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Quantity does matter. Juvenile hormone and the onset of vitellogenesis in the German cockroach

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Abstract

We aimed to elucidate why cockroaches do not produce vitellogenin in immature stages, by studying the appearance of vitellogenin mRNA in larvae of *Blattella germanica*. Treatment of female larvae in any of the last three instars with 1 μg of juvenile hormone (JH) III induces vitellogenin gene transcription, which indicates that the fat body is competent to transcribe vitellogenin at least from the antepenultimate instar larvae. In untreated females, vitellogenin production starts on day 1 after the imaginal molt, when corpora allata begin to synthesize JH III at rates doubling the maximal of larval stages. This coincidence suggests that the female reaches the threshold of JH production necessary to induce vitellogenin synthesis on day 1 of adult life. These data lead to postulate that larvae do not synthesize vitellogenin simply because they do not produce enough JH, not because their fat body is incompetent.

Keywords: Vitellogenin; Juvenile hormone; German cockroach; *Blattella germanica*; Reproduction; Metamorphosis; Ecdysteroids

1. Introduction

In practically all insect species, vitellogenesis and oocyte growth are restricted to the adult stage. In addition to rather exceptional cases of paedogenesis and neoteny (see Sehnal et al., 1996), other exceptions are found in the female larvae of Ephemeropterans, which are able to mature eggs before metamorphosis (Sehnal et al., 1996). Also, several species of moths initiate vitellogenesis in the last larval instar (as in the gypsy moth, *Lymantria dispar*) or in prepupal or early pupal stages (as in the silkmoths *Hyalophora cecropia* and *Bombyx mori*, respectively) (Wyatt and Davey, 1996; Bellés, in press). Selective pressures derived from the short adult life of these species have led to a subtle interplay between juvenile hormone (JH) and ecdysteroids, which has allowed them to mature oocytes just when the female reaches the adult stage. However, this does not occur in most insect groups, which start vitellogenesis after the imaginal molt. Why, then, do immature insects not produce vitellogenin? It is because the genes coding for vitellogenin cannot be expressed in larvae but are switched on at metamorphosis? Or is it because the levels of the vitellogenic hormone, JH, are below the threshold of induction of vitellogenin gene expression?

Recent studies on insect metamorphosis, mostly conducted at morphological and structural levels but also dealing with functional aspects, have focused on holometabolous species, like the fruit-fly *Drosophila melanogaster*, the Sphingid moth *Manduca sexta* and the silkmoth *B. mori* (see Gilbert et al., 1996). However, less modified, hemimetabolous insects may afford more suitable models to answer the above questions, given their relative simplicity compared with the holometabolous species. Among the hemimetabolous insects, cockroaches have been favorite experimental animals, especially regarding the onset of vitellogenesis, a process that has been considered as a convenient model of functional metamorphosis (Kunkel, 1981).

Kunkel (1981) was able to induce vitellogenin production (measured in terms of protein by

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immunoelectrophoresis) in larvae of *Blattella germanica* treated with JH III, and concluded that the fat body of the German cockroach could be a useful model to examine the development of a JH response. We have cloned the vitellogenin cDNA in *B. germanica* (Martín et al., 1998; Comas et al., 2000), which has allowed us to re-explore the subject by measuring vitellogenin expression in terms of mRNA. With these tools, we have re-assessed whether the production of vitellogenin exclusively in the adult derives from a metamorphic event involving a change in the adult fat body to perform a new function or a change in the adult corpora allata allowing the production of the high amounts of JH necessary to induce the expression of the vitellogenin gene. Our results rather point to the second possibility.

2. Materials and methods

2.1. Insects

Specimens of *B. germanica* were obtained from a colony reared in the dark at 30 ± 1 °C and 60–70% r.h. Freshly ecdysed fifth or sixth instar larvae or adult females were selected under red light and used at the appropriate ages. All dissections and tissue sampling were carried out on carbon dioxide-anaesthetized specimens. In the conditions stated above, the first reproductive cycle of the female lasts 7 days, after which takes place choriogenesis and oviposition.

2.2. Quantification of ecdysteroid concentration in the hemolymph

Hemolymph (2–5 μl) samples were extracted with methanol (200 μl) and then centrifuged at 13,000 *g* for 5 min. The pellet was resuspended in methanol (200 μl) and centrifugation was repeated. The supernatants were pooled, and stored at −20 °C. Ecdysteroids were quantified by ELISA, following the procedure described by Porcheron et al. (1989), and adapted to *B. germanica* by Pascual et al. (1992) and Romañá et al. (1995). 20-Hydroxyecdysone (Sigma) and 20-hydroxyecdysone–acetylcholinesterase (Cayman) were used as standard and enzymatic tracer, respectively. The antiserum (AS 4919, supplied by Prof. P. Porcheron), was used at a dilution of 1/50,000. Absorbances were read at 450 nm using a Multiscan Plus II Spectrophotometer (Labsystems). The ecdysteroid antiserum used has the same affinity for ecdysone and 20-hydroxyecdysone (Porcheron et al., 1989), but since the standard curve was obtained with the latter compound, results are expressed in pg of 20-hydroxyecdysone equivalents.

2.3. Incubation of corpora allata and quantification of juvenile hormone synthesis

Individual corpora cardiaca–corpora allata complexes were incubated in 100 μl of TC199 medium (Sigma), containing L-methionine (0.1 mM), Hank's salts, Hepes (20 mM) plus Ficoll (20 mg/ml), to which L-[^{3}H-methyl] methionine (Amersham) had been added to achieve a final specific activity of 7.4 Gbq/mmol. JH III, which is the native JH in the adult female of *B. germanica* (Camps et al., 1987), was quantified in standard 3 h incubation periods. At the end of the incubation period, JH III synthesis was determined by organic solvent extraction and thin layer chromatography, following Piulachs and Couillaud (1992).

2.4. Treatments with juvenile hormone

Larvae of different ages or freshly ecdysed adult females of *B. germanica* were topically treated with JH III (Sigma) at doses of 0.01, 0.1 or 1 μg per animal, in 1 μl of acetone. Controls received the same volume of acetone. Vitellogenin mRNA levels were studied 6 or 10 h after JH treatment.

2.5. Estimation of vitellogenin mRNA levels with RT-PCR and Southern blot

Total RNA was isolated from fat bodies, brains, ovaries and midguts dissected from four to six females at various stages using the GenElute™ Mammalian Total RNA kit (Sigma). One microgram of each RNA preparation was DNAse treated (Promega) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen) and random hexamers (Promega). For vitellogenin detection, these cDNA samples were subjected to PCR amplification with 47 cycles at 94 °C (30 s), 60 °C (30 s) and 72 °C (1 min). As a reference, the samples of the same cDNAs were subjected to 35 cycles of PCR with specific primers to the hydroxymethyl-glutaryl Coenzyme A (HMG-CoA) reductase and 21 cycles of PCR for the ribosomal 16S subunit. In all cases, PCR procedures were carried out within the linear range of amplification. Southern blot probes were generated by PCR with the same primer pair using plasmid DNA containing the corresponding cDNA clone as a template. The primers for vitellogenin were, forward: 5′-TGAAATGCGAAGGAAAGCCAA-3′, reverse: 5′-CCTGTCAAGACCTGAAATGTAT-3′; for HMG-CoA reductase, forward: 5′-CACTTGCAACAACTGAG GGC-3′, reverse: 5′-GAAGGCATGGTGCAGGATAC-3′; and for 16S, forward: 5′-TTACGCTGTTATCCC TTA-3′, reverse: 5′-CGCCTGTTTATCAAAAACAT-3′. The probes were labeled with fluorescein using the Gene Images random prime labeling module (Amersham Biosciences). RT-PCR followed by Southern blotting of

total RNA without reverse transcription did not result in amplification, indicating that there was no genomic contamination.

3. Results and discussion

3.1. Hemolymph ecdysteroids during the two last larval instars and the adult stage

The concentration of ecdysteroids in the hemolymph was measured in females in the penultimate (fifth) and last (sixth) larval instars, and compared with that reported in the adult during the first gonadotrophic cycle by Romañá et al. (1995) (Fig. 1A). In larvae, the concentration of ecdysteroids was maximal approximately 3 days before the corresponding molt. The peak concentration was higher in the penultimate than in the last larval instar, but the secretion of ecdysteroids was more prolonged in the latter. This pattern is typical of a number of hemimetabolous species, like the cockroach *Nauphoeta cinerea* (Lanzrein et al., 1985), the cricket *Gryllus bimaculatus* (Gerstenlauer and Hoffmann, 1995) and the locust *Schistocerca gregaria* (Tawfik et al., 1996). In hemimetabolous species, the more prolonged secretion of ecdysteroids, or the occurrence of a double peak of ecdysteroids, prior to the imaginal molt has been related to the commitment to metamorphosis (Nijhout, 1994). The concentrations of ecdysteroids in the penultimate and last larval instars of *B. germanica* were similar to those measured in *G. bimaculatus* (Gerstenlauer and Hoffmann, 1995) and *S. gregaria* (Tawfik et al., 1996), but one order of magnitude lower than those measured in *N. cinerea* (Lanzrein et al., 1985). The contrast between the high amounts of circulating ecdysteroids in larvae (produced by the prothoracic glands) and the low amounts present in the adult female (produced by the ovaries) (Pascual et al., 1992; Romañá et al., 1995) (Fig. 1A) is readily apparent.

3.2. Juvenile hormone production during the last two larval instars and the adult stage

The rates of JH synthesis by corpora allata incubated in vitro were measured in females in the penultimate and last larval instars, and during the first 3 days of the adult stage. Data on adult females were complemented with those reported by Maestro et al. (1994) (Fig. 1B). In the larvae studied, the maximal rates of JH III production (ca. 50 fmol/h × pair CA) were observed on day 2 of the penultimate larval instar. In contrast, the rates in the last instar were very low, with maximal values around 10 fmol/h × pair CA. In the adult, the rates spectacularly increased. On day 1 they reached ca. 100 fmol/h × pair CA, thus doubling the maximum of the penultimate

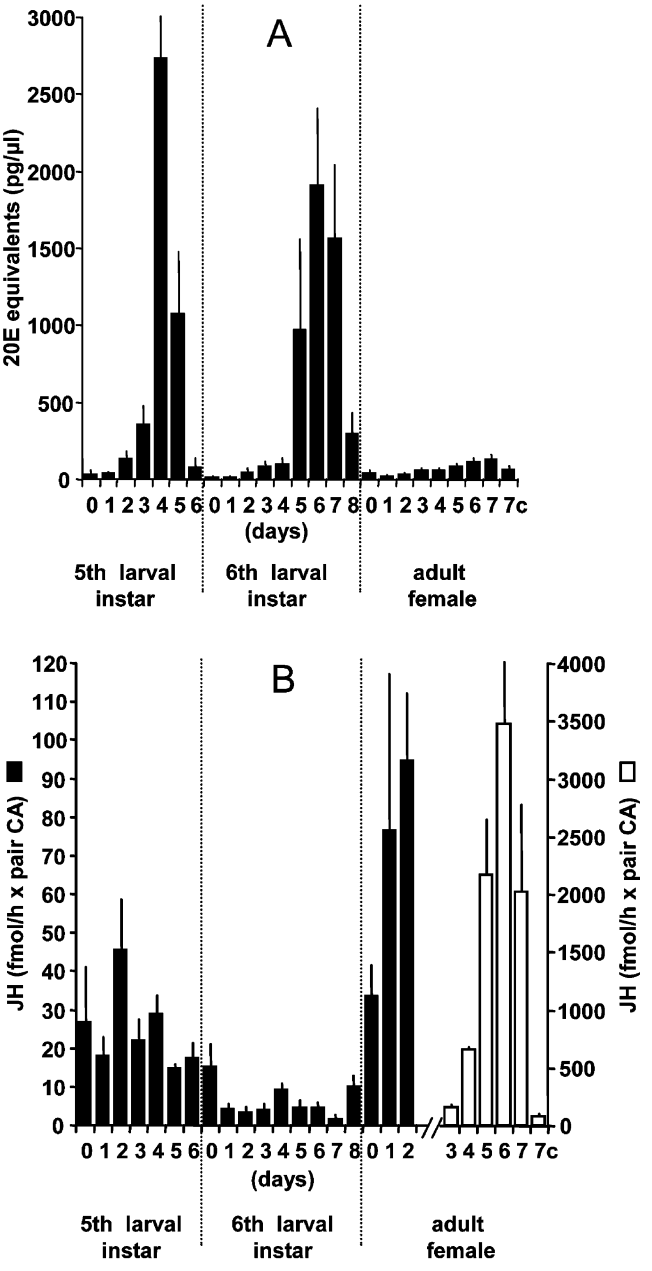

Fig. 1. Ecdysteroids and juvenile hormone (JH) during the penultimate (fifth) and last (sixth) larval instars and the adult stage of *B. germanica* females. (A) Hemolymph ecdysteroids. Data on the adult stage are from Romañá et al. (1995), although new measurements were made during the present study (days 2, 4 and 6 of adult life, $n = 2$ each), to make sure that the concentrations were within the ranges reported in 1995. Results are expressed in pg of 20-hydroxyecdysone (20E) equivalents. (B) Rates of juvenile hormone (JH) synthesis by corpora allata (CA) incubated in vitro. Data on days 3–7c of the adult stage are from Maestro et al. (1994) (note the change of scale for these days), although new measurements were made during the present study (day 5, $n = 7$), to make sure that the concentrations were within the ranges reported in 1994. In all cases, vertical bars indicate the S.E.M. ($n = 7$–15). The day "7c" means 7-day-old with chorionated oocytes.

larvae, and at the peak on day 6, they reached ca. 3500 fmol/h × pair CA.

This general pattern of moderate rates in the penultimate larval instar, very low in the last one, and very high in the adult female, has been reported in all species studied to date, e.g., among the hemimetabolous species, the desert locust *S. gregaria* (Injeyan and Tobe, 1981) and the cockroach *Diploptera punctata* (Szibbo et al., 1982). In terms of JH concentration in the hemolymph, the pattern is also similar, as described for the cockroaches *N. cinerea* (Lanzrein et al., 1985) and *D. punctata* (Tobe et al., 1985). These data point to a correlation between the pattern of synthetic rates and that of hemolymph concentration. In *B. germanica*, the concentrations of JH in the hemolymph during the first reproductive cycle have been reported by Sevala et al. (1999). According to these authors, the concentration rose from ca. 7 pg/µl in early vitellogenesis to ca. 80 pg/µl in mid vitellogenesis and peaked (ca. 170 pg/µl) in late vitellogenesis, but rapidly declined until ca. 7 pg/µl at oviposition time.

The pattern of JH in the two last larval instars is related to metamorphosis, whereas the high amounts produced in the adult are related to vitellogenesis. In the adult female, the patterns of JH (Bellés et al., 1987; Maestro et al., 1994; Fig. 1B of this paper) and vitellogenin (Martín et al., 1995a, b) are parallel during the first half of the vitellogenic cycle, but become uncoupled in the second half, when vitellogenin production begins to decrease (Martín et al., 1995a, b, 1998) while JH production continues to increase (Bellés et al., 1987; Maestro et al., 1994). This suggests that something other than decline in JH concentration regulated the fall of vitellogenin synthesis.

The dependence of *B. germanica* on JH to produce vitellogenin has also been shown at the level of gene expression. Experiments in vivo (Comas et al., 1999) and in vitro (Comas et al., 2001) have revealed that JH induces vitellogenin gene transcription. In addition, the first signs of vitellogenin mRNA in a normal reproductive cycle appear on day 1 of adult life (Martín et al., 1998), just when JH production begins to reach rates far above those of previous stages (Fig. 1B). This raises the hypothesis that the larval fat body does not produce vitellogenin simply because it does not receive enough JH, not because it is incompetent to make this protein. The question is: would the larval fat body be able to transcribe the vitellogenin gene if JH were present at appropriate concentrations?

3.3. Induction of vitellogenin expression in larvae. Influence of the juvenile hormone dose

The above-mentioned issue was addressed by experiments carried out with females on day 4 of the last larval instar, which is characterized by low levels of ecdysteroids and low production of JH (Fig. 1). Doses of 0.01, 0.1 and 1 µg of JH III per animal were administered topically, and vitellogenin mRNA was studied 6 h later. Slight signs of vitellogenin mRNA were detected in the fat body from specimens treated with 0.1 µg of JH III, whereas the transcript was clearly apparent in 1 µg-treated specimens (Fig. 2). No vitellogenin mRNA was detected in brain, ovary or gut tissues of specimens treated with 1 µg of JH III (Fig. 2), which indicates that the mechanisms regulating the tissue specificity of vitellogenin expression in the fat body also operate in immature stages. In addition, the mRNA levels of HMG-CoA reductase, an enzyme in the mevalonate pathway expressed in the fat body of *B. germanica* (Casals et al., 1997; Zapata et al., 2002), were not altered by treatment with JH III. Thus, the response of the vitellogenin gene to JH III is specific and not the result of an overall increase in fat body transcriptional activity.

The results show that vitellogenin gene expression can be induced by JH in the fat body of last larval instar in a dose-dependent manner. Interestingly, the minimal effective dose (0.1 µg of JH III) is the same required by an allatectomized adult female to start vitellogenin gene transcription (Comas et al., 1999).

3.4. Induction of vitellogenin expression in larvae and newly emerged adult. Influence of the stage

The next step was to investigate the induction of vitellogenin gene expression at other stages. Therefore, studies were carried out in specimens from mid penultimate larval instar, in early, mid and late last larval instar, and

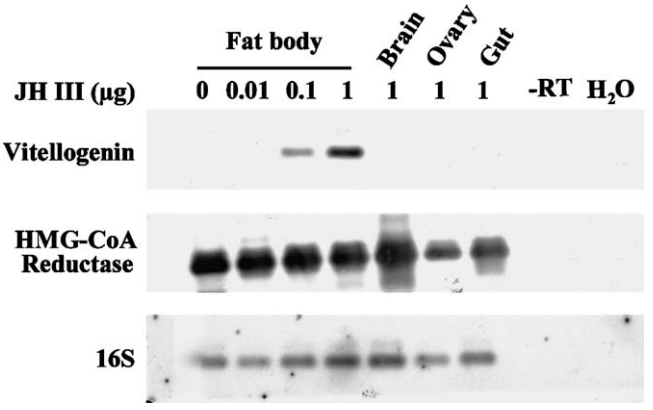

Fig. 2. Tissue-specificity and dose-dependency in vitellogenin transcription induced by juvenile hormone III (JH III) in *B. germanica* female larvae. Increasing doses of JH III were topically applied to 4-day-old last larval instar females of *B. germanica*, and the fat body, brain, ovary and gut were dissected out 6 h later. Semiquantitative RT-PCR was used to detect the transcripts. JH III-inducibility was transcript (vitellogenin)-specific, since the level of the HMG-CoA reductase transcript was unaffected by hormone treatment. Amplification of 16S transcript was used as a control of sample loading. RT-PCR of various samples without reverse transcriptase (-RT) or with water as a template (H_2O) did not yield any signal.

in newly emerged adults. A dose of 1μg of JH III was applied topically and vitellogenin mRNA in fat body was measured 6 and 10 h later.

JH induced vitellogenin gene transcription at all stages studied (Fig. 3). In general, mRNA levels were higher after 10 h than after 6 h. Normalization of the vitellogenin mRNA values with those of 16S transcript indicates that the freshly ecdysed adult female was the most sensitive stage, followed by mid (day 4) last instar larvae and late (day 7) last instar larvae, mid (day 3) penultimate instar larvae and early (day 0) last instar larvae. Finally, in a preliminary experiment following the same methodology but carried out with the mid antepenultimate (4th) larval instar, vitellogenin mRNA was detected at very low levels after 24 h of the administration of JH (data not shown).

Our results measuring vitellogenin mRNA are similar to those obtained by Kunkel (1981), who quantified vitellogenin by immunoelectrophoresis and suggested that the fat body gradually increases the capacity to respond to the vitellogenic action of JH during the last larval instars. The responses are clear and comparable after 6 h of JH treatment, which indicates that there are not relevant stage differences, if any, in the lag time of appearance of vitellogenin mRNA, as occurs when the experiments are monitored in terms of vitellogenin (Kunkel, 1981). Vitellogenin mRNA appears as early as 2 h after treatment of allatectomized adult females with 1 μg of JH III, as reported elsewhere (Comas et al., 1999). When data are normalized with respect to the 16S transcript, a curious decrease in vitellogenin mRNA is observed in late last instar larvae in comparison with mid instar (Fig. 3). A similar decrease of vitellogenin was described by Kunkel (1981), who claimed that it could be due to an antagonistic effect of the high concentrations of ecdysteroids in that period (see Fig. 1A). Although exogenous ecdysteroids can inhibit early vitellogenesis in cockroaches (Engelmann, 2002), an alternative explanation for Kunkel's observations is that the high levels of esterases occurring at pre-adult stages (Tobe et al., 1985) may lead to a much faster metabolism of the JH administered in the experiments, thus lowering vitellogenin expression.

4. Concluding remarks

In control females, vitellogenin production starts on day 1 of adult life (Martín et al., 1998), just when JH production shows rates well above those of previous stages, practically doubling the maximal rates observed in penultimate larval instar (Fig. 1B). It seems that the female of *B. germanica* reaches the threshold of JH production, and hence of titer, necessary to induce vitellogenin synthesis on day 1 of adult life. Moreover, treatment of female larvae in the last three instars with 1 μg-dose of JH III induces vitellogenin gene transcription, indicating that the fat body is competent to transcribe vitellogenin as early as the antepenultimate stadium. The most parsimonious explanation of why larvae do not produce vitellogenin is because they do not synthesize enough JH.

Our results also suggest that the capacity of the fat body to produce vitellogenin increases with time, possibly owing to the progressive structural development of the tissue, mainly related to polyploidy and proliferation of organelles involved in protein synthesis (Wyatt and Davey, 1996). This suggests that larval fat body would need even more JH than the freshly emerged adult to start vitellogenesis. Indeed, the progressive vitellogenin transcriptional capabilities of the fat body from the fourth to the sixth larval instars and the adult stage seem to be in the frame of the ontogenetic process of gradual organic growth, rather than being a functional metamorphosis of the fat body characterized by qualitative stage phenotypes. Therefore, although cockroaches may still provide useful and relatively simple models of genuine metamorphosis, such as the disappearance of prothoracic glands in the adult, vitellogenin transcription seems not to be useful as a metamorphic marker.

Another datum emerging from the present and previous studies on insect vitellogenesis is the relatively

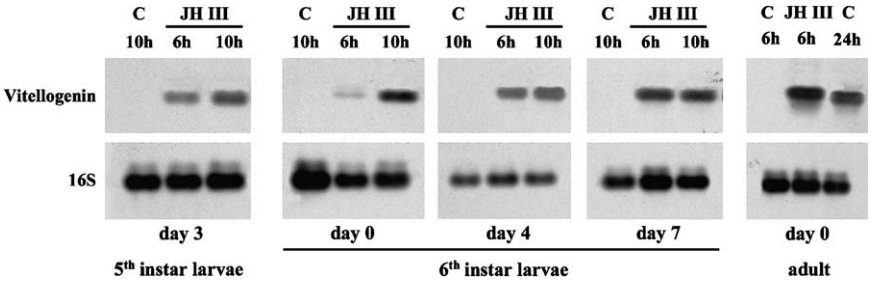

Fig. 3. Juvenile hormone III (JH III) induction of vitellogenin transcript in staged fat bodies from female larvae and adult *B. germanica*. A dose of 1 μg of JH III was topically applied to females in 1 μl acetone, and the fat body was dissected out 6 and 10 h later. Controls (C) received the same volume of acetone. Vitellogenin transcript levels were measured by semiquantitative RT-PCR. Amplification of 16S transcript was used as a control of sample loading.

high doses of JH necessary to induce vitellogenin gene expression. *B. germanica* is one of the most sensitive species to the vitellogenic action of JH, given that the effective doses of the hormone are in the order of 1 μg in vivo (Comas et al., 1999), but in other species, like the cockroach *Leucophaea maderae* (della-Cioppa and Engelmann, 1984) and the locust *Locusta migratoria* (Wyatt et al., 1987), effective doses of the native hormone are two and three orders of magnitude higher, respectively. The scarce sensitivity of the vitellogenin gene to JH action seems quite general, and may have been an evolutionary solution to prevent the onset of vitellogenesis in larval stages while significant concentrations of JH are needed to repress metamorphosis at the same time.

Therefore, the production of vitellogenin exclusively in the adult seems to derive from the capacity of the corpora allata to produce significantly higher amounts of JH after the imaginal molt. The allatotropic process that lead to such a dramatic increase of corpora allata activity in the adult female remains enigmatic. Another enigma is the further tremendous increase in JH synthesis until oviposition (Fig. 1A), because it seems no longer necessary to maintain the vitellogenic cycle (Maestro et al., 1994). In this context, it is worth noting that the follicle cells produce increasing amounts of ecdysteroids (Pascual et al., 1992; Romañá et al., 1995), which, after reaching a threshold concentration, induce choriogenesis (Bellés et al., 1993). We would also like to highlight that ecdysteroids are involved in the activation of apoptosis, whereas JH protects against cell death (Soller et al., 1999), especially in processes related to metamorphosis (Lockshin, 1985; Dai and Gilbert, 1998). Thus, a plausible explanation for the huge amounts of JH produced in the second half of the vitellogenic cycle is that JH prevents the onset of apoptosis in the follicle cells and resorption of the growing oocytes (Lockshin, 1985).

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