Production and Extraovarian Processing of Vitellogenin in Ovariectomized *Blattella germanica* (L.) (Dictyoptera, Blattellidae)

DAVID MARTÍN,* MARIA-DOLORS PIULACHS,* XAVIER BELLÉS*†

The fat body produces vitellogenic proteins which are released to the haemolymph and incorporated into developing oocytes, where they are processed to vitellins. Endocrine and other physiological cues regulate vitellogenesis in a complex homeostatic pattern, in which the ovary may have a pivotal role. The present paper describes the effects of ovariectomy on vitellogenin production in the cockroach *Blattella germanica*. The results show that the absence of ovaries leads to a rising and saturable accumulation of vitellogenic proteins, both in the haemolymph and in the fat body, whereas in intact females the production of these proteins is cyclic. This suggests that the ovary is involved in terminating vitellogenesis in intact females. We also report that vitellogenin is processed in the haemolymph of ovariectomized females in a similar manner to that of vitellin in the ovary of intact specimens

Fat body   Vitellogenin   Vitellin   Juvenile hormone   *Blattella germanica*   Cockroaches

INTRODUCTION

The fat body is a key organ in insect vitellogenesis. It produces vitellogenic proteins which are released to the haemolymph and incorporated into developing oocytes, where they are processed to vitellins (Engelmann, 1979; Keeley, 1985; Kunkel and Nordin, 1985). In turn, endocrines and other physiological cues regulate vitellogenesis in a quite complex homeostatic pattern, in which the ovary may have a pivotal role in feedback regulatory loops (Engelmann, 1983; Valle, 1993).

Surgical ovariectomy has been used as an experimental approach to clarify the exact role of the ovary in this context. Among insect orders, cockroaches have often been chosen as models, because they exhibit discrete vitellogenic cycles, which suggests the mediation of fine-tuned mechanisms of regulation, susceptible to conceptual dissection and experimental study. In respect of endocrine cues, experiments of ovariectomy in cockroaches have been conducted to study the effects on juvenile hormone (JH) (Maestro *et al.*, 1994 and references therein) and on ecdysteroids (Romañá *et al.*, 1995

and references therein). Similar experiments have also been used to study the effects on the synthesis of vitellogenic proteins, for example in *Periplaneta americana* (Bell, 1969), *Leucophaea maderae* (Engelmann, 1978), *Nauphoeta cinerea* (Lanzrein *et al.*, 1981) or *Blattella germanica* (Kunkel, 1981).

B. germanica is an ovoviviparous cockroach in which oocyte maturation proceeds in cycles separated by periods of ootheca transport, during which the development of the new batch of basal oocytes remains arrested. Therefore, it offers a good model for such studies. In this species, we have recently reported the effects of ovariectomy on JH and ecdysteroid production (Maestro *et al.*, 1994; Romañá *et al.*, 1995, respectively). In addition, we have designed an immunoenzymatic assay to quantify vitellogenic proteins, and described vitellogenin and vitellin patterns throughout the first gonadotropic cycle of this species (Martín *et al.*, 1995).

The present paper deals with the effects of ovariectomy on the production of vitellogenic proteins, again in *B. germanica*. The results show that the absence of ovaries leads to a rising and saturable accumulation of vitellogenic proteins, not only in the haemolymph but also in the fat body. In addition, we report, for the first time, that vitellogenin is processed in the haemolymph of ovariectomized females in a similar manner to vitellin in the ovary of intact specimens.

*Department of Agrobiology (C.I.D., C.S.I.C.), Jordi Girona 18, 08034 Barcelona, Spain.

†To whom all correspondence should be addressed.

MATERIAL AND METHODS

Insects

Virgin females of *B. germanica* of different ages were used in all experiments. They were obtained from a colony fed on Panlab dog chow and water, and reared in the dark at 30 ± 1°C and 60–70% r.h.

Ovariectomy

Ovariectomy was performed in the last larval instar as described elsewhere (Maestro *et al.*, 1994). Females which underwent a normal imaginal moult (*ca* 90% of the specimens operated) were used in the experiments. Absence of ovaries was assessed by dissection before collecting the samples for vitellogenic protein studies.

Preparation of samples for protein quantification and ELISA

Haemolymph and abdominal fat body samples were obtained as previously described (Martín *et al.*, 1995). The haemolymph was diluted in NaCl 0.4 M (1 mM PMSF) and stored at −20°C. Fat body tissue was homogenized in NaCl 0.4 M (1 mM PMSF) and the cellular debris was pelleted by centrifugation (20,000 *g*, 20 min). The layer just above the pellet was collected and stored at −20°C. Fat body, and haemolymph soluble proteins were quantified according to Bradford (1976), by using bovine serum albumin as standard. Levels of vitellogenic proteins were determined by ELISA (see below).

ELISA quantification of vitellogenic proteins

The procedure and materials were as described by Martín *et al.* (1995). Vitellogenic proteins from haemolymph and fat body samples were dissolved in carbonate buffer (0.05 M, pH 9.6), and the resulting solutions (100 μl) were absorbed to wells of 96-well ELISA microplates (NUNC-Immuno Plate Maxisorp 96F, Roskilde, Denmark) by incubation at 4°C overnight. The ELISA was conducted using secondary peroxidase labelling revealed with 3,3′,5,5′-tetramethylbenzidine (Sigma, Madrid, Spain), and the antiserum against vitellogenin–vitellin previously reported (Martín *et al.*, 1995). Absorbance was read at 450 nm with a Titertek Multiscan Plus MKII spectrophotometer (Labsystems, Helsinki, Finland). Haemolymph and fat body vitellogenic proteins were expressed as $\mu g^{-1}/\mu l^{-1}$ of haemolymph and $ng^{-1}/\mu g^{-1}$ of fat body proteins respectively.

Electrophoretic separations and Western blotting procedures

SDS–PAGE electrophoresis of vitellogenin and vitellin was carried out using the method of Laemmli (1970) with 7.5% polyacrylamide slab gels (see Martín *et al.*, 1995 for details). Western blotting was also performed as previously described (Martín *et al.*, 1995) following the method of Towbin *et al.* (1979). Vitellogenin and vitellin separated by SDS–PAGE electrophoresis were transferred to a nitrocellulose membrane, proteins were

visualized by Ponceau S stain, and standard proteins were located and marked. Then the filter was blocked with 5% non-fat dried milk, and incubated with the antiserum against vitellogenin–vitellin (1:8,000). The filter was washed and incubated with secondary goat anti-rabbit antibody conjugated to peroxidase (Sigma, Madrid, Spain) at a dilution of 1:4,000. After additional washing, the remaining bound secondary antibody was visualized by incubating the filter with 4-chloro-1-naphthol (Sigma, Madrid, Spain).

RESULTS

Vitellogenic proteins and total protein contents in the haemolymph

Measurements of vitellogenic proteins and total protein contents in the haemolymph from ovariectomized females were compared to those previously observed in intact females.

Until day 2, levels of vitellogenic proteins were similar in both cases (Fig. 1). From day 2 onwards, however, levels of vitellogenic proteins in ovariectomized specimens showed a steady increase, to reach a concentration 11-fold (around 90 μg μl⁻¹) that of intact females (8 μg μl⁻¹) on day 6 (Fig. 1). These high levels were maintained on days 7, 8 and 12, and did not show the cyclic pattern observed in intact females (Fig. 1).

Similar results were obtained when measuring haemolymph protein contents (Fig. 1 inset). Both ovariectomized and intact females showed similar levels until day 2, but thereafter those corresponding to ovariectomized specimens steadily increased until day 6, when they remained stable. Circulating vitellogenic proteins in ovariectomized females of the later age account for about 80% of total proteins in the haemolymph, which is cle-
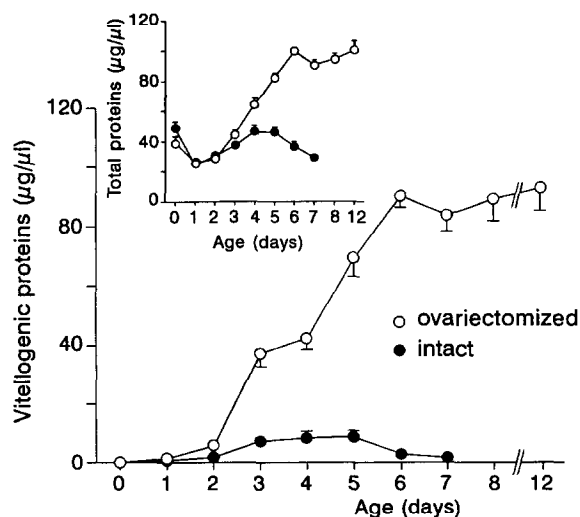

FIGURE 1. Concentration of vitellogenic proteins in the haemolymph of ovariectomized females of *B. germanica* during the first days of adult life. Values are expressed as the mean ± SEM (*n* = 9–14). The inset shows the dynamics of total protein contents along the same period. The corresponding values for intact females, according to Martín *et al.* (1995), are also shown.

arly higher than that calculated in intact females, where vitellogenic proteins represent only 20% of total proteins.

Vitellogenic proteins and total protein contents in the fat body

Fluctuations in vitellogenic proteins in the fat body, both in intact and in ovariectomized females (Fig. 2), showed a similar pattern to that observed in the haemolymph. From day 2, vitellogenic proteins increased steadily to reach a plateau on day 7 in ovariectomized specimens, whereas they maintained lower values and showed a cyclic profile in intact females. Since vitellogenic proteins are expressed in relative terms, i.e. with respect to total proteins, it is easily seen that when considering maximal values (approx. 500 ng μg^{-1}, days 6, 7, 8 and 12) vitellogenic proteins account for 50% of total soluble proteins. This percentage is much higher than that measured in intact females (approx. 10%).

Electrophoretic pattern of haemolymph vitellogenic proteins

SDS–PAGE electrophoresis had revealed that the haemolymph vitellogenin of intact females is composed of two subunits with molecular weights of 160 and 102 kDa (Wojchowski et al., 1986; Martín et al., 1995). When incorporated into the developing oocyte, vitellogenin is transformed into vitellin, for which the 160 kDa subunit is processed to yield three subunits of 95, 60 and 50 kDa. Considering the 60 kDa subunit as a transient form of the 50 kDa one (Purcell et al., 1988), mature vitellin comprises three subunits of 102, 95 and 50 kDa (Wojchowski et al., 1986; Martín et al., 1995).

In ovariectomized females, the two subunits of vitellogenin (160 and 102 kDa) appear in the haemolymph on day 2 (Fig. 3). From day 3, however, 3 additional bands with mol.wt of 95, 60 and 50 kDa appeared, which suggests that vitellogenin is processed in the haemolymph

FIGURE 3. 7.5% SDS–PAGE electrophoresis of haemolymph from ovariectomized *B. germanica* of different ages. The same amount of haemolymph proteins (5 μg) was loaded in each lane. Molecular weight markers are indicated to the left, and subunits of vitellogenin and vitellin are indicated to the right.

in a similar manner to that of vitellin in the ovary of intact females. The vitellogenic nature of these bands was assessed by Western blotting, by using a polyclonal antibody against vitellogenin–vitellin previously reported (Martín et al., 1995). Results (Fig. 4) revealed that the bands with mol.wt of 160, 102, 95, 60 and 50 kDa immunoreact with the antiserum, the immunoblot analysis [Fig. 4(B)] giving a pattern identical to that of ovarian vitellin as reported by Martín et al. (1995). Referring to
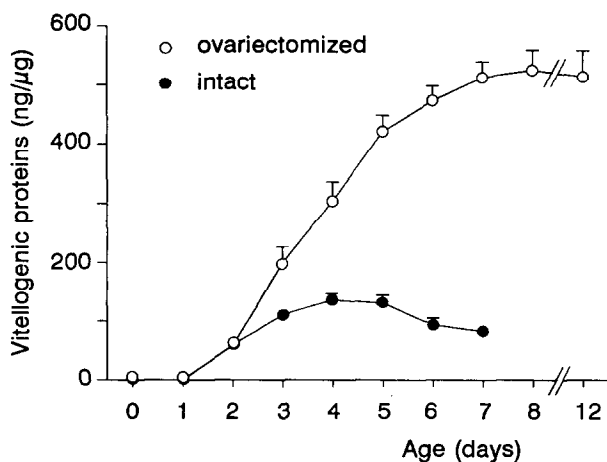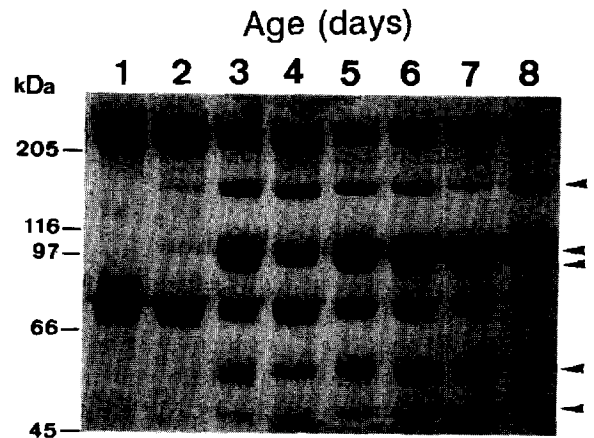

FIGURE 2. Concentration of vitellogenic proteins in the fat body of ovariectomized females of *B. germanica* during the first days of adult life. Values are expressed as the mean ± SEM (n = 9–12). The corresponding values for intact females, according to Martín et al. (1995), are also shown.
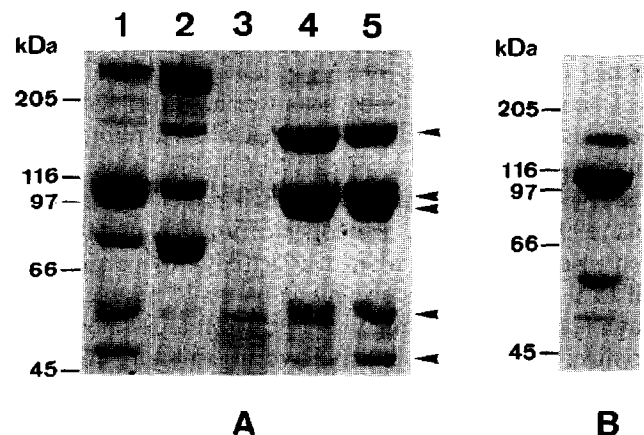

FIGURE 4. Immunodetection on Western blots of vitellogenin and vitellin in the haemolymph from ovariectomized females of *B. germanica*. (A) 7.5% SDS–PAGE electrophoresis of: haemolymph from 5-day-old ovariectomized females (lane 1); haemolymph from 5-day-old intact females (lane 2); ovarian soluble proteins from 2-day-old females (lane 3); ovarian soluble proteins from 4-day-old females (lane 4); and ovarian soluble proteins from 5-day-old females (lane 5). The same amount of proteins (5 μg) was loaded in each lane. The arrows to the right indicate the subunits of vitellogenin and vitellin. (B) Immunoblot analysis with antiserum against vitellogenin–vitellin (haemolymph from 5-day-old ovariectomized females). The antiserum reveals five bands (160, 102, 95, 60 and 50 kDa) corresponding to the subunits of vitellogenin–vitellin. Note that 102 and 95 kDa bands are practically fused, and that mobility of the bands in (B) was different with respect to (A) (see molecular weight markers indicated to the left of each figure).

Fig. 3, it is worth noting that the 102, 95 and 60 kDa bands progressively increase in intensity from day 3. This suggests that the processing of vitellogenin in the haemolymph starts on day 3 (just when the abnormal accumulation begins) and proceeds steadily.

DISCUSSION

Ovariectomy in B. germanica results in an increase in vitellogenic protein levels, not only in the haemolymph but also in the fat body itself. Qualitatively similar results have been reported for circulating vitellogenin in ovariectomized specimens of this species by Kunkel (1981), as well as in other cockroaches, like P. americana (Bell, 1969), L. maderae (Engelmann, 1978) and N. cinerea (Lanzrein et al., 1981).

Ovariectomy leads to an increase in total protein contents in the haemolymph, but there is a differential effect in vitellogenic proteins. In ovariectomized specimens, from day 3, vitellogenic proteins account for 70–80% of total soluble proteins, whereas in intact females the percentage of vitellogenic proteins remains low throughout the gonadotropic cycle, representing between 18 and 19% of total haemolymph proteins on the days of maximal concentration (days 3–5) (see also Martín et al., 1995). Similar figures have been reported in other cockroaches after ovariectomy, as in L. maderae, where vitellogenin represents about 70% of haemolymph proteins, whereas in intact females it only accounts for 5–6% (Engelmann, 1978), or in N. cinerea, where concentration of vitellogenin in the haemolymph of ovariectomized specimens is up to 4-fold that in intact females (Lanzrein et al., 1981).

Concerning the fat body, it is worth noting that intact females do not accumulate vitellogenin. Therefore, fluctuations of vitellogenic proteins in the fat body reflect the dynamics of production of such proteins (Martín et al., 1995). However, vitellogenic proteins in fat body increase dramatically after ovariectomy, to approx. 50% of total proteins. This accumulation could be due to the high levels of circulating vitellogenic proteins in ovariectomized females, which may have an inhibitory effect on their release and synthesis.

Vitellin contents in the ovary during the first gonadotropic cycle have been reported by Martín et al. (1995). They are undetectable in freshly emerged and 1-day-old females, but increase thereafter from approx. 50 μg ovary pair on day 2 to approx. 4 mg on day 6 (150 μg on day 3, 700 μg on day 4 and 2.8 mg on day 5). Therefore, the total amount of vitellogenic proteins produced during the first 6 days of imaginal life is roughly the same (i.e. some 4 mg) in intact females (Martín et al., 1995) and in ovariectomized specimens (present results). However, whereas in intact females the first significant increase in production was observed between days 3 and 4 (approx. 650 μg produced during this interval: Martín et al., 1995), in ovariectomized specimens this occurs between days 2 and 3 (approx. 800 μg produced during

this interval: present results). In addition, the production is clearly cyclic in intact females, with a peak between days 4 and 5 (Martín et al., 1995), whereas in ovariectomized specimens it increases steadily until day 6 and then stabilizes (present results).

Our previous studies have shown that virgin ovariectomized specimens of B. germanica produce low levels of JH during the first 9 days of imaginal life (Maestro et al., 1994). In spite of this, the present results indicate that ovariectomized females steadily increase the production of vitellogenic proteins during these days. This suggests that rising rates of JH synthesis observed during the gonadotropic cycle of intact females (Bellés et al., 1987; Gadot et al., 1989; Maestro et al., 1994) are not necessary to modulate the parallel increase in the production of vitellogenic proteins.

On the other hand, the production of vitellogenic proteins in intact females begins to decrease from days 4 to 6 (Martín et al., 1995), whereas the rates of JH synthesis by the corpora allata are still rising in this period, and do not decrease until day 7, when the ootheca is being formed (Bellés et al., 1987; Maestro et al., 1994). Moreover, JH contents in the haemolymph increase from days 3 to 5 and remain high until day 7 (Camps et al., 1987). These data had suggested (Martín et al., 1995) that a factor or mechanism other than the decrease in JH may be involved in the termination of vitellogenesis in the fat body. Termination of vitellogenesis may be simply due to nutritional exhaustion that could occur towards the end of oocyte maturation. However, the results reported herein indicate that ovariectomy leads to a rising and saturable accumulation of vitellogenic proteins, both in the haemolymph and in the fat body itself. This rather suggests the occurrence of a specific factor involved in terminating vitellogenesis in intact females and points to the ovary as a possible site in which to search such a factor.

Our results show, for the first time, that vitellogenin is processed in the haemolymph of ovariectomized females in a similar way to that of vitellin in the ovary of intact specimens. The occurrence of large amounts of vitellogenic proteins accumulated in the haemolymph of ovariectomized specimens has certainly facilitated the detection of extraovarian processing. Thus, the possibility that this phenomenon also occurs in intact females cannot be excluded, although it would be more difficult to observe due to the fast turnover of these proteins in the haemolymph. In any case, however, extraovarian processing of vitellogenic proteins suggests that enzymes involved in the transformation of vitellogenin into vitellin are present in the haemolymph and that could be produced and released by the fat body. Then, they may be incorporated into the growing oocytes in intact females, simultaneously to vitellogenin.

This hypothesis recalls the phenomenon reported in the mosquito Aedes aegypti, were Raikhel and associates have recently described the occurrence of a proenzyme, named vitellogenic carboxypeptidase (VCP), which is

released by the fat body, and then incorporated into the oocyte (Hays and Raikhel, 1990). The proenzyme is accumulated within the oocyte until its activation and use in the hydrolysis of yolk proteins during embryogenesis (Cho *et al.*, 1991). Production of VCP is ecdysteroid-dependent (Deitsch and Raikhel, 1993), and the corresponding cDNA and gene have recently been cloned and sequenced (Cho *et al.*, 1991; Deitsch and Raikhel, 1993, respectively).

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