgin and fertilized females should be reflected in changes in the intensity of both RNA and protein synthesis in the fat body of the two groups of the insects studied. The rate of RNA synthesis, measured as labelled uridine incorporation into total RNA during a 3-h incubation *in vitro*, showed in both groups similar pattern except for the differences seen on days 13–14 (Fig. 4). In virgin females the rate of RNA synthesis slowed down during adult development, whereas in mated females a slight increase in RNA synthesis, corresponding to the second gonadotropic cycle, can be seen. A more distinct difference in the rate of protein synthesis in the fat body of mated and virgin females was obtained during a 6-h incubation period using [^{14}C]leucine *in vitro* (Fig. 5). On day 9 and 15 the fat body of mated females synthesized protein with an intensity equal to the rate of synthesis observed during the first gonadotropic cycle. In contrast, the intensity of protein synthesis in the fat body of virgin females diminished and remained very low even on day 20. A comparison between protein syn-

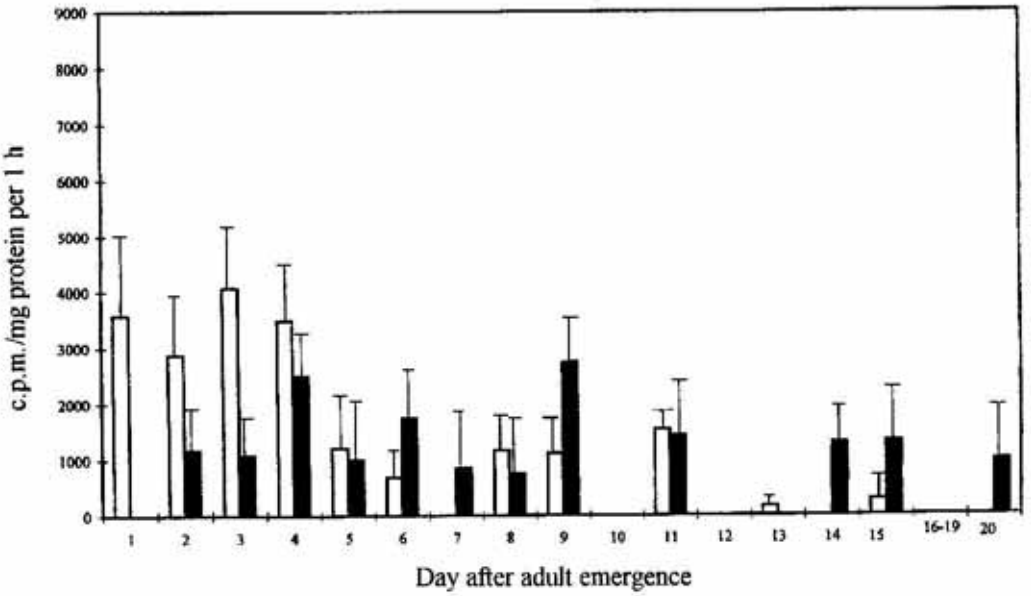

Fig. 4. The rate of RNA synthesis in the fat body of Tenebrio molitor virgin (white bars) and mated (black bars) females measured as [^{3}H]uridine incorporation during a 3-h incubation in vitro.
The values are means (±SD) of 4–25 and 5–41 measurements for virgin and mated females, respectively. Incorporation of the isotope was expressed as c.p.m./mg of protein present in the tissue incubated in 100 µl of medium.

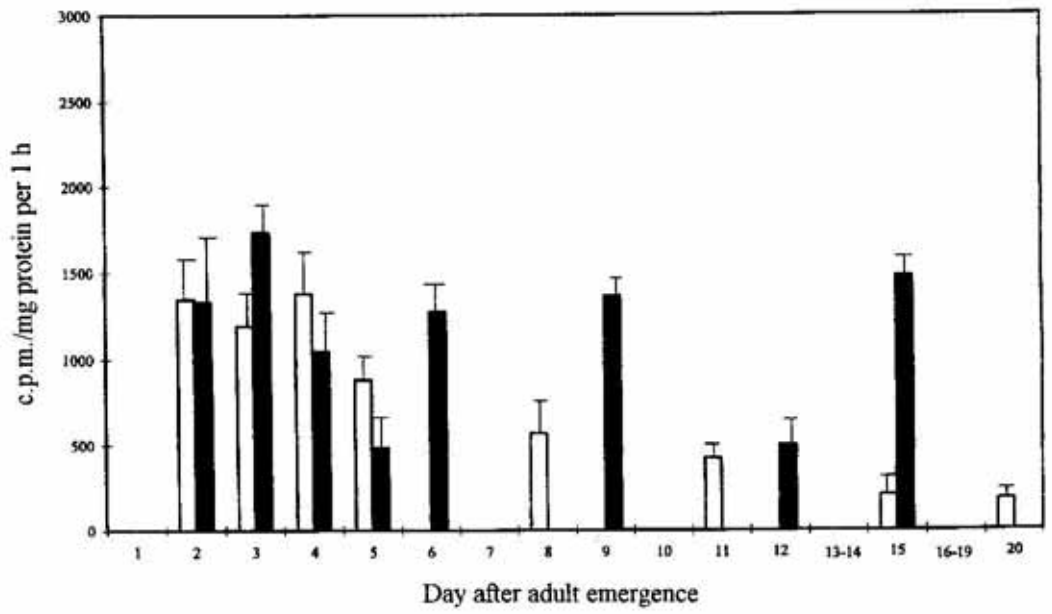

Fig. 5. The rate of protein synthesis in the fat body of virgin (white bars) and mated (black bars) females measured as [^{14}C]leucine incorporation during a 6-h incubation in vitro.

thesis in the fat body of mated and virgin females, expressed as the rate of labelled leucine incorporation into total protein during a 6-h incubation *in vivo*, is presented in Fig. 6. In both groups the total protein synthesis reached a maximum on day 6, and was more than three times as high as the synthesis observed on day 2, when the oogenesis started.

Figure 7 illustrates the protein pattern of the oocytes and haemolymph taken from adult males and females of *Tenebrio molitor*. The presence of one large (158 kDa) and two small (56 kDa and 45 kDa) vitellogenin subunits can be seen only in female haemolymph on consecutive days of adult development. These proteins are present neither in the haemolymph of

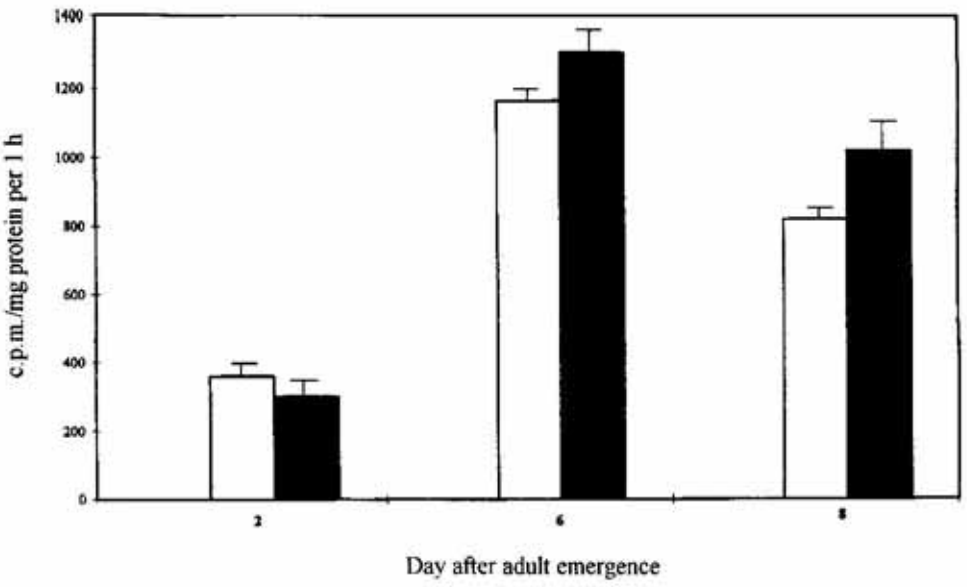

Fig. 6. The rate of protein synthesis in the fat body of virgin (white bars) and mated (black bars) females of Tenebrio molitor after injection of 0.25 μCi of [^{14}C]leucine.
Radioactivity incorporated into protein was measured 6 h after the injection. The values are means (±SD) from 4–21 measurements for virgin and 5–11 measurements for mated females.

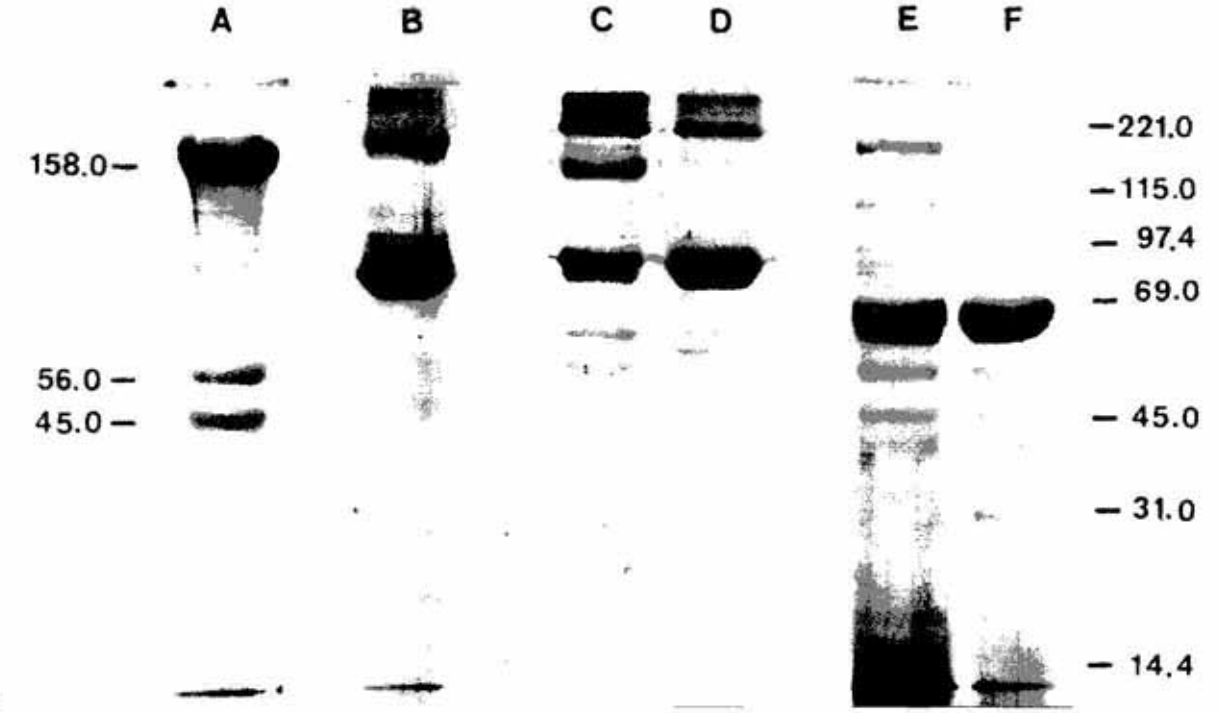

Fig. 7. SDS/PAGE analysis of oocytes and haemolymph of females during adult development.
A, Oocyte proteins. Haemolymph proteins: B, 2-day-old female; C, 3-day-old female; E and F, respectively, 5 and 7-day-old females. Molecular mass is given in kdaltons. The haemolymph pattern from 3-day-old males (D) is included for comparison.

3-day-old males, nor at any stage of their development (not shown). However, proteins of about 300 kDa, 80 kDa and 60 kDa are present in the haemolymph of both females and males. Their mass was evaluated by comparison with standards. The appearance of these proteins, their presence in total haemolymph proteins, and the level of large subunits change considerably during adult development. The latter, expressed as percentage of total haemolymph proteins, are presented in Fig. 8A and B. As can be seen, the level of the 158 kDa protein subunits in virgin females did not change substantially over days 2–10 after adult emergence and fluctuated around 20% of total haemolymph protein (Fig. 8A). However, in mated females the level of the vitellogenin large subunit dropped on days 5 and 7, i.e. at the end of the first gonadotropic cycle, and then remained rather low. High concentration of the 80 kDa protein could be observed at the beginning of adult development in both mated and virgin

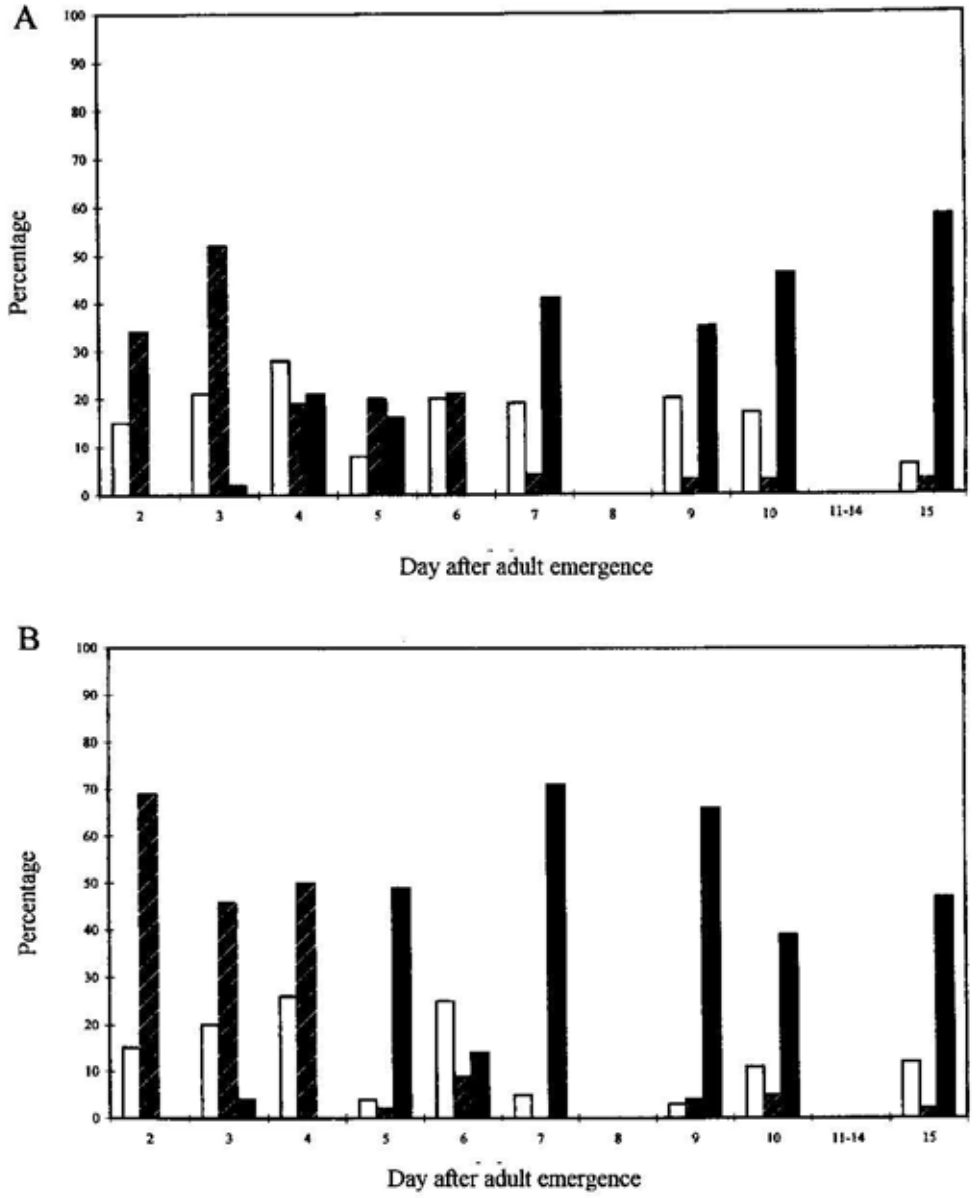

Fig. 8. Densitometric analysis of major haemolymph proteins in adult virgin (A) and mated (B) females of Tenebrio molitor.

The units are arbitrary and represent peak areas from 4–5 electrophoreses performed in 10% SDS/polyacrylamide gel. White bars, large vitellogenin subunit of 158 kDa; hatched bars, 80 kDa protein; black bars, 60 kDa protein.

females. This protein was abundant during 4 days in mated, and up to 6 days in virgin females. A protein of 60 kDa appeared in adult females on day 3 and its concentration progressively increased, reaching 60–70% of the total haemolymph protein. Neither the 80 kDa nor 60 kDa protein is similar to vitellogenins. They are not recognised by antibodies against *Tenebrio molitor* vitellogenins (not shown), so they must be involved in other metabolic pathways.

DISCUSSION

Hormonal regulation of vitellogenesis and the appearance of characteristic protein molecules in insect fat body and haemolymph have been described in many insect species [2, 22]. Some observations have been also made on hormonal requirements for the proper time course of these processes in *Tenebrio molitor* [14, 23]. In

this study, special attention has been focused on the differences between three stages of the complex process of vitellogenesis, which depend on mating. These steps include 1) RNA and protein synthesis in the fat body, 2) the appearance of special age-dependent classes of haemolymph proteins in females, and 3) vitellin sequestration in the oocytes. Oocyte growth in females of *Tenebrio molitor* measured as differences in their size parameters (length, width and volume) points to the importance of mating factor in the efficiency of yolk protein deposition (see Fig. 1). In mated females, the volume of oocytes increased up to the 7th day after adult emergence and the cycle is repeated every 8 days. Unlike in virgins, large oocytes are absent from the oviducts of fertilized females; this is caused by the initiation of egg deposition. In virgin females the oocytes grow at a slower rate, they reach their maximum size on day 9 (two days later than in mated ones). After laying a few eggs the virgins do not repeat their gonadotropic cycle and the oocytes remain small. Apparently, in agreement with previous findings [15], mating is not required for the initiation of vitellogenesis, but enhances the rate of oocyte maturation and egg laying. In mated females, large oocytes are released from oviduct on day 8, i.e. during the egg laying period, when the first gonadotropic cycle is completed. In contrast, in virgin females no increase in oocyte size can be observed after the 9th day of adult development.

The rate of yolk protein synthesis during a 6-h incubation *in vivo* with labelled leucine, and the appearance of labelling in the oocytes show that at the beginning of vitellogenesis (on day 2) both synthesis and deposition proceed in virgin and mated females with a similar intensity (see Fig. 3). The differences between these processes become apparent in the second part of the oogenic cycle, especially on days 6 and 8. In mated females the rate of deposition of the newly synthesized yolk proteins in their oocytes is twice as intense as in virgin females.

These differences are not very much affected by changes in the rate of RNA synthesis in the fat body measured by the *in vitro* incorporation of labelled uridine into total RNA during a 3-h period (see Fig. 4). An increase in RNA synthesis in mated females coincides with their entering the second oogenic cycle. Similar changes in protein synthesis in the fat body of virgin and mated females measured as leucine incorporation during a 6-h incubation *in vivo* were also observed. During the first gonadotropic cycle the rate of protein synthesis in both groups of animals, between day 2 and days 6–8, was most intense. During the second oogenic cycle we observed significant differences between protein synthesis in the fat body of virgin and mated females.

Interestingly enough, there was a relatively low percentage of the large vitellogenin subunit compared to three other haemolymph proteins of about 300 kDa, 80 kDa and 60 kDa. We believe that these proteins might be lipophorins, well known as carriers involved in transport of diet-derived hydrophobic nutrients such as sterols, phospholipids, or glycerolipids *via* the haemolymph to the fat body and other tissues. This group of carrier proteins can be observed at larval, prepupal, pupal, and adult life stages in *Manduca sexta* [24]. In addition to haemolymph lipid transport, lipophorins are involved in uptake of lipids by developing oocytes. Lipid content, and composition of lipoproteins vary from species to species and reflect the role of lipophorins in a particular species (for review see [25]). Three lipid transfer molecules, considerably different in the lipid part of the molecule, which were found in *Manduca sexta* [26], *Periplaneta americana* [27] and *Locusta migratoria* [28], did not differ much in size of the apoprotein part of the molecule. ApoLTP-I of 310–320 kDa, ApoLTP-II of 85–94 kDa, and ApoLTP-III of 55–68 kDa found in these insect species do not differ very much from the abundant haemolymph proteins found in this study in *Tenebrio molitor*. We estimate the molecular mass of these proteins to be about 300 kDa, 80 kDa, and 60 kDa.

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