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Effects of juvenile hormone and ecdysone on the timing of vitellogenin appearance in hemolymph of queen and worker pupae of *Apis mellifera*

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Abstract

The caste-specific regulation of vitellogenin synthesis in the honeybee represents a problem with many yet unresolved details. We carried out experiments to determine when levels of vitellogenin are first detected in hemolymph of female castes of *Apis mellifera*, and whether juvenile hormone and ecdysteroids modulate this process. Vitellogenin levels were measured in hemolymph using immunological techniques. We show that in both castes the appearance of vitellogenin in the hemolymph occurs during the pupal period, but the timing was different in the queen and worker. Vitellogenin appears in queens during an early phase of cuticle pigmentation approximately 60h before eclosion, while in workers the appearance of vitellogenin is more delayed, initiating in the pharate adult stage, approximately 10h before eclosion. The timing of vitellogenin appearance in both castes coincides with a slight increase in endogenous levels of juvenile hormone that occurs at the end of pupal development. The correlation between these events was corroborated by topical application of juvenile hormone. Exogenous juvenile hormone advanced the timing of vitellogenin appearance in both castes, but caste-specific differences in timing were maintained. Injection of actinomycin D prevented the response to juvenile hormone. In contrast, queen and worker pupae that were treated with ecdysone showed a delay in the appearance of vitellogenin. These data suggest that queens and workers share a common control mechanism for the timing of vitellogenin synthesis, involving an increase in juvenile hormone titers in the presence of low levels of ecdysteroids.

Keywords: honey bee, vitellogenin, juvenile hormone; ecdysone

Introduction

Juvenile hormone and ecdysteroids are the key players involved in vitellogenin synthesis in many insects, acting in concert with sex determination genes and dietary components (Bownes, 1994; Bitondi and Simões, 1996). Male last instar larvae and adult females of *Locusta migratoria* respond positively to the application of the juvenile hormone analogues methoprene and pyriproxyfen, showing increases in vitellogenin gene expression (Dhadialla et al., 1987). Also in *Blattella germanica* females, vitellogenin gene expression is induced by topical application of juvenile hormone (Comas et al., 1999). In Lepidopterans, the expression of this protein shows different grades of requirement for juvenile hormone and ecdysteroids, related to their diverse reproductive strategies (Ramaswamy et al., 1997). In Diptera it has been shown that that juvenile hormone, as well as ecdysone, are responsible for the induction of yolk protein gene expression (Hagedorn, 1994; Bownes, 1986, 1994).

In highly social insects, one or few highly fertile queens generally monopolize colony reproduction, while workers are subfertile or even completely sterile. The regulation of vitellogenin synthesis in these alternative phenotypes has been targeted by several studies to promote understanding of the mechanisms underlying the establishment of reproductive dominance hierarchies. The regulation of vitellogenin production has been studied in the honeybee *Apis mellifera* during the last decade using immunological and SDS-PAGE techniques (Engels et al., 1990; Bitondi and Simões, 1996; Pinto et al., 2001). Traces of vitellogenin were detected in queens approximately ten hours before eclosion, followed by a sharp increase rapidly thereafter. In workers, significant vitellogenin titers were only detected a few days after eclosion (Engels et al., 1990).

The hormonal regulation of vitellogenin synthesis in honeybees remains unresolved in many details. Application of low doses of juvenile hormone to adult workers led to an increase in vitellogenin levels, whereas high doses of this hormone or its synthetic analogues, inhibited this response (Rutz et al., 1976; Engels et al., 1990; Pinto et al., 2000). In contrast, juvenile hormone had little effect on levels of vitellogenin in adult queens (Engels et al., 1990). Juvenile hormone action in the honeybee has been reviewed by Robinson and Vargo, 1997.

During late pupal development, as well as during the first days after eclosion, ecdysteroid titers decline and remain at relatively low levels in queens and in workers (Feldlaufer et al., 1985; Hartfelder et al., 2001). However, caste differences are observed in the juvenile hormone titer, which is elevated in queens (Rembold, 1987). Here, we tested whether the appearance of hemolymph vitellogenin in *A. mellifera* pupae depends on a combination of these two hormones, the end result of which is the caste-specific program of vitellogenin synthesis observed in adult bees. Utilizing a more sensitive approach to determine the onset of vitellogenin appearance in the hemolymph, we obtained evidence that this process may occur already during the late pupal stages and is hormone dependent.

Materials and Methods

Workers and queens of Africanized honeybees, *Apis mellifera*, were collected from hives of the Experimental Apiary of the Department of Genetics, Faculty of Medicine in Ribeirão Preto, University of São Paulo, Brazil. To obtain pupae of uniform age, queens were periodically confined for 6h on combs without brood, where they laid eggs. At appropriate age, worker pupae were removed from the brood frames and maintained in an incubator (34 ºC and 80% relative humidity), where development progressed normally. To obtain queen pupae, first instar larvae were transferred to queen cells (with a drop of royal jelly) and reared in a queenless colony until the pupal stage. Staging followed the criteria established by Michelette and Soares (1993), used to identify aging pupae according to eye color and absence or presence and intensity of cuticle pigmentation (Table 1).

Newly emerged workers were separated into cohorts of 150-200 bees that were confined in wooden cages (8x11x13cm). Newly emerged queens were individually confined in 20ml glass vials. Adult bees received water and food (40% of pollen and sugar, with sufficient water to obtain a pasty consistency) ad libitum.

Hormone and actinomycin D treatments and hemolymph sampling

Pupae were either treated topically on the abdominal cuticle with 2 µl of a juvenile hormone-III solution (Sigma, 5 mg/ml in acetone), or received an injection of 1 µl ecdysone solution (Sigma, 5mg/ml in ethanol/Ringer solution 1:4). Control experiments were performed with the respective solvents. At least 3 groups consisting of 3-6 individuals each (for a total of 9-18 bees) were used in each experiment. In studies on transcriptional regulation, actinomycin D (10 µg in DMSO/Ringer's solution 1:1) was injected 1h prior to juvenile hormone application. All treatments were performed at one developmental stage prior to the initiation of hemolymph sampling at the next stage, e.g., when juvenile hormone was applied at the Pbm I-stage, the hemolymph sampling started with Pbm II.

Hemolymph was obtained from pupae from a small incision in the dorsal cuticle between the 2nd and 3rd tergite, or, in adults by cutting off the wings at their bases. After adding a few crystals of phenylthiourea to prevent melanization, the hemolymph pools were centrifuged (3000g for 10 min at 4 ºC) and the supernatants were stored at -20 ºC.

Rocket immunoelectrophoresis, SDS-PAGE, and Western blotting

Vitellogenin in hemolymph was measured according to Bitondi and Simões (1996) with a few modifications. Briefly, antibodies against vitellogenin were obtained by homogenizing 0-6 h queen eggs in 1.5 ml Ringer's saline. The homogenate was centrifuged at 5000 g for 15 min at 10 ºC. The supernatant was emulsified in 2 ml Freund's complete adjuvant. Two subcutaneous 1 ml injections were administered to rabbits at a one-week interval. A third injection without adjuvant was given a week later. The rabbits were bled at the fourth week. For electrophoresis the obtained serum was used at a concentration of 1% in a 1% agarose solution in 0.06 M Tris-HCl buffer, pH 8.6. This same buffer, at a concentration of 0.3 M was used in the electrode compartments. Electrophoresis was run at 25 ºC for 16 h, at 0.08 V/gel cm, using volumes of hemolymph containing 3 µg of total protein. The hemolymph protein concentration were determined using the Bradford (1976) assay. After electrophoresis, the gels were washed in 0.9% NaCl and dried for subsequent staining with Coomassie Brilliant Blue R-250.

Soluble hemolymph proteins (0.7-1 µg) were separated by SDS-PAGE on 7.5% acrylamide gels. The transfer of proteins from the polyacrylamide gels to a polyvinylidene fluoride membrane (Millipore, Immobilon 0.45 µ) was performed by Western blotting in 20 mM Tris, 192mM glycine and 20% methanol. Membranes were assayed utilizing a rabbit antiserum (1:500), raised against honeybee egg proteins (Bitondi and Simões, 1996), followed by incubation with a peroxidase-anti peroxidase system (Dako, Denmark, Z113) and diaminobenzidine (Vector SK-4100) as chromogen.

Table 1. Abbreviations of developmental stages studied, characteristics and time to eclosion, in hours.

Hours	Stages	Characteristics
190	Pw	white-eyed pupae, unpigmented cuticle
170	Pp	pink-eyed pupae, unpigmented cuticle
160	Pb	brown-eyed pupae, unpigmented cuticle
85	Pbl	brown-eyed pupae, light pigmented cuticle
60	Pbm*	brown-eyed pupae, intermediary pigmented cuticle
30	Pbd	brown-eyed pupae, dark pigmented cuticle
10	Pha	pharate adult (pupae that look like adults)
0	Ne	newly emerged
	1d-3d	days after emergence

*in some experiments, Pbm was subdivided in I, II and III, according to the progressing cuticle pigmentation

Results

Vitellogenin appears in hemolymph at an earlier pupal stage in queens and at a higher concentration than in workers

Using rocket immunoelectrophoreses we corroborated previous data showing that vitellogenin appears earlier in queens than in workers. Queen hemolymph samples presented a small peak of immunoreactive vitellogenin in hemolymph from brown-eyed pupae with dark pigmented cuticle (Pbd), increased slightly in the next stage (pharate adult; Pha), and reached elevated levels in newly emerged and 1 to 3 day old bees (Fig. 1A). Until the 3rd day after eclosion, worker hemolymph exhibited only low reaction peaks, compared to those of the queen (Fig. 1B). These data show that vitellogenin levels in queens, are 10 times higher than that observed in workers during the first days of adult life.

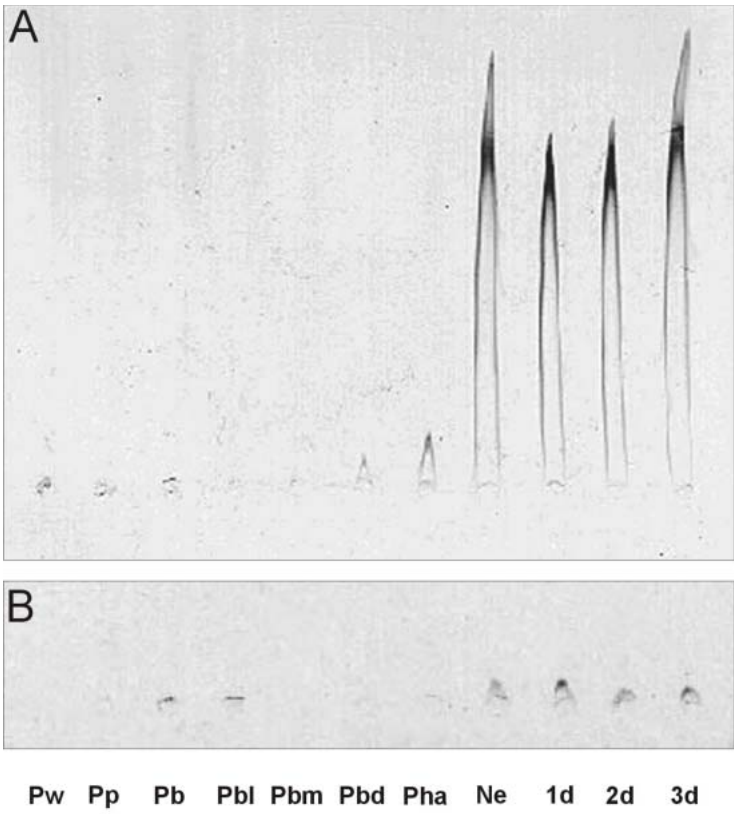

Figure 1. Vitellogenin titers in hemolymph of pupal stages and young adults of *Apis mellifera* queens (A) and workers (B). Rocket immunoelectrophoreses with 3µg hemolymph proteins per well were carried out in 1% agarose gels containing 1% rabbit antiserum raised against honeybee egg proteins. Pw: white-eyed pupae; Pp: pink-eyed pupae; Pb: brown-eyed pupae, all of them with still unpigmented cuticle; Pbl: brown eyed pupae initiating cuticular pigmentation; Pbm: brown eyed pupae showing intermediary grade of cuticular pigmentation; Pbd: brown eyed pupae showing dark pigmented cuticle; Pha: pharate adult; Ne: newly emerged bee; 1d-3d: days after eclosion.

Utilizing Western blots as a more sensitive assay we detected vitellogenin in queen hemolymph at the Pbm stage, approximately 60h before eclosion (Fig.2). In worker hemolymph vitellogenin was detected later at the Pha stage, about10h before eclosion (Fig. 2). It was occasionally possible to detect vitellogenin in the worker in an earlier developmental stage (Pbd, Fig. 5A).

Juvenile hormone advances vitellogenin appearance in both castes

Exogenous juvenile hormone applied to pink- and brown-eyed pupae had a pronounced effect on vitellogenin levels in both castes, advancing the onset of hemolymph vitellogenin appearance to earlier pupal stages. Western blot analyses indicated that in queen pupae treated with juvenile hormone, vitellogenin is expressed in PbmI, with increasing levels by Pbd, while in control pupae vitellogenin appeared only in PbmII, and remained at low levels (Fig. 3A). A similar effect was observed in worker pupae treated with juvenile hormone, which responded with an earlier appearance of vitellogenin in the Pbm II stage (Fig. 3B). Detectable vitellogenin levels were not seen in control worker pupal hemolymph during corresponding stages (Fig. 3B). Levels of total hemolymph protein concentration (Fig. 4A,B) were not affected by the juvenile hormone treatments (Mann-Whitney test >0.05).

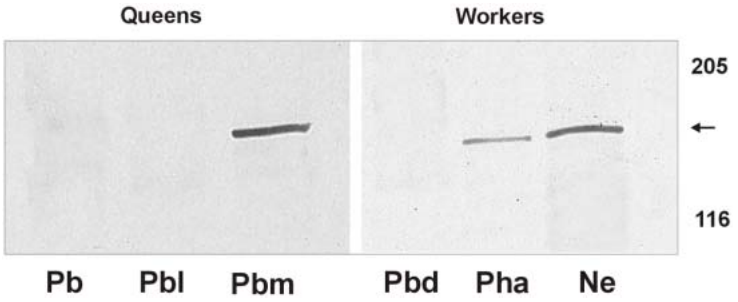

Figure 2. Western blot analysis to determine the onset of vitellogenin synthesis in honeybee queen and worker pupae. SDS-PAGE (7.5%) was performed with 1µg of hemolymph proteins. After blotting to a PVDF membrane vitellogenin was detected by a rabbit antiserum raised against honeybee egg proteins and revealed by a PAP-DAB system. Pb: brown-eyed pupae with unpigmented cuticle; Pbl, Pbm and Pbd: brown-eyed pupae showing, light, intermediary, or dark pigmented cuticle, respectively; Pha: pharate adult; Ne: Newly emerged bee; 205, 116: molecular weight references (kDa); Arrow shows the 180 kDa vitellogenin band.

Juvenile hormone stimulates transcription of the vitellogenin gene

Juvenile hormone could either induce vitellogenin gene transcription, or trigger translation from a previously transcribed vitellogenin mRNA. To distinguish between these two possibilities, worker pupae received a single injection of 10µg of actinomycin D, which inhibits gene transcription, 1h before the topical application

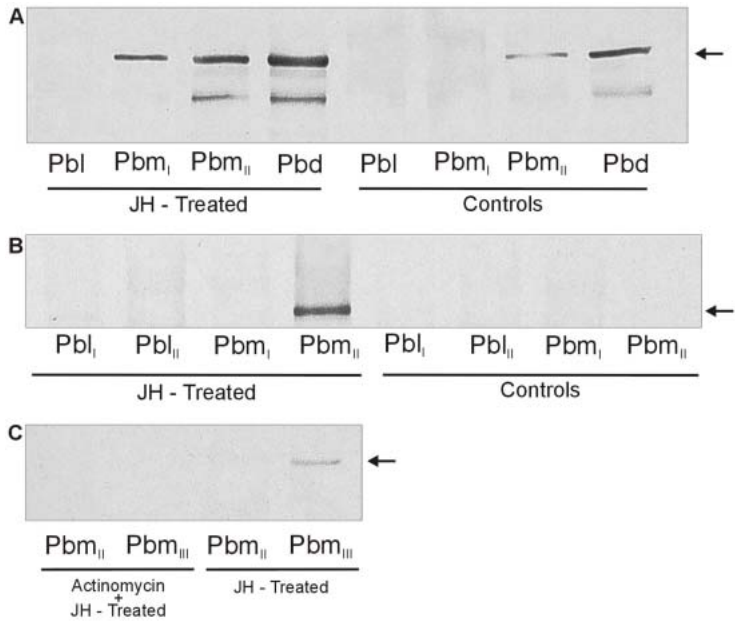

Figure 3. Western blot analysis to determine the onset of vitellogenin synthesis in honeybee queen and worker pupae. SDS-PAGE (7,5%) was performed with 1µg of hemolymph proteins. After blotting to a PVDF membrane vitellogenin was detected by a rabbit antiserum raised against honeybee egg proteins in combination with the PAP-DAB detection system. A) queen and B) worker pupae treated as brown-eyed pupae (Pb) with 10 µg juvenile hormone dissolved in acetone (treated), or with acetone (controls). C) Hemolymph from worker pupae of Apis mellifera treated, in Pb phase, first with a single injection of 10 µg of actinomycin D, and 1h later, with 10 µg of juvenile hormone-III. Controls received 10 µg of juvenile hormone-III. Pbl, Pbm and Pbd: brown-eyed pupae showing, light, medium, or dark pigmented cuticle, respectively. Pbm I , Pbm II and Pbm III are progressive ages within Pbm phase. Arrows indicate the 180 kDa vitellogenin band.

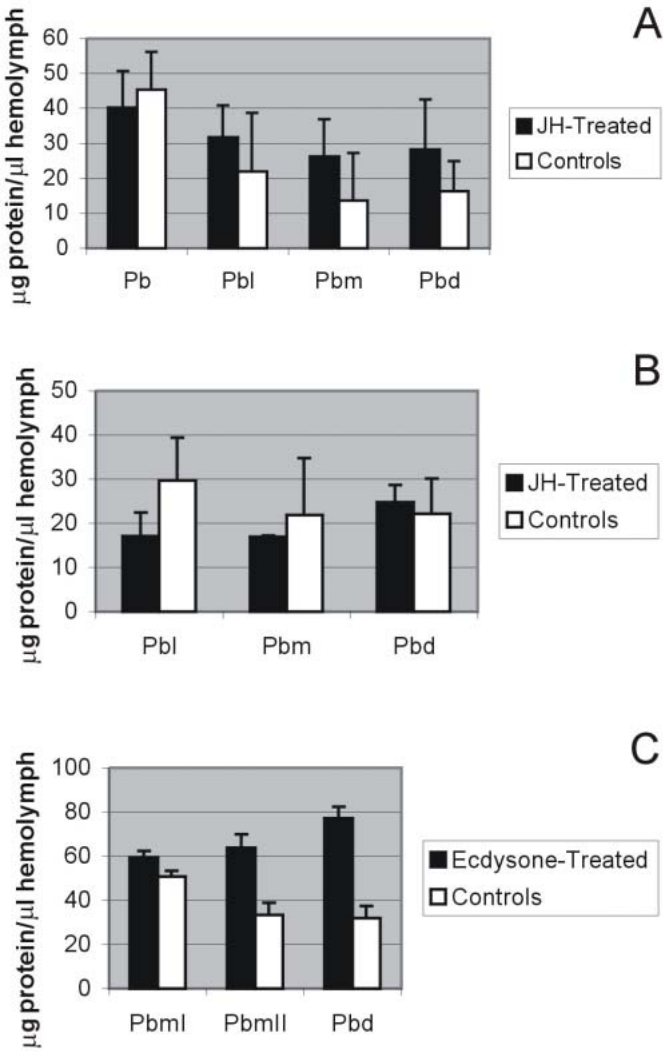

Figure 4. Protein concentrations (means SEM) in the hemolymph of A) queen and B) worker pupae of *Apis mellifera* treated with either 10 µg of juvenile hormone (in 2 µl acetone), or with 2 µl of acetone (solvent control) during the pink-eyed pupal phase (Pp). n=3 (hemolymph pools). C) Hemolymph protein concentrations in worker pupae of Apis mellifera injected with 5 µg of ecdysone diluted in ethanol/Ringer's solution 1:4 (treated), or with ethanol/Ringer's solution (controls) during the brown-eyed pupal phase (Pbl). n=3 (hemolymph pools); Statistical analysis: Pbm II and Pbd-ecdysone treated differ from controls, p <0.05 (Mann-Whitney test). Pb: brown-eyed pupae with unpigmented cuticle; Pbl, Pbm and Pbd: brown-eyed pupae showing light, intermediary or dark pigmented cuticle, respectively.

of 10µg of juvenile hormone-III. Hemolymph from Pbm workers treated with actinomycin and juvenile hormone was vitellogenin free, as judged by the Western blot assays, while hemolymph of control pupae, which received juvenile hormone but no actinomycin, had this protein in the Pbm III stage as expected (Fig. 3C). These results suggest that juvenile hormone regulates vitellogenin synthesis primarily at the transcriptional level during the pupal stages.

High levels of ecdysteroids inhibit the appearance of vitellogenin synthesis in honeybee pupal hemolymph

Injection of 5 µg of ecdysone at the Pbl stage, delayed the appearance of vitellogenin in queen and worker pupae. Ecdysone-treated worker pupae did not exhibit detectable vitellogenin levels at an advanced Pbd stage, when this protein can occasionally be detected in controls (Fig. 5A). Similarly, in ecdysone-treated queens, vitellogenin became apparent only in the pharate adult stage (Pha), representing almost a 48h delay as compared to its normal onset in the hemolymph (Fig. 5B). Total protein levels were significantly higher in the hemolymph of ecdysone-treated worker pupae than in control worker pupae (Mann-Whitney test <0.05; Fig. 4C).

Discussion

Caste-specific timing of vitellogenin synthesis

Previous reports on honeybee vitellogenin synthesis and its regulation suggest that vitellogenin first appears in the hemolymph a few hours before eclosion in queens, and after eclosion in workers (Engels et al., 1990). By refining the detection methods, we have shown that vitellogenin is detectable in both castes prior to eclosion during the late pupal period. In queens, vitellogenin first appears in Pbm II pupae, while in workers, vitellogenin appears later, in the darker pupae (Pbd) or pharate adult stage (Pha) which corresponds to approximately 60 and 10 hours before adult eclosion, respectively. Since queens and workers develop at different rates, with queens eclosing earlier, we used eye and cuticle pigmentation characteristics to account for differences in developmental rates. This allowed us to conclude that vitellogenin synthesis initiation in queens is precocious relative to workers, when the physiological state is considered rather than the absolute time required to complete preimaginal development. Upon eclosion, vitellogenin levels in queen hemolymph were higher than in workers. These differences in vitellogenin levels are most obviously related to the respective reproductive capacity of queens and workers. Queens are capable of producing 500-2000 eggs per day, and for oogenesis to proceed adequately, this apparently requires that the fat body initiate vitellogenin synthesis early despite the fact that yolk deposition does

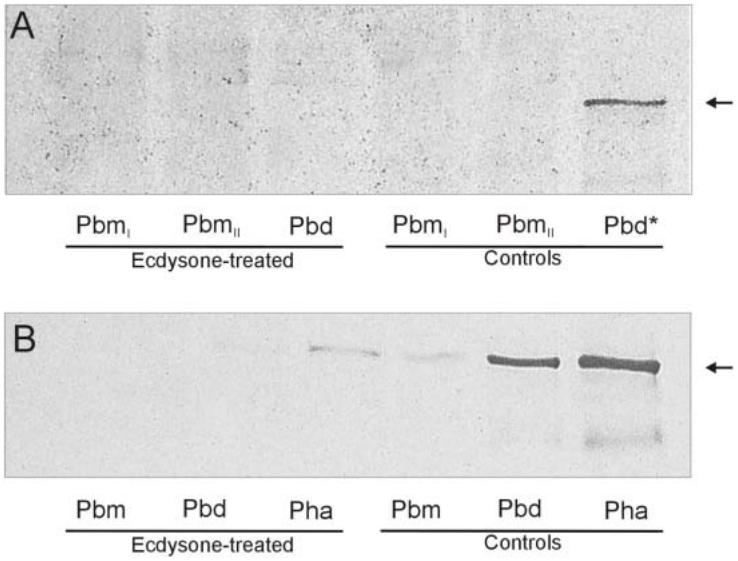

Figure 5. Western blot analysis to determine the onset of vitellogenin synthesis in honeybee queen and worker pupae, showing the effect of ecdysone on vitellogenin expression. A) workers, and B) queens treated in Pbl stage with 5 µg of ecdysone (in ethanol/Ringer's solution 1:4), or with ethanol/Ringer's solution (controls). Pbm and Pbd: brown-eyed pupae showing, intermediary or dark pigmented cuticle, respectively. Pbm I and Pbm II: progressive ages within Pbm phase. Pha: pharate adult * Advanced brown-eyed dark ,pigmented cuticle pupae. Arrows indicate the 180kDa vitellogenin band.

not start until after mating (Engels, 1974). It is, then, surprising that vitellogenin expression initiates during pupal development also in workers. This could indicate a shared mechanism for vitellogenin synthesis initiation, since under normal conditions only a small percentage of workers lay eggs, amounting to about 0.01% of the workers (Visscher, 1989; 1996).

Just after eclosion, queen hemolymph contains small amounts of vitellogenin, however, the titer rises rapidly thereafter to 70% of the total protein of the hemolymph. After mating the vitellogenin titer declines slightly, apparently because vitellogenesis (yolk deposition) has started. If mating flights are prevented vitellogenin titers continue to rise up to 90% of total protein. The same occurs if egg production is interrupted (Engels, 1974).

Juvenile hormone and ecdysteroid effects on the initiation of vitellogenin synthesis

Our results show that the onset of vitellogenin synthesis both in queens and in workers coincides with a slight increase in endogenous levels of juvenile hormone detected by Rembold (1987) at the end of pupal development. By topical application of this hormone we could confirm that this is not only a temporal but also a functional correlation. We could also confirm that juvenile hormone apparently acts as a trigger for the caste-specific initiation of vitellogenin synthesis. The clearest effect of exogenous juvenile hormone was the advance of vitellogenin appearance in both castes. The fact that even after juvenile hormone application the caste-specific differences in the timing of vitellogenin appearance were maintained, and not advanced to a common point, indicates that additional factors need to be considered. Taken together, our results demonstrate that juvenile hormone promotes vitellogenin synthesis during the final stages of queen and worker pupal development, as is the case in several other insects (Comas et al., 1999; Dhadialla et al., 1987; Wyatt, 1988). The lack of vitellogenin synthesis in pupae treated with actinomycin corroborates that this hormone acts at the transcriptional level, as also observed for others insects (Hagedorn et al., 1973; Pan and Wyatt, 1976; Engelmann, 1983; Satyanarayana et al., 1994).

In our experiments, queen and worker pupae that were submitted to ecdysone treatment showed a delay in the appearance of hemolymph vitellogenin. Considering that the ecdysteroid titer profile during pupal development (Feldlaufer 1985; Hartfelder and Engels, 1998) exhibits decreasing levels in the final pupal stages, we conclude that the high levels of ecdysteroids, caused by ecdysone-injection, inhibited the appearance of vitellogenin, perhaps by an effect on vitellogenin gene expression. Ecdysteroid-mediated inhibition of vitellogenin synthesis also occurs in other insects. In the pyralid moth *Diatraea grandiosella*, vitellogenin synthesis requires the presence of juvenile hormone, together with decreasing ecdysteroid levels (Shu et al., 1997), just like in *A. mellifera*. Lepidopterans are an interesting group for such comparative studies, because diverse mechanisms of vitellogenin synthesis regulation can be correlated with differences in life history strategies (Ramaswamy et al., 1997). In species that exhibit egg development during larval stages or during the larval/pupal transition, vitellogenin expression generally depends on ecdysteroids, as shown for *Lymantria dispar* (Davis et al., 1990; Hiremath and Jones, 1992) and *Bombyx mori* (Ohnishi, 1987). However, in species initiating oogenesis only after adult eclosion, such as *Danaus plexippus* (Pan and Wyatt, 1976), the locust (Wyatt, 1988) and *Pseudaletia unipuncta* (Cusson et al., 1994), vitellogenin expression is controlled by juvenile hormone. In many aspects, the regulation of vitellogenin synthesis in *A. mellifera* exhibits striking similarity to those Lepidopterans in which vitellogenin synthesis starts during the pupal stages, e.g. in *D. grandiosella* (Shu et al., 1997) and *Plodia interpunctella* (Shirk et al., 1990). In the tobacco hornworm, *Manduca sexta*, vitellogenin is first detectable in the prepupal stage and its synthesis was enhanced by the juvenile hormone analog methoprene (Satyanarayana et al., 1994). Interestingly, the acquisition of competence to respond to methoprene required prior exposure to juvenile hormone acid-II, in addition to 20-hydroxyecdysone (Ismail et al., 1998; Ismail et al. 2000).

In our experiments, exogenous ecdysteroids prevented the decay in total hemolymph protein levels that normally occurs at the end of the pupal stage. Engels (1990) observed higher protein synthesis in fat body of queens incubated in vitro in the presence of ecdysone. The declining endogenous ecdysteroid titer at the end of the pupal stage (Zufelato et al., 2000) is correlated with a decreasing protein levels in hemolymph (Michelette and Engels, 1995). These results suggest a double mode of action of ecdysteroids on the pupal fat body. Ecdysone may prolong or enhance the expression of several pupal-specific hemolymph proteins and specifically inhibit that of vitellogenin.

Evidence for distinct mechanisms controlling initiation and maintenance of vitellogenin synthesis in the honeybee castes

The striking differences in egg production between adult queens and workers have long attracted attention to the underlying physiological control mechanisms. Many of the earlier experiments, conducted under the paradigm of juvenile hormone control of vitellogenin synthesis in adult insects, produced results that on first view appeared contradictory. Allatectomy performed on young workers prevented the appearance of vitellogenin that was restored after juvenile hormone-III application (Imboden et al., 1976). Yet increasing vitellogenin levels during the two first weeks after eclosion was not accompanied by continuously increasing titers of juvenile hormone in worker hemolymph. After the second week, when juvenile hormone titers start to increase considerably, vitellogenin levels diminish, simultaneously with changes in worker behavior (Rutz et al., 1976; Fluri et al., 1982; Engels et al., 1990; Huang et al., 1991; Hartfelder and Engels, 1998). These observations are consistent with experiments where application of low juvenile hormone-III doses induced an increase in vitellogenin levels in 4 day-old workers (Rutz et al., 1976). An inhibitory effect on vitellogenin expression by high doses of juvenile hormone was reported for workers (Rutz et al., 1976; Pinto et al., 2000). In conclusion, low juvenile hormone titers appear to play at least a permissive role in maintaining vitellogenin synthesis in adult workers. This hypothesis is reinforced by the fact that foraging workers had high levels of juvenile hormone, but nursing workers, laying workers and queens had relatively low levels of this hormone (Robinson et al., 1991; Fahrbach et al., 1995).

While vitellogenin synthesis in adult workers respond to fluctuating juvenile hormone levels, at least to some extent, it appears to be completely independent of the effect of juvenile hormone in

adult queens. Allatectomy and juvenile hormone application did not modulate vitellogenin levels in queens, except for extremely high doses of juvenile hormone-I and II, which repressed vitellogenin synthesis (Engels et al., 1990). Instead, a heat-labile head factor has been proposed to maintain vitellogenin synthesis at high levels in adult queens (Kaatz, 1988; Engels et al., 1990).

Our results indicate that the initiation of vitellogenin expression in the late pupal stages is governed by a different regulatory mechanism. Its timing correlates with an increase in late pupal juvenile hormone titer, and application of juvenile hormone III caused a caste-specific shift to earlier stages. These results may appear to contradict the earlier studies by Kaatz (1988), who carried out carefully timed allatectomy, decapitation, and juvenile hormone rescue experiments on queen pupae. This author did not detect significant effects of allatectomy or juvenile hormone application on vitellogenin synthesis, because the incorporation rates of ^{3}H-leucine into vitellogenin, and into hemolymph proteins in general, were very low during the late pupal stages. With a more sensitive immunological assay system we have shown that queens and workers appear to share a common control mechanism for vitellogenin induction. Juvenile hormone plays a dominant role in this mechanism, possibly assisted by declining ecdysteroid endogenous titer. Figure 6 summarizes the main results obtained on juvenile hormone and 20E- regulation of onset of vitellogenin synthesis in *A. mellifera* queens and workers.

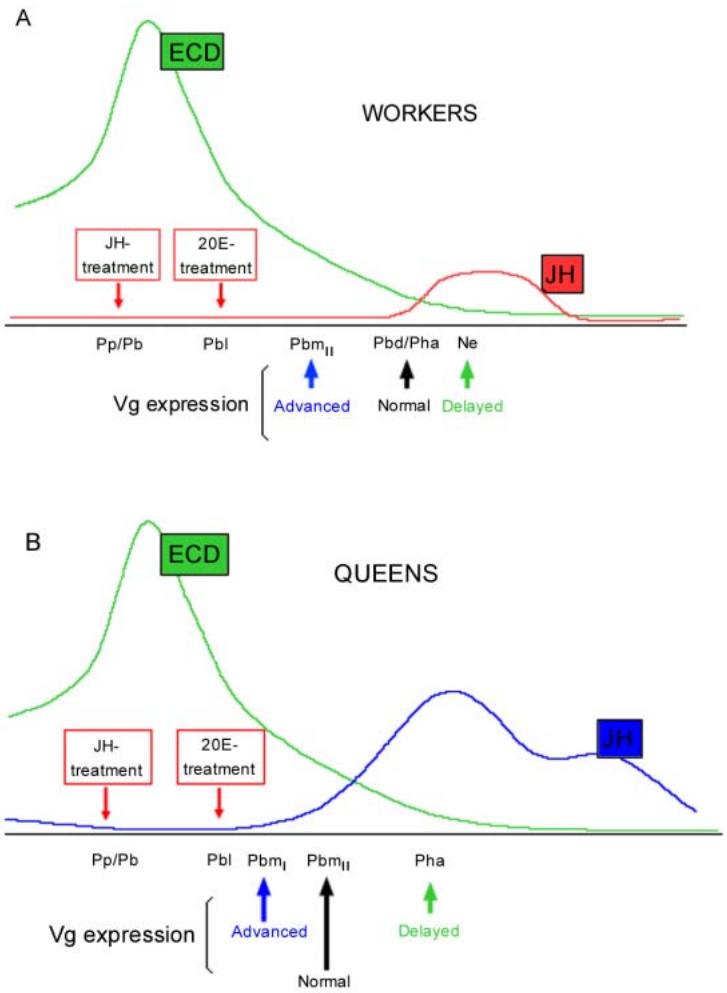

Figure 6. Hormone-dependent initiation of vitellogenin expression in honeybee (A) workers and (B) queens. Curves represent ecdysteroid (ECD) and juvenile hormone (JH) caste-specific titers during pupal and early adult stages. Red arrows indicate the stages when hormone treatments were performed. The black arrows mark the normal onset of vitellogenin expression, which is advanced in juvenile hormone-treated (blue arrows) or delayed in 20E-treated bees (green-arrows). Pp, Pb: pink or brown-eyed pupae with unpigmented cuticle; Pbl, Pbm, Pbd: brown-eyed pupae showing light, intermediary or dark pigmented cuticle; Pbm I, Pbm II: progressive ages within Pbm phase; Pha: pharate adult. Hormonal titers data: Feldlaufer et al. (1985), Rembold (1987), Hartfelder and Engels (1998) and Pinto et al. (unpublished data).

The participation of endogenous ecdysteroids in the regulation of vitellogenin synthesis in honeybee pupae needs further investigation. Present information on pupal ecdysteroid titers (Feldlaufer et al., 1985; Hartfelder et al., 2001) suggests that the initiation of vitellogenin synthesis coincides with declining ecdysteroid titers. Ecdysteroid levels remain at low levels during adult life in honeybees, independent of caste and social conditions in the colony Hartfelder et al., 2001). The apparent ecdysteroid independence of the reproductive physiology of highly eusocial bees contrasts with observations on the primitively eusocial bumble bee, *Bombus terrestris*, where ecdysteroid titers are elevated in egg laying queens and in queenless workers (Bloch et al., 2000).

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