3.9 Hormonal Control of Reproductive Processes

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3.9.1. Introduction

The years following the 1985 publication of *Comprehensive Insect Physiology, Biochemistry and Pharmacology* have been marked by stunning developments in insect science. A technological revolution in biochemistry, molecular biology, and genetics has swept all areas of biological science, and has profoundly influenced insect science as well. With this technological revolution, the small size of insects is no longer a barrier to discovering their biochemical make-up, cloning or characterizing genes, or uncovering genetic and hormonal signals governing their functions. Sequencing of insect genomes, particularly that of *Drosophila melanogaster*, has led to the identification of previously unknown genes and to the development of functional genomic approaches that lead to further elucidation of genetic regulatory networks.

During this period, insect endocrinology has shifted its focus from physiology and biochemistry to molecular biology and genetics. The latter are the subjects of the present volume. This chapter specifically reviews the progress that has been achieved since 1985 in our understanding of the hormonal control of insect reproduction. Particular attention is paid to those areas where significant progress has been made at the molecular and genetic levels.

There are a number of recent reviews on hormonal regulation of yolk protein (*yp*) genes (Raikhel *et al.*, 2003; Belles, 2004; Bownes, 2004; Wang *et al.*, 2004); vitellogenins and their processing (Sappington *et al.*, 2002; Telfer, 2002; Tufail *et al.*, 2004); nonvitellogenin yolk proteins (Bownes and Pathirana, 2002; Telfer, 2002; Masuda *et al.*, 2004; Yamahama *et al.*, 2004), and the cell biology of the insect fat body (Giorgi *et al.*, 2004).

3.9.2. Endocrine Control of Female Reproduction

3.9.2.1. Evolution of Endocrine Control of Vitellogenesis and Egg Development

The insect female reproductive system consists of the ovaries, which contain ovarioles, oviducts, spermatheca, accessory glands, vagina, and ovipositor (Davey, 1985; Chapman, 1998). Ovarioles are the egg producing units in the ovary. Typically, the insect ovary has four to ten ovarioles; however, some species contain many more ovarioles. Ovarioles are tubular and consist of both somatic and germline cells. At the apex of each ovariole, a germarium houses the primary germline cells. The follicles or egg chambers form within the germarium

and continue to mature along the ovariole tube. There are three types of ovaries in insects (Büning, 1994). Ovaries of primitive groups of insects contain panoistic ovarioles with egg chambers consisting of the oocytes surrounded by the follicular epithelium (panoistic ovarioles). In more advanced insects, where the structure of ovarioles is more complex (meroistic), some germ cells are set aside to form nurse cells that produce massive amounts of numerous products for the developing oocyte. In polytrophic meroistic ovarioles, each egg chamber contains its own group of nurse cells connected to the oocyte. Follicle cells surround the oocyte and nurse cells. In telotrophic meroistic ovaries, each ovariole contains the trophic chamber with large groups of nurse cells. The trophic chamber is connected to the egg chambers by nutritive cords. Egg chambers of telotrophic ovarioles contain only the oocyte surrounded by the follicle cells.

Endocrine control of female reproduction is governed by different types of hormones: neuropeptides (see **Chapter 3.10**), juvenile hormones (JHs; see **Chapter 3.7**), and ecdysteroids (see **Chapters 3.3** and **3.5**). In keeping with common practice among researchers in the field, ecdysteroid is used as the generic term for steroidal insect molting hormones, reserving the term ecdysone for the specific chemical compound 2β, 3β, 14α, 22R, 25-pentahydroxy-5β-cholest-7-en-6-one, originally known as α-ecdysone. The abbreviation 20E will be used to refer to 20-hydroxyecdysone (2β, 3β, 14α, 20R, 22R, 25-pentahydroxy-5β-cholest-7-en-6-one), the ecdysone metabolite believed to serve as the active hormone in most well-characterized responses. Individually, or in concert, the regulation of particular events during female reproduction by these hormones has evolved along with their effects on molting and metamorphosis. Control of female reproduction in Apterygota remains the most enigmatic. Studies of the firebrat, *Thermobia domestica* (Zygentoma), have shed some light on the hormonal regulation of vitellogenesis and ovarian development in primitive apterous insects (Bitsch and Bitsch, 1984; Bitsch *et al.*, 1986). In this insect, molting occurs continually into the adult stage, and oogenesis is coordinated with the ecdysteroid regulated adult molting cycle (Bitsch *et al.*, 1986). Treatment with the anti-allatal drug precocene blocks ovarian maturation, which indicates its dependence upon juvenile hormone secreted by the corpora allata (CA) (Bitsch and Bitsch, 1984; Bitsch *et al.*, 1986). Further studies are required in order to understand the precise roles of JH and ecdysteroids in regulating vitellogenesis and oogenesis in apterous insects.

It is well established that major events of reproduction in all insect orders with incomplete metamorphosis (Hemimetabola) are governed by JH. In the orders with complete metamorphosis (Holometabola), control strategies have evolved differently. In beetles (Coleoptera), JH remains the major regulatory hormone of reproductive events. In Hymenoptera, the role of JH is elaborated in eusocial species having a single or a few reproductive females in a colony (see **Chapter 3.13**). In Lepidoptera, female reproduction is regulated either by JH or ecdysteroids. In many such species, egg maturation occurs during the pharate adult stage and requires coordination with hormonal signals controlling metamorphosis. In dipteran insects, mosquitos, and flies, ecdysteroids have the leading role as hormonal regulators, but JH has an important role as a regulator in dipteran females in preparing reproductive tissues for ecdysteroid mediated events, such as vitellogenesis. For all insects, neuropeptides play a key role regulating the production of JH and ecdysteroids. A summary of the primary hormones involved in reproduction according to the phylogeny of insect orders is presented in Figure 1; for some orders, this is not known.

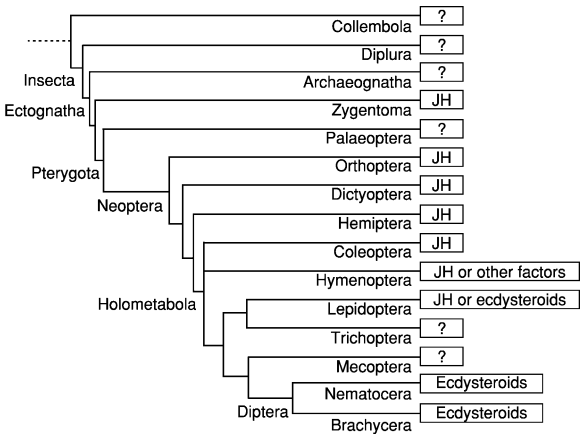

Figure 1 Utilization of juvenile hormone or ecdysteroids as major regulators of vitellogenesis and reproduction among insect orders. Some hemipterans show incomplete dependence on JH. In hymenopterans, JH plays a vitellogenic role in nonsocial and primitive social groups, but not in advanced groups (see **Chapter 3.13**). In lepidopterans, those species that begin vitellogenesis after adult emergence are JH dependent, and those in which vitellogenesis proceeds between pupal and adult stages are partially dependent on JH, whereas species in which vitellogenesis proceeds within or before the pupal stage are independent of JH. Dipterans, in general, are ecdysteroid dependent, although JH may play an accessory role in vitellogenesis (Modified from Belles, X., **2004**. Vitellogenesis directed by juvenile hormone. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part B: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth, UK, pp. 157–198).

3.9.2.1.1. Juvenile hormone directed female reproduction In the adult females of Hemimetabola (Dictyoptera to Hemiptera) and Coleoptera, JH is the main regulator and pleiotropically controls most aspects of female reproduction (**Figure 2**). The major role of JH in reproduction is to regulate vitellogenin (Vg) gene expression in the fat body, generally, and in ovarian follicular epithelium (Wyatt and Davey, 1996; Engelmann, 1983, 2003; Belles, 2004). Some gryllid and hemipteran species show an incomplete dependence upon JH (Wyatt and Davey, 1996; Strambi *et al.*, 1997).

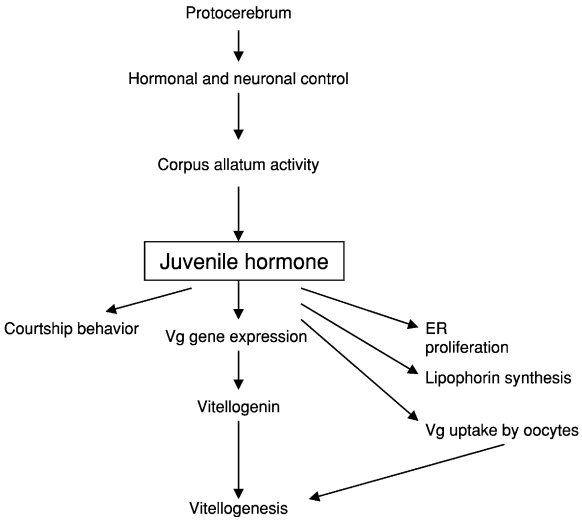

Figure 2 The pleiotropic role of juvenile hormone in insect reproduction. ER, endoplasmic reticulum; Vg, vitellogenin. (Reproduced with permission from Engelmann, F., **2003**. Juvenile hormone action in insect reproduction. In: Henry, H.L., Norman, A.W. (Eds.), Encyclopedia of Hormones, Vol. 2. Academic Press, San Diego, pp. 536–539.)

Cockroaches (Dictyoptera) are the classical model for studies of JH dependent vitellogenesis. In the oviparous *Periplaneta americana* (Blattidae), Weaver and Edwards (1990) have shown that allatectomy or treatment with inhibitors of JH synthesis blocks Vg production, oocyte growth, and ootheca formation, whereas JH treatment restores these processes. The regulation of vitellogenesis by JH has been most thoroughly studied in *Blattella germanica* (Blattellidae). In this typical JH dependent species, in which the females carry the ootheca externally until the first inside larvae emerge, JH production (Belles *et al.*, 1987), Vg titers (Martín *et al.*, 1995), and Vg mRNA levels (Comas *et al.*, 1999) show cyclical and approximately parallel patterns during the reproductive cycle (**Figure 3**). The pseudoviviparous cockroach, *Leucophaea maderae*, has been one of the favorite models for biochemical studies of JH regulation of reproduction. Effective doses to induce vitellogenesis in females *in vivo* range from 1 μg of JH I to 25 μg of JH III *in vivo*. Methoprene, a potent JH analog (JHA), induces vitellogenesis in adult males, as well (Don-Wheeler and Engelmann, 1997). In another pseudoviviparous cockroach, *Blaberus discoidalis*, allatectomy prevents ovarian maturation that can be restored by JH III treatment (Keeley and McKercher, 1985). In decapitated adult females, methoprene or JH III induces Vg protein synthesis (Keeley *et al.*, 1988) and a 6.5 kb mRNA, presumably corresponding to the Vg transcript (Bradfield *et al.*, 1990). The neuropeptide, hypertrehalosemic hormone, enhances the vitellogenic

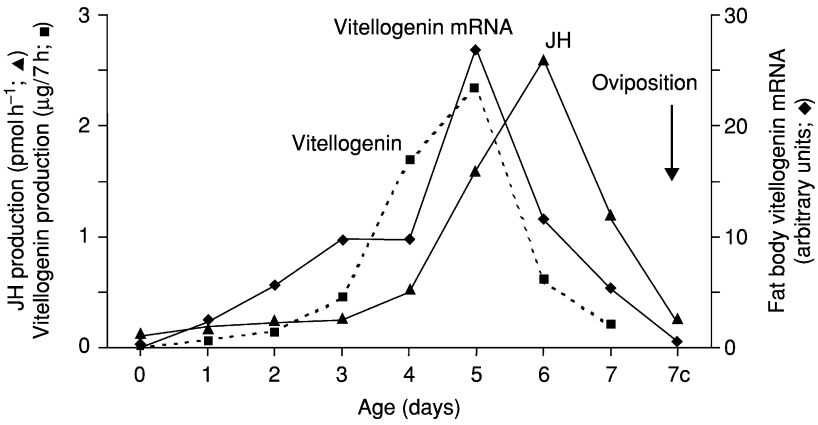

Figure 3 Production rates of juvenile hormone (JH) from the corpora allata, vitellogenin (Vg) from the periovarial fat body, and fat body Vg mRNA during the first reproductive cycle in the cockroach *Blattella germanica*. (Based on data from Belles, X., Casas, J., Messenguer, A., Piulachs, M.D., **1987**. *In vitro* biosynthesis of JH III by the corpora allata of *Blattella germanica* (L.) adult females. *Insect Biochem. 17*, 1007–1010; Martin, D., Piulachs, M.D., Belles, X., **1995**. Patterns of hemolymph vitellogenin and ovarian vitellin in the German cockroach, and the role of juvenile hormone. *Physiol. Entomol. 20*, 59–65; and Martin, D., Comas, D., Piulachs, M.D., Belles, X., **1998**. Isolation and sequence of a partial vitellogenin cDNA from the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae), and characterization of the vitellogenin gene expression. *Arch. Insect Biochem. Physiol. 38*, 137–146.)

effects of JH (Lee and Keeley, 1994a). In *Nauphoeta cinerea*, in which hemolymph Vg levels show a cyclic pattern during the gonadotropic cycle, JH I, II, and III can induce Vg production (Buschor and Lanzrein, 1983).

In Dermaptera, the CA are required for reproduction in female earwigs, *Anisolabis maritima*, as first shown by Ozeki (1951; see Rankin *et al.*, 1997), and JH is the main gonadotropic hormone in female *Euborellia annulipes*, which have vitellogenic cycles (Rankin *et al.*, 1997). Rouland *et al.* (1981) reported that allatectomized females *Labidura riparia* do not produce vitellogenic proteins, and 0.1 μg of farnesol, a precursor of JH, restores vitellogenesis.

In Orthoptera, the regulation of vitellogenesis by JH has been demonstrated in a number of locusts and grasshoppers (Acrididae) through classical experiments involving allatectomy and JH treatment (Wyatt, 1991; Wyatt and Davey, 1996). The most thoroughly studied orthopteran species has been *Locusta migratoria*, in which Vg synthesis is cyclic (Chinzei and Wyatt, 1985). Induction of Vg synthesis in female *L. migratoria* by JH homologs is only achieved with repeated doses or with high doses coinjected with a JH esterase inhibitor (Wyatt *et al.*, 1987), whereas synthetic JHAs at doses between 2 and 30 μg have a much more potent vitellogenic action (Edwards *et al.*, 1993; Zhang *et al.*, 1993). Neuropeptides may also be involved, since a brain factor enhances vitellogenesis induced by JH in fat body incubated *in vitro* (Glinka *et al.*, 1995). JH titer, and Vg protein and mRNA levels have been described during the reproductive cycle of the locust *Schistocerca gregaria* (Mahamat *et al.*, 1997) and the grasshopper *Romalea microptera* (= *Romalea guttata*) (Borst *et al.*, 2000).

In contrast to other orthopteroids, the phasmid *Carausius morosus* is independent of JH for vitellogenesis (Bradley *et al.*, 1995). Allatectomy does not prevent production of viable eggs in this insect, as first reported by Pflugfelder in 1930, and later confirmed with antibodies against *C. morosus* Vg that detected its production in allatectomized and intact females (Bradley *et al.*, 1995). However, it is not clear whether the independence from JH for vitellogenesis applies to all Phasmida.

In crickets (Gryllidae), both JH and ecdysteroids are involved in the control of vitellogenesis (review: Strambi *et al.*, 1997). In *Acheta domesticus*, the CA are necessary for oocyte growth, and JH restores vitellogenesis in allatectomized or decapitated females (Renucci *et al.*, 1987; Loher *et al.*, 1992). Conversely, female *Teleogryllus commodus* emerging from allatectomized larvae can still produce eggs (Loher and Giannakakis, 1990). Although allatectomy of *Gryllus bimaculatus* reduced the rate of egg production, oocytes in allatectomized females contained a similar amount of yolk protein as oocytes in controls; JH or JHA restored egg production to a variable extent (Hoffmann and Sorge, 1996). Notably, low doses of ecdysteroids stimulated oocyte growth in females of this species (Chudakova *et al.*, 1982; Behrens and Hoffmann, 1983). These results suggest that in crickets in which allatectomy does not completely suppress oocyte growth, ecdysteroids may play a vitellogenic role (Hoffmann and Sorge, 1996).

For Hemiptera, the CA were shown to be necessary for vitellogenesis in *Rhodnius prolixus* by V.B. Wigglesworth in the 1930s, and this observation has since been confirmed for several other hemipteran species (Wyatt and Davey, 1996; Davey, 1997; Belles, 2004). Later studies demonstrated that allatectomy of *R. prolixus* does not totally abolish Vg synthesis, but JH treatment does restore normal production (Wang and Davey, 1993). Female *Oncopeltus fasciatus* chemically allatectomized with precocenes produced the Vg precursor, but its conversion to mature Vg, which is incorporated into the oocytes, did not take place, as shown with electrophoresis of native proteins and immunodiffusion (Kelly and Hunt, 1982). A later study of this species found that the synthesis of two female specific Vg components, 200 and 170 kDa, was inhibited in precocene treated specimens and restored by administration of JH (Martinez and Garcerá, 1987). This discrepancy may be due to differences in the precocene treatment. Only one study has demonstrated a role for JH in a homopteran species. In the black bean aphid, *Aphis fabae*, ovarian development begins prenatally in this parthenogenic species, in such a manner that oocytes differentiate in the embryonic germaria, and at emergence each ovariole of a first instar aphid already contains one or two developing embryos. Precocene treatment of aphid nymphs inhibited oocyte development in embryos inside the parental ovaries, whereas JH reversed this inhibition (Hardie, 1987).

For Coleoptera, JH is the main gonadotropic hormone, but surprisingly little is known about the reproductive endocrinology of this holometabolous order with the greatest number of insect species. A long day regimen for the Colorado potato beetle, *Leptinotarsa decemlineata*, leads to reproductive activity and a short day induces diapause. JH or JHA (pyriproxyfen) treatment of short-day females induces Vg synthesis, and the JHA also induces Vg production in last instar larvae (de Kort *et al.*, 1997). Similarly, females of *Coccinella septempunctata*

reared on suboptimal artificial diets fail to reproduce, but Vg synthesis can be induced by treatment with JHA (Zhai *et al.*, 1984, 1987; Guan, 1989). In the spruce weevil, *Pissodes strobi*, treatment of previtellogenic females with JH III induces the precocious appearance of Vg transcripts, as determined by the identification of Vg mRNA (Leal *et al.*, 1997). This study also showed that Vg mRNA accumulates slower in beetles feeding on Sitka spruce trees (*Picea sitchensis*), which are resistant to *P. strobi* attack, thus suggesting that this plant contains anti-juvenoid compounds.

3.9.2.1.2. From nonsocial sawflies to eusocial ants and bees Within the Hymenoptera, JH appears to play a role in female reproduction among nonsocial and eusocial species and through this action may affect caste determination in the eusocial groups (see **Chapter 3.13**). In the primitive suborder Symphyta, only *Athalia* sawflies have received attention. JH applied to male *Athalia rosae* induced an increase in Vg production, and ovaries implanted into these males incorporated the Vg (Hatakeyama and Oishi, 1990). Later, it was shown that ovaries of *Athalia rosae* implanted into males of the closely related species, *Athalia infumata*, incorporated the heterospecific Vg that was induced in the host by JH III treatment (Hatakeyama *et al.*, 1995). Although these studies did not examine vitellogenesis in females, the results suggest a role for JH in females.

The regulation of reproduction by JH varies among the suborder Apocrita, which encompasses a range of species from solitary types to highly social groups like ants and honeybees (for reviews of JH action in social Hymenoptera see Robinson and Vargo, 1997; Hartfelder, 2000; **Chapter 3.13**). In the primitive eusocial paper wasps (Polistidae), *Polistes dominulus* (= *Polistes gallicus*) and *P. metricus*, JH acts as a gonadotropin. Queens or dominant females of *Polistes* show high JH titers associated with growing ovaries, whereas subordinate females have low JH titers. In general, allatectomy of dominant females leads to a reduction in the dominance status. The bumble bee, *Bombus terrestris* (Apidae), shows a relatively primitive social structure, and JH titers are high in egg laying queens. A regulatory role for JH is substantiated by the positive correlation between ovary development and rate of JH production in queenless workers (Block *et al.*, 2000). The role of JH remains unresolved among the studies of ants and bees with a complex social structure. JH treatment of isolated virgin queens of the fire ant, *Solenopsis invicta* (Myrmicinae) leads to wing shedding and oviposition, which is prevented by allatectomy. Subsequent JH treatment of allatectomized queens induces these phenomena (see Robinson and Vargo, 1997). Conversely, JH titers are low in reproductive female ants in the genus *Diacamma* (Ponerine; Sommer *et al.*, 1993). In the highly eusocial honeybee, *Apis mellifera* (Apidae), Vg synthesis in laying queens was only slightly reduced by allatectomy, and JH treatment increased ovary development only weakly (see Robinson and Vargo, 1997). Exogenous JH administered to queen and workers of *A. mellifera* advances the timing of vitellogenin appearance (Barchuk *et al.*, 2002); together, these results indicate that JH does not play a primary role in honeybee reproduction.

3.9.2.1.3. Butterflies and moths: endocrine compatibility of metamorphosis and vitellogenesis In Lepidoptera, some species begin vitellogenesis after adult emergence, and studies of Papilionoidea (e.g., *Pieris brassicae*, *Nymphalis antiopa*, *Polygonia caureum*, *Vanessa cardui*, and *Danaus plexippus*) and Noctuoidea (e.g., *Heliothis virescens, Helicoverpa zea*, and *Pseudaletia unipuncta*) show that JH stimulates vitellogenesis (see Cusson *et al.*, 1994a; Ramaswamy *et al.*, 1997). In *P. unipuncta*, the release rate of JH from CA *in vitro* is positively correlated with Vg synthesis (Cusson *et al.*, 1994b). In this species and *H. virescens*, vitellogenesis is abolished in decapitated females, and JH treatment restores it (Cusson *et al.*, 1994a; Ramaswamy *et al.*, 1997).

For other groups of Lepidoptera, egg development proceeds between the pupal and pharate adult stages. Studies of species in the Tortricoidea have shown that decapitation of female *Choristoneura fumiferana* and *C. rosaceana* reduces egg production, but Vg remains in the hemolymph (Delisle and Cusson, 1999). Treatment of the decapitated females with methoprene restores egg production. Similarly, treatment of female codling moths (*Cydia pomonella*) with fenoxycarb, a JHA, did not affect protein yolk content, but it did stimulate chorionation (Webb *et al.*, 1999). In this species, ecdysteroids may have a priming effect on vitellogenesis, since treatment with ecdysteroid agonists (tebufenozide and methoxyfenozide) increased levels of circulating Vg but did not affect its incorporation into the oocytes (Sun *et al.*, 2003). These results suggest that vitellogenesis is not completely dependent on JH and that it plays a primary role in Vg uptake by the oocyte and in chorionation in this family.

In the Sphingid (Bombycoidea) *Manduca sexta*, vitellogenesis starts 3–4 days before adult emergence and proceeds in the absence of the pupal CA; thereafter, JH is necessary to complete oocyte growth and chorionation (Satyanarayana *et al.*,

1994). Vg was present in prepupae of both sexes at low levels, had disappeared by pupal ecdysis, and reappeared in the late pharate adult females. Methoprene treatment induced Vg synthesis and Vg mRNA in prepupae and freshly molted pupae, and simultaneous administration of ecdysteroids abolished the vitellogenic action of JH (Satyanarayana *et al*., 1994). The pyraloid moths studied to date have a similar reproductive physiology. In *Plodia interpunctella*, the declining ecdysteroid titer triggers vitellogenesis in early pupae, and ecdysteroid treatment inhibits vitellogenin uptake by the oocytes (Shirk *et al*., 1992). Similarly, vitellogenesis in *Diatrea grandiosella* proceeds in the absence of ecdysteroids, whereas choriogenesis is completed with JH in the pharate adult (Shu *et al*., 1997).

In non-Sphingid bombycoids, such as *Bombyx mori* (Bombycidae), *Hyalophora cecropia* (Saturniidae), and *Malacosoma pluviale* (Lasiocampidae), and in the lymantrid (Noctuoidea) *Lymantria dispar*, vitellogenesis proceeds before adult emergence in the absence of JH. In these groups, vitellogenesis seems totally independent of JH, as shown by allatectomy and JH treatment, which did not influence oocyte growth (see Wyatt and Davey, 1996 for references). Other studies of *L. dispar*, in which vitellogenesis starts as early as day 3 of the last larval instar, found that treatment with JHAs on day 2 of this instar selectively prevents the production of Vg (Fescemyer *et al*., 1992) and Vg mRNA (Hiremath and Jones, 1992).

3.9.2.1.4. Mosquitos and flies: the shift to ecdysteroid mediated vitellogenesis For the Diptera, edysteroids have the primary role of regulating reproduction, as demonstrated in detail almost exclusively in mosquitos and flies. JH plays an important priming role in these females by preparing reproductive tissues for ecdysteroid mediated processes. Regulation of Vg production by ecdysteroids was first demonstrated in the yellow fever mosquito, *Aedes aegypti*. Vg synthesis in the mosquito fat body is stimulated by a blood meal and inhibited by removal of ovaries prior to blood feeding (Hagedorn and Fallon, 1973). The ovaries secrete the factor required for Vg production by the fat body, and this was found to be ecdysone (E), which is converted into the active form of the hormone 20-hydroxyecdysone (20E) (Hagedorn *et al*., 1975). Remarkably, mosquitos and flies are the only insects known to use ecdysteroids as the key regulator of reproduction. How or why this shift away from JH- to ecdysteroid-mediated reproduction occurred in Diptera remains an enigma.

The endocrinology of vitellogenesis in mosquitos (suborder Nematocera) has been reviewed in detail for *A. aegypti* (Hagedorn, 1985; Raikhel, 1992a; Dhadialla and Raikhel, 1994; Sappington and Raikhel, 1998a; Raikhel *et al*., 2003; Wang *et al*., 2004) and investigated in *Culex pipiens*, *Aedes atropalpus*, and *Anopheles stephensi* (Hagedorn, 1985; Klowden, 1997). In *A. aegypti*, vitellogenesis is separated into four phases: previtellogenic (PV) preparation, arrest, yolk protein (YP) synthesis (vitellogenesis), and termination of vitellogenesis (Raikhel, 1992b; Dhadialla and Raikhel, 1994). A newly emerged female needs about 3 days to become competent for the physiological demands of intense vitellogenesis. During this phase, the fat body and ovary acquire responsiveness to 20E and become competent for blood meal-activated vitellogenesis and oogenesis, respectively (Flanagan *et al*., 1977; Li *et al*., 2000; Zhu *et al*., 2003a). After 3 days of PV preparation, the fat body and ovary enter a state of arrest that persists until a blood meal is taken. During the YP synthesis stage, proteins are produced by the fat body and accumulated by developing oocytes (Raikhel, 1992a; Raikhel *et al*., 2002). This massive synthesis peaks at around 24 h post-blood meal (PBM), then drops sharply, and terminates by 36–42 h PBM. The fat body undergoes remodeling, and the first batch of eggs completes chorion formation and is oviposited.

Titers of JH III and ecdysteroids in female *A. aegypti* are presented in **Figure 4**. During PV preparation, JH III titer increases and stabilizes during the arrest phase, and the CA remains active (Shapiro *et al*., 1986). JH is required for the fat body to attain competence for YP synthesis in response to 20E. Following blood feeding, the JH III titer drops as a result of a rapid decrease in CA activity and elevation of JH esterase titer in the hemolymph (Shapiro *et al*., 1986). Blood feeding triggers the release of the ovarian ecdysteroidogenic hormone (OEH) from the medial neurosecretory cells of the brain for up to 12 h postfeeding (**Figure 5**; Lea, 1972; Brown *et al*., 1998). In response to OEH, the ovary produces ecdysteroids, and the hemolymph titer of ecdysteroids in female mosquitos is correlated with the rate of YP synthesis in the fat body (Hagedorn *et al*., 1975). The ecdysteroid titers are only slightly elevated at 4 h PBM, rise sharply at 6–8 h PBM to a maximum level at 18–20 h PBM, and then decline to previtellogenic levels by 30 h PBM (Hagedorn *et al*., 1975).

Numerous studies have clearly established that ecdysteroid control of YP synthesis is a central event in the blood meal-activated regulatory cascade

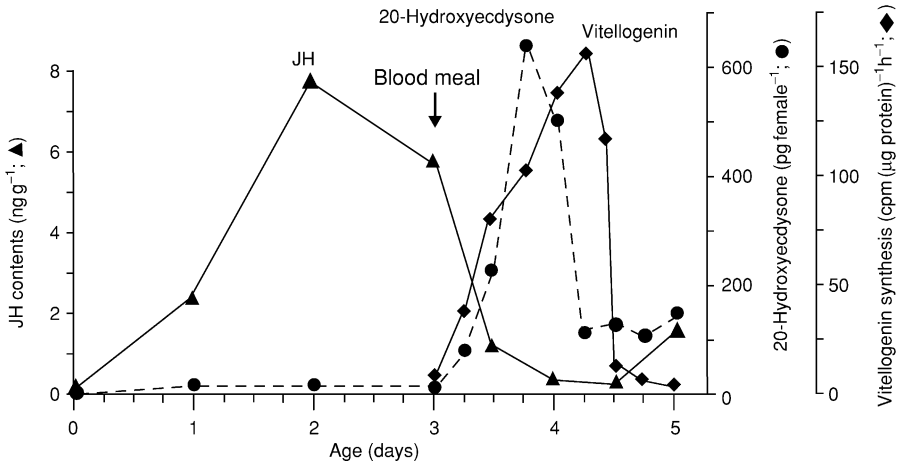

Figure 4 Juvenile hormone (JH) and 20-hydroxyecdysone titers and the level of Vg during the first reproductive cycle in the mosquito *Aedes aegypti*. (Based on data from Hagedorn, H., **1985**. The role of ecdysteroids in reproduction. Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 8. Pergamon, Oxford, pp. 205–261; and Dhadialla, T., Raikhel, A.S., **1994**. Endocrinology of mosquito vitellogenesis. In: Davey, K.G., Peter, R.E., Tobe, S.S. (Eds.), Perspectives in Comparative Endocrinology. National Research Council of Canada, Ottawa, pp. 275–281.)

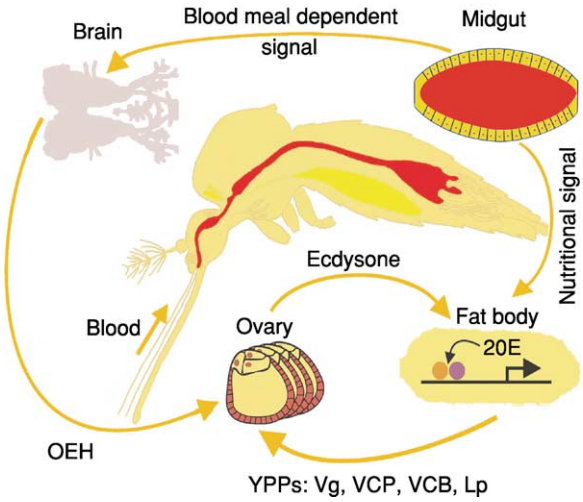

Figure 5 Schematic representation of the nutritional and hormonal activation of vitellogenesis in the mosquito *Aedes aegypti*. A blood meal results in a direct signal to the fat body, which is required to initiate yolk protein precursor (*YPP*) gene expression. The brain also receives a signal from the midgut, which activates medial neurosecretory cells to release a peptide hormone, ovarian ecdysteroidogenic hormone (OEH). OEH activates follicular cells of the primary follicles to produce ecdysone. Ecdysone is converted in the fat body to the active steroid hormone, 20-hydroxyecdysone (20E), which activates *YPP* gene expression via an ecdysone hierarchy. Yolk protein precursors, vitellogenin (Vg), vitellogenic carboxypeptidase (VCP), vitellogenic cathepsin B (VCB), and lipophorin (Lp) are secreted by the fat body into the hemolymph and selectively accumulated by developing oocytes.

leading to successful egg maturation (reviews: Hagedorn, 1985; Raikhel, 1992b; Dhadialla and Raikhel, 1994; Wang *et al*., 2004). Consistent with the proposed role of 20E in activating mosquito YP synthesis, experiments using fat body *in vitro* have shown that physiological doses of 20E (10^{-6} M) activate yolk protein precursor (*YPP*) genes, which are described below (Deitsch *et al*., 1995; Cho *et al*., 1999).

Molecular studies have demonstrated that although the ecdysteroid triggered regulatory hierarchies, such as those implicated in the initiation of metamorphosis, are reiteratively utilized in the control of mosquito vitellogenesis, the unique interplay of hierarchical factors is determined by the mosquito's biology (Raikhel *et al*., 2002, 2003; Wang *et al*., 2004). Of particular importance are the cyclicity of vitellogenic response and its dependence upon blood feeding. The demands for a very high level of expression of *YPP* genes, especially *Vg*, put additional restraints on the hormonal regulation of these genes.

The endocrine control of vitellogenesis in higher flies (suborder Cyclorrhapha) has been studied extensively in several species (reviews: Bownes, 1986, 1994, 2004; Raikhel *et al*., 2003) and is altered according to whether the species lays eggs in batches or continuously. In species laying eggs in batches, the maturation of a synchronous group of oocytes is controlled by changes in hormone levels, as described best for the housefly, *Musca domestica* (Adams and Filipi, 1983; Adams *et al*., 1985).

In this species, the fat body starts to synthesize YPs after the female fly feeds on a protein meal, and YP uptake also commences in the ovary. This phase is controlled by the circulating ecdysteroids, and the fat body later shuts down YP synthesis as egg development is completed. There is a strong correlation

between ecdysteroid titers in the hemolymph and the progress of vitellogenesis, as well as between ecdysteroid titers and the amount of YP produced in the fat body (Adams *et al.*, 1985). Ovaries produce ecdysteroids *in vitro*, and ovariectomized flies have reduced ecdysteroid levels early in the cycle (Adams *et al.*, 1985). In the autogenic strain of *M. domestica*, decapitation of females blocks Vg gene expression in fat body, but 20E restores accumulation of Vg mRNA. Moreover, males were induced to produce YPs in response to the hormone (Agui *et al.*, 1991). JH acts at an early stage to prime the fat body for YP synthesis and ensures that the oocytes are arrested in a previtellogenic stage (Adams, 1974, 1981). Interestingly, Adams *et al.* (1989) also have shown that the response to 20E in male houseflies was enhanced by application of JH. Many aspects of the regulation of vitellogenesis in the housefly are quite similar to those described for *A. aegypti*.

In the blowfly, *Calliphora vicina*, YP-containing secretory granules become visible in the fat body when vitellogenesis is initiated by ingestion of a protein meal, but the granules are not detected in the fat body of ovariectomized females (Thomsen and Thomsen, 1974, 1978). Their appearance can be restored in ovariectomized females by injection of 20E (Thomsen *et al.*, 1980). These observations have suggested the involvement of the ovary and ecdysteroids in the initiation of YP synthesis in the fat body of *C. vicina* and is reminiscent of the response of *A. aegypti* to a blood meal, which triggers the ovary to produce ecdysteroids and initiate a cycle of Vg production in the fat body.

In the blowflies, *Sarcophaga* and *Phormia*, non-protein-fed females respond to 20E by inducing YP synthesis in fat body (Huybrechts and De Loof, 1982). Unlike *C. vicina*, ovariectomized houseflies and blowflies still produce YPs indicating additional complexity of hormonal regulation in these insects (Engelmann and Wilkens, 1969; Jensen *et al.*, 1981; Huybrechts and De Loof, 1982). Interestingly, male *Sarcophaga*, *Phormia*, and *Lucilia* also produce YPs in fat body in response to 20E injection (Huybrechts and De Loof, 1982). JH alone cannot induce YP synthesis in males in *Musca* or *Calliphora* (Adams, 1974; Huybrechts and De Loof, 1982; Adams *et al.*, 1989).

In those species continuously laying eggs, each individual egg chamber differentiates, and its progress through vitellogenesis is modulated by hormonal signals connecting its development to environmental factors such as mating and food intake. This type of vitellogenesis is characteristic of *D. melanogaster*, and many related species. The regulation of vitellogenesis in *D. melanogaster* has been the subject of numerous studies using molecular, genetic, developmental, and physiological approaches (Bownes, 2004). In this species, adult sex determination (see **Chapter 1.5**) is the primary factor for correct expression of yolk protein genes. Once the genes are active in the female, the level of expression is modulated by ecdysteroids and JH in a complex way (Bownes *et al.*, 1993; Bownes, 2004). JH seems crucial for vitellogenesis, especially in the ovary (Bownes *et al.*, 1996; Soller *et al.*, 1999), but it appears that JH does not directly affect the transcription of *yp* genes in the fat body. More recent data reported by Richard *et al.* (1998, 2001) points to a more prominent role for ecdysteroids in regulating vitellogenesis. It is interesting that in the Caribbean fruit fly, *Anastrepha suspensa*, in which the YPs are only produced in the ovary, there is no evidence of hormonal regulation by JH or 20E in females, and 20E injection is unable to stimulate YP synthesis in males (Handler, 1997).

3.9.2.2. Yolk Protein Precursors

3.9.2.2.1. Sites of yolk protein production In most insects, the fat body is the exclusive site for production of yolk protein precursors (YPPs) and, in others, the ovary is a complementary vitellogenic organ. **Table 1** provides a list of species in which fat body and ovarian YPP production have been reported, and in cyclorrhaphan Diptera, YPP synthesis occurs in both the fat body and the ovary. In some dipteran species, such as the stable fly *Stomoxys calcitrans*, *Anastrepha suspensa* and perhaps the tsetse fly *Glossina austeni*, the ovary is the exclusive site of YPP synthesis (review: Wyatt and Davey, 1996). Insect YPs are the subject of several recent reviews (Bownes and Pathirana, 2002; Sappington *et al.*, 2002; Telfer, 2002; Tufail *et al.*, 2004; Masuda *et al.*, 2004; Yamahama *et al.*, 2004).

3.9.2.2.2. The fat body In female insects, the main function of the fat body is to produce massive amounts of YPPs. Multiple lobes of this organ are distributed mainly in the abdomen and to a lesser extent in the thorax and head. In many insects, fat body consists of a single cell type, the trophocyte (= adipocyte), and in cockroaches and some other insects, mycetocytes and urate cells are also present (Kelly, 1985; Locke, 1998). The multilobed structure of this tissue enhances its interaction with hemolymph. Also, the fat body is responsible for intermediary metabolism; storage of carbohydrates,

Table 1 Vitellogenic organs in insects

Order–suborder	Species	Vitellogenic organ		Type of ovarioles
		Fat body	Ovary	
Zygentoma	*Thermobia domestica*[a]	X	X	Panoistic
Dictyoptera	*Blattella germanica*[b]	X		Panoistic
Dictyoptera	*Leucophaea maderae*[b]	X		Panoistic
Orthoptera–Caelifera	*Locusta migratoria*[b]	X		Panoistic
Orthoptera–Ensifera	*Acheta domesticus*[b]	X		Panoistic
Phasmida	*Bacillus rossius*[b]	X		Panoistic
Hemiptera	*Rhodnius prolixus*[c]	X	X	Telotrophic meroistic
Coleoptera	*Leptinotarsa decemlineata*[b]	X	X	Telotrophic meroistic
Coleoptera	*Coccinella septempunctata*[b]	X	X	Telotrophic meroistic
Hymenoptera	*Apis mellifera*[d]	X	X	Polytrophic meroistic
Lepidoptera	*Plodia interpunctella*[b]	X		Polytrophic meroistic
Lepidoptera	*Hyalophora cecropia*[b]	X		Polytrophic meroistic
Lepidoptera	*Manduca sexta*[b]	X		Polytrophic meroistic
Diptera–Nematocera	*Rhyncosciara americana*[b]	X		Polytrophic meroistic
Diptera–Nematocera	*Aedes aegypti*[b]	X		Polytrophic meroistic
Diptera–Brachycera	*Dacus oleae*[b]	X	X	Polytrophic meroistic
Diptera–Brachycera	*Drosophila melanogaster*[b]	X	X	Polytrophic meroistic
Diptera–Brachycera	*Musca domestica*[b]	X	X	Polytrophic meroistic
Diptera–Brachycera	*Sarcophaga bullata*[b]	X		Polytrophic meroistic
Diptera–Brachycera	*Calliphora vicina*[b]	X	X	Polytrophic meroistic
Diptera–Brachycera	*Stomoxys calcitrans*[b]		X	Polytrophic meroistic
Diptera–Brachycera	*Anastrepha suspensa*[e]		X	Polytrophic meroistic
Diptera–Brachycera	*Glossina austeni*[b]	?	X	Polytrophic meroistic

[a]Rousset and Bitsch (1993).
[b]See Valle (1993) for references.
[c]Melo *et al.* (2000).
[d]K.R. Guidugli, M.D. Piulachs, X. Belles, and Z.L.P. Simões, unpublished data.
[e]Handler (1997).

lipids, and proteins; and synthesis of hemolymph proteins. These functions are hormonally controlled and successively change in accordance with the demands of different life stages.

Trophocytes are equipped with abundant cytoplasmic organelles that accomplish a great variety of synthetic and secretory functions. The basal lamina enveloping the fat body allows diffusion of large oligomeric proteins with molecular sizes of over 300 000 (even up to 500 000 Da) and keeps the apical plasma membrane of trophocytes structurally differentiated. This apical membrane is highly infolded and resembles a plasma membrane reticular system in which secretory granules are being released by exocytosis (Locke and Huie, 1983; Raikhel and Lea, 1983; Dean *et al.*, 1985; Raikhel and Snigirevskaya, 1998; Mazzini *et al.*, 1989; Giorgi *et al.*, 2004).

3.9.2.2.3. Fat body derived yolk protein precursors

3.9.2.2.3.1. Vitellogenins In most female insects, the major constituent of protein yolk is Vg, a large, conjugated protein that is taken into oocytes and stored as vitellin (Vn). The amino acid sequence, structure, and composition of Vg are sufficiently conserved between insects and other oviparous animals to indicate origin from a common ancestral protein (Chen *et al.*, 1994; Sappington and Raikhel, 1998a; Sappington *et al.*, 2002) and may share homology with other, more distantly related lipoproteins (Blumenthal and Zucker-Aprison, 1987; Spieth *et al.*, 1991; Sappington *et al.*, 2002). Insect Vgs are encoded by mRNAs of 6–7 kb that are translated as primary products of ~200 kDa. Vg primary products have been characterized at the molecular level for Hemimetabola and Holometabola species (Sappington *et al.*, 2002; Tufail *et al.*, 2004).

The primary pre-proVg is cleaved into subunits (apoproteins), ranging from 50 to 180 kDa, by subtilisin-like endoproteases and proprotein convertases (Barr, 1991; Rouille *et al.*, 1995). These enzymes recognize a consensus motif (R/K)XX(R/K) preceding the cleavage site, and the motif is found in all known insect Vgs (Chen *et al.*, 1994; Sappington and Raikhel, 1998a; Sappington *et al.*, 2002; Tufail *et al.*, 2004). Vg convertase (VC) has been characterized from the vitellogenic fat body of

A. aegypti (Chen and Raikhel, 1996) and is a homolog of human and *D. melanogaster* furins and a *D. melanogaster* convertase (Barr *et al.*, 1991; Roebroek *et al.*, 1991, 1992a, 1992b; Hayflick *et al.*, 1992).

In hemimetabolous insects like *L. maderae*, *P. americana*, and *Riptortus clavatus*, the Vg primary product is cleaved into large and small polypeptides, including polypeptides of ~80–110 kDa (**Figure 6**) (Della-Cioppa and Engelmann, 1987; Hirai *et al.*, 1998; Tufail *et al.*, 2001; Tufail and Takeda, 2002). It has also been reported that Vgs from some hemimetabolous insects, such as *L. maderae* and *R. clavatus*, are processed further in the oocyte (Hirai *et al.*, 1998; Tufail and Takeda, 2002).

In holometabolous insects, the Vg primary product is cleaved into a single large and small polypeptide (**Figure 6**) (Dhadialla and Raikhel, 1990; Chen *et al.*, 1994, 1996). As with other apocritan Hymenoptera, Vg is not cleaved in the parasitic wasp, *Pimpla nipponica* (**Figure 6**; Nose *et al.*, 1997). In the fall armyworm moth, *Spodoptera frugiperda*, only a single Vg apoprotein was detected in hemolymph and ovarian extracts, although other lepidopteran Vgs normally are processed into two subunits (Hiremath and Lehtoma, 1997; Sorge *et al.*, 2000).

Following extensive co- and posttranslational modifications, Vg subunits form high molecular weight oligomeric phospholipoglycoproteins (400–600 kDa) that are secreted into the hemolymph of females (Osir *et al.*, 1986a; Wojchowski *et al.*, 1986; Dhadialla and Raikhel, 1990; Sappington and Raikhel, 1998a; Giorgi *et al.*, 1998; Tufail *et al.*, 2004). Mature vitellogenins generally exist as oligomers, but monomeric molecules of about 300 kDa may exist in the cockroach *N. cinerea* (Imboden *et al.*, 1987).

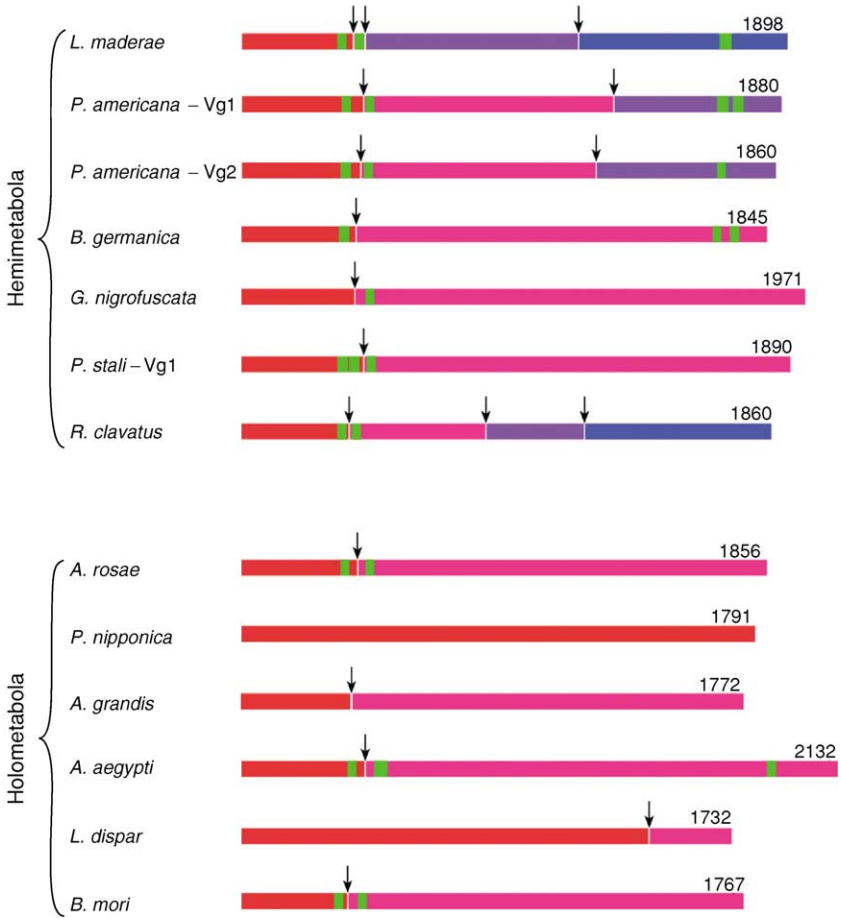

Figure 6 Schematic representation of the cleavage sites and polyserine domains in vitellogenins from 12 insect species. The arrows and white lines indicate the putative or determined cleavage sites following the consensus RXXR cleavage site sequence. The green segments show the polyserine stretches. Numbers indicate the amino acid residues deduced from the N-termini (excluding the signal peptides). Color code is used to indicate the number of vitellogenin subunits resulting from proteolytic cleavage. (Modified from Tufail, M., Raikhel, A.S., Takeda, M., **2004**. Biosynthesis and processing of insect vitellogenins. In: Raikhel, A.S., Sappington, T.W. (Eds.), Reproductive Biology of Invertebrates, Vol. 12, Part B: Progress in Vitellogenesis. Science Publishers, Enfield, USA/Plymouth, UK.)

One to several *Vg* genes have been identified in different insect species (reviews: Telfer, 2002; Tufail *et al.*, 2004). In the silkworm *B. mori*, *Vg* is encoded by a single gene (Tufail *et al.*, 2004), and there are five *Vg* genes in the mosquito *A. aegypti* (Romans *et al.*, 1995). Regulatory regions of *Vg* genes that govern hormone dependent expression have been characterized in great detail for *A. aegypti* (Kokoza *et al.*, 2001) and only partially for *L. migratoria* and *B. germanica* (Wyatt *et al.*, 1984; Locke *et al.*, 1987; Belles, 2004).

3.9.2.2.3.2. Yolk polypeptides of the cyclorrhaphous Diptera In higher Diptera, the number of YPs ranges from one to five, and they are different from the Vg of other insects (review: Bownes and Pathirana, 2002). There are three major YPs of 46, 45, and 44 kDa (Barnett *et al.*, 1980; Bownes *et al.*, 1993) in *D. melanogasti* and up to five YPs ranging from 40 to 51 kDa in *C. erythrocephala* (Fourney *et al.*, 1982), *Neobellieria* (=*Sarcophaga*) *bullata* (Huybrechts and De Loof, 1982), *M. domestica* (Adams and Filipi, 1983), *A. suspensa* (Handler, 1997), *Phormia regina* (Zou *et al.*, 1988), *Ceratitis capitata* (Rina and Savakis, 1991), and eight other species of *Drosophila* (Bownes, 1980). The primary translation products of cyclorrhaphan YPs are close to the size of each mature polypeptide. Their post-translational modification includes glycosylation and phosphorylation and tyrosine sulfation (Brennan *et al.*, 1980; Minoo and Postlethwait, 1985; Baeuerle and Huttner, 1985).

Yolk protein genes have been characterized for *D. melanogaster* (Hung and Wensink, 1983; Garabedian *et al.*, 1987; Yan *et al.*, 1987), *C. capitata* (Rina and Savakis, 1991), and *C. erythrocephala* (Martinez and Bownes, 1994). Deduced amino acid sequences for the three YPs of *D. melanogaster* show that they are related to each other and to other cyclorrhaphan YPs. These YPs are not lipoproteins (see **Chapter 4.6**), likely constitute subunits of a larger native protein (Fourney *et al.*, 1982; Adams and Filipi, 1983; Zou *et al.*, 1988), and more closely resemble a family of vertebrate lipases and not the insect Vgs (Bownes *et al.*, 1988; Terpstra and Ab, 1988).

3.9.2.2.3.3. Additional yolk proteins secreted by the fat body A number of supplemental proteins are secreted by the fat body of vitellogenic females and selectively accumulated by developing oocytes. Typically, these YPs are minor yolk components but in some species can be as abundant as Vg (Telfer, 2002; Masuda *et al.*, 2004). Most are female-specific products, but some are found in the hemolymph of both sexes. They are likely to serve specialized functions necessary for embryonic development and, as yet, it is difficult to draw any unifying conclusions, since very few of these YPs have been characterized for insects.

3.9.2.2.3.3.1. Microvitellogenin Microvitellogenin (mVg) is a small yolk protein of 30 kDa (Pan, 1971; Kawooya *et al.*, 1986; Telfer and Pan, 1988; Pan *et al.*, 1994). In lepidopteran species, mVg is synthesized and secreted by the fat body (Cole *et al.*, 1987) and incorporated by ovarian follicles (Telfer and Kulakosky, 1984; Kulakosky and Telfer, 1989). *Manduca sexta* mVg is a monomeric protein with no detectable carbohydrate, lipid, or phosphate (Kawooya *et al.*, 1986). *Bombyx mori* produces multiple 30 kDa proteins that are the principal components of both male and female hemolymph during late larval and pupal stages (Izumi *et al.*, 1981) and constitute 35% of the egg total soluble protein (Zhu *et al.*, 1986). Deduced amino acid sequences from five *B. mori* mVg cDNA clones revealed their high similarity (Sakai *et al.*, 1988), and *M. sexta* mVg shares 70% sequence similarity with a *B. mori* mVg (Wang *et al.*, 1989). Furthermore, antigenic similarity between mVgs in *M. sexta* and *H. cecropia* (Kawooya *et al.*, 1986) supports the homology of lepidopteran mVgs.

3.9.2.2.3.3.2. Lipophorin as a yolk protein Lipids are a critical source of energy during insect embryogenesis (Beenakkers *et al.*, 1985) and can represent as much as 30–40% of the egg's dry weight (Troy *et al.*, 1975; Kawooya and Law, 1988; Briegel, 1990). Lipophorins (Lp) are lipid transport proteins in insects (see **Chapter 4.6**) and play a prominent role in the accumulation of lipids in insect oocytes (review: Antwerpen van *et al.*, 2004). Kinetic analyses have indicated that lipid transfer is affected by a saturable mechanism in both *M. sexta* (Kawooya and Law, 1988) and *H. cecropia* (Kulakosky and Telfer, 1990), thus indicating that lipid uptake occurs via receptor-mediated endocytosis. Specific ovarian receptors for Lp have been cloned from *L. migratoria* (Dantuma *et al.*, 1997), *A. aegypti* (Cheon *et al.*, 2001), and *B. germanica* (Ciudad, Piulachs, and Belles, unpublished data). In saturniid and sphingid moths, Lp is the second most abundant YP (Chino *et al.*, 1977b; Telfer *et al.*, 1991; Telfer and Pan, 1988), and it has been found in the yolk of the fall webworm *H. cunea* (Arctiidae) (Yun *et al.*, 1994). In mosquito eggs, Lp makes up only 3% of total egg proteins (Sun *et al.*, 2001). In *B. germanica*, Lp facilitates hydrocarbon uptake by maturing oocytes (Fan *et al.*, 2002).

3.9.2.2.3.3.3. Pro-proteases Yolk proteins are essential reserves of amino acid and other nutrients for the developing insect embryos, and sequential cleavage of YPs into smaller molecules occurs throughout embryogenesis (Zhu *et al*., 1986; Yamashita and Indrasith, 1988; Masetti and Giorgi, 1989; Yamamoto and Takahashi, 1993; Izumi *et al*., 1994; Takahashi *et al*., 1996; Cho *et al*., 1999; Yamahama *et al*., 2004). This proteolytic degradation of YPs is probably regulated through a battery of proteases (see **Chapter 4.7**).

At present, two female-specific proenzymes are known to be deposited in the protein yolk of *A. aegypti*. One is a serine carboxypeptidase, vitellogenic carboxypeptidase (VCP), and it is a glycosylated 53 kDa protein secreted by the female fat body in synchrony with vitellogenin (Hays and Raikhel, 1990). An antigenically similar protein of the same size occurs in the yolk, and the enzyme is activated during embryogenesis by cleavage to a 48 kDa polypeptide (Cho *et al*., 1991). A 44 kDa fat body protein, vitellogenic cathepsin B (VCB), with sequence similarity to vertebrate cathepsin B is also incorporated into the yolk of *A. aegypti* (Cho *et al*., 1999). It is converted to 42 kDa after internalization in developing oocytes, and then to 33 kDa in developing embryos, coinciding with the onset of YP degradation. The 33 kDa protein degrades mosquito YPs *in vitro*. Secretory pathways for VCP and VCB in the fat body and their endocytosis in the oocyte were shown by immunocytochemistry to be the same as those of vitellogenin (Snigirevskaya *et al*., 1997a). Notably, they are deposited in the amorphous, peripheral layer of the yolk bodies surrounding the central core of the vitellin crystal (Snigirevskaya *et al*., 1997b).

An acid cysteine proteinase (see **Chapter 4.7**) was first purified from *Bombyx* eggs (Kageyama and Takahashi, 1990) and named *Bombyx* cysteine proteinase (BCP). It has broad substrate specificity and hydrolyzes various protein substrates, including *B. mori* yolk proteins. Since BCP accumulates in hemolymph, it is secreted by the fat body as shown by Northern blots. Ovarian follicle cells also synthesize the enzyme, as established by Northern blot and its synthesis from ovarian RNA (Yamamoto *et al*., 2000).

3.9.2.2.3.3.4. Other fat body products used as yolk proteins For several insects, there are reports of other proteins that are incorporated into yolk. In *L. migratoria* females, 21 kDa and 25 kDa proteins are produced by the fat body in synchrony with the synthesis of Vg (Zhang *et al*., 1993; Zhang and Wyatt, 1996) and accumulate in oocytes along with Vg. These additional yolk proteins are significant constituents of the yolk in *L. migratoria* eggs. In the lepidopteran *M. sexta*, a blue biliprotein (insecticyanin) composed of four 21.4 kDa subunits is a component of larval hemolymph and is present in eggs (Chino *et al*., 1969; Kang *et al*., 1995). An arylphorin-like cyanoprotein that is a hemolymph storage hexamer is also deposited in eggs of the bean bug *R. clavatus* (Chinzei *et al*., 1990; Miura *et al*., 1994). The incorporation of these pigmented proteins may help to conceal eggs from predators (Chino *et al*., 1969). The iron transport protein, transferrin (see **Chapter 4.10**) has been shown to be selectively deposited in yolk of the flesh fly *Sarcophaga peregrina* (Kurama *et al*., 1995) and the bean bug *R. clavatus* (Hirai *et al*., 2000). Hemolymph and oocytes of *Rhodnius prolixus* also contain a 15 kDa heme binding protein (Oliveira *et al*., 1995). Eggs of the stick insect *C. morosus* contain a minor yolk protein that is secreted by the fat body and sulfated by the follicle cells (Giorgi *et al*., 1995).

3.9.2.2.4. Yolk proteins synthesized by ovarian follicle cells Irrespective of insect ovary type, only the follicle cells in egg chambers engage in the production of proteins that are utilized as YPs. As demonstrated for several insects, follicle cells secrete a protein that has a similar antigenic reactivity and subunit composition as the Vg produced by fat body. In the firebrat *T. domestica*, there is the dual origin of YP from fat body and ovaries (Rousset and Bitsch, 1993) (**Table 1**). The synthesis of YPs by both tissues in adult females of this order, where molting and reproduction alternate, indicates that it could be an ancestral condition. YP production has also been observed in ovarian follicles of the heteropteran *R. prolixus* (Melo *et al*., 2000) and two coleopterans, *C. septempunctata* (Zhai *et al*., 1984) and *L. decemlineata* (Peferoen and De Loof, 1986). In the honeybee, *A. mellifera*, Vg expression has been reported in the fat body (Piulachs *et al*., 2003), but a more recent study has revealed that it is also expressed in the ovaries (K.R. Guidugli, M.D. Piulachs, X. Belles, and Z.L.P. Simões, unpublished data). These findings in *R. prolixus* and *A. mellifera* suggest the importance of reassessing the vitellogenic role of the ovary in more species.

In higher flies (suborder Cyclorrhapha), the ovarian origin of YPs was first detected in *D. melanogaster* (Bownes and Hames, 1978), and in this and other species, these YPs have been investigated extensively (see Section 3.9.2.2.3.2). Follicle cells were identified as the site of synthesis in *D. melanogaster*

by their ability to secrete YPs when manually separated from follicles labeled with [^{35}S]methionine, and by *in situ* hybridization to contain YP mRNAs (Brennan *et al.*, 1982). Follicle cells are implicated in production of YPs in *C. erythrocephala* (Rubacha *et al.*, 1988) and in *S. bullata* (Geysen *et al.*, 1986). In the stable fly, *S. calcitrans* (Houseman and Morrison, 1986; Chen *et al.*, 1987), the Caribbean fruit fly *A. suspensa* (Handler and Shirk, 1988; Handler, 1997), and the tsetse fly, *glossina austeni* (Huebner *et al.*, 1975), female specific proteins do not occur in the hemolymph of reproductive adults, thus indicating that YPs are synthesized only in the ovaries.

In some cyclorrphan flies, YP genes are expressed differently in the fat body and follicular epithelium. In *C. erythrocephala*, ovaries produced 51 and 49 kDa YPs, while fat body secreted a 46 kDa YP and possibly a different 49 kDa YP (Fourney *et al.*, 1982). In *D. melanogaster*, the two tissues secrete the same three YPs, but the smallest one,YP3, is underrepresented in ovarian synthesis (Brennan *et al.*, 1982; Isaac and Bownes, 1982). Ovarian and fat body YPs are mixed in the eggs of these flies.

In several species of Lepidoptera, follicle cells secrete proteins that are incorporated into yolk and may account for up to 25% of the total soluble protein, as in mature eggs of *B. mori* (Zhu *et al.*, 1986). In the moth *H. cecropia*, a 55 kDa protein is present in the intercellular spaces and yolk bodies (Bast and Telfer, 1976). Follicles of *B. mori* produce an ovary specific protein of 225 kDa with three 72 kDa subunits (Ono *et al.*, 1975; Indrasith *et al.*, 1988; Sato and Yamashita, 1991a); one of which is converted to a 64 kDa polypeptide by egg maturation. Sequencing of a cDNA clone revealed its similarities to human gastric and rat lingual lipases, especially to a noncatalytic lipid binding domain (Inagaki and Yamashita, 1989; Sato and Yamashita, 1991b). Two follicle specific YPs have also been isolated from *M. sexta* (Tsuchida *et al.*, 1992). One is a 130 kDa protein consisting of two glycosylated and phosphorylated 65 kDa subunits, similar to those of the follicle cell derived yolk protein of *B. mori*. The second is a slightly larger 140 kDa protein that is glycosylated but not phosphorylated. In the pyralid moth *P. interpunctella*, two yolk polypeptides originate in the follicle cells, and the 235–264 kDa protein with subunits of 69 and 33 kDa is an ortholog of the egg specific protein in *B. mori* (Shirk *et al.*, 1984; Bean *et al.*, 1988; Shirk and Perera, 1998). Sequences for a cDNA clone encoding YP4 of *P. interpunctella* (Perera and Shirk, 1999) showed it to be an ortholog of a follicular product in *G. mellonella* (Rajaratnam, 1996).

3.9.2.3. Mechanisms of Juvenile Hormone Action in Vitellogenesis

3.9.2.3.1. Induction of vitellogenin gene expression Vitellogenic action of JH is one of the hallmarks of insect reproduction. Although JH is known to initiate vitellogenesis in many insects (see **Chapter 3.7**), its precise mechanism of action is poorly understood. Studies of the induction of *Vg* gene transcription by JH have focused on two species: the migratory locust, *L. migratoria*, and the German cockroach, *B. germanica* (Wyatt, 1991, 1997; Belles, 2004).

Two *Vg* genes have been partially characterized in *L. migratoria*, and studies at the molecular level have demonstrated the absence of detectable Vg mRNA in the fat body of JH deprived specimens (Dhadialla *et al.*, 1987). Transcription of both *Vg* genes is coordinately induced *in vivo* by JH or JHA (Dhadialla *et al.*, 1987; Glinka and Wyatt, 1996), but allatectomized locust females have a low sensitivity to JH, since doses up to 100 μg of JH III do not induce Vg production. JHAs, such as methoprene or pyriproxyfen, are more potent and stable (Wyatt *et al.*, 1996). When JHA is administered to females chemically allatectomized with the allatocide precocene within 1 day of adult emergence, there is a lag of 12–24 h before Vg mRNA can be detected (Edwards *et al.*, 1993). This lag period can be shortened by prior administration of a sub-effective dose of JH or JHA that is insufficient by itself to induce *Vg* gene transcription. This dose likely primes the fat body cells for an accelerated response to a subsequent effective dose (**Figure 7**) (Edwards *et al.*, 1993; Wyatt *et al.*, 1996). The lag period between JHA treatment and vitellogenesis can be extended by inhibition of protein synthesis with cycloheximide (**Figure 7**) (Edwards *et al.*, 1993). These results suggest that the action of JH on *Vg* genes in locust females is indirect and requires the synthesis of protein factors involved in transcription. In this respect, the priming action of JH on fat body for a vitellogenic response is similar to that in mosquitos in which the molecular nature of this JH action has been recently elucidated (Zhu *et al.*, 2003a).

Studies on the regulation of vitellogenesis in *B. germanica* also were facilitated by the cloning of a Vg cDNA (Martin *et al.*, 1998; Comas *et al.*, 2000). Experiments *in vivo* with allatectomized females have shown that Vg mRNA can be detected as early as 2 h after treatment with 1 μg of JH III, whereas Vg protein can be detected 2 h later (**Figure 8**). In addition, dose–response studies show that doses of 0.1, 1, and 10 μg of JH III induced Vg

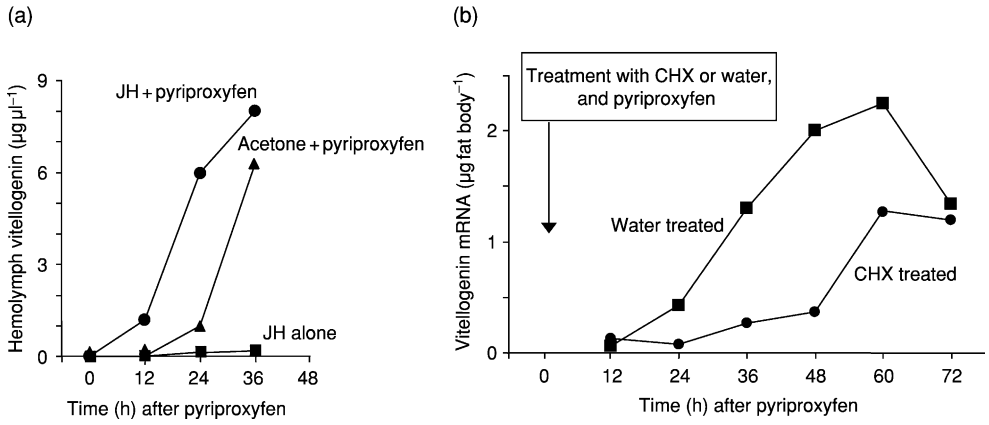

Figure 7 Induction of vitellogenin transcription in chemically allatectomized females of the migratory locust, *Locusta migratoria*. (a) Shortening of the response lag time by administration of a subeffective dose of JH. Specimens pretreated with low doses of JH III in acetone (four applications of 10 μg each, over 48 h) and then treated with 10 μg of pyriproxyfen, synthesize vitellogenin earlier (circles) than those receiving an equivalent treatment of acetone alone and pyriproxyfen (triangles). Equivalent pretreatment with JH III, but no pyriproxyfen, did not induce detectable vitellogenin synthesis (squares). (Data from Wyatt, G.R., Braun, R.P., Zhang, J., **1996**. Priming effect in gene activation by juvenile hormone in locust fat body. *Arch. Insect Biochem. Physiol. 32*, 633–640.) (b) Inhibitory effects of cycloheximide (CHX) upon vitellogenesis induced by pyriproxyfen. Insects were treated with cycloheximide (62 μg) in water or with water alone, and 1 h later treated with 10 μg pyriproxyfen. Cycloheximide delayed vitellogenin transcription by about 1 day, which is approximately equal to the duration of inhibition of protein synthesis. (Reproduced with permission from Edwards, G.C., Braun, R.P., Wyatt, G.R., **1993**. Induction of vitellogenin synthesis in *Locusta migratoria* by the juvenile hormone analog, Pyriproxyfen. *J. Insect Physiol. 39*, 609–614.)

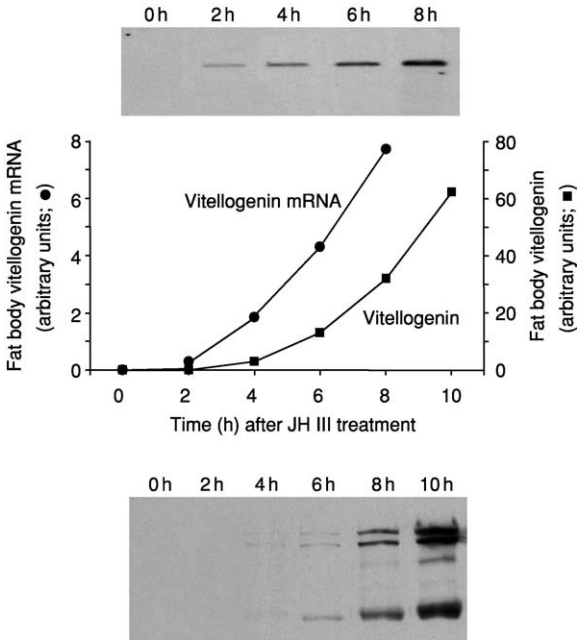

Figure 8 Production of vitellogenin mRNA and vitellogenin protein *in vivo* after JH treatment of allatectomized females of *Blattella germanica*. A dose of 1 μg of JH III was topically applied to 48 h old allatectomized females; the fat body was dissected 2, 4, 6, 8, or 10 h later, and analyzed for vitellogenin mRNA (Northern blot, above), or vitellogenin protein (Western blot, below). (Reprinted with permission from Comas, D., Piulachs, M.D., Belles, X., **1999**. Fast induction of vitellogenin gene expression by juvenile hormone III in the cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae). *Insect Biochem. Mol. Biol. 29*, 821–827, with permission from Elsevier.)

synthesis, but not 0.01 μg (Comas *et al.*, 1999, 2001). Cycloheximide applied to fat body *in vitro* abolishes the vitellogenic effects of JH (Comas *et al.*, 2001), again suggesting that the effect of JH on the *Vg* gene involves the synthesis of protein factors involved in transcription.

3.9.2.3.2. Potential response elements in JH-dependent genes related to vitellogenesis Results from the above studies of JH action on vitellogenesis in the locust and cockroach led to the hypothesis that JH may affect proteins belonging to the nuclear hormone receptor superfamily (see **Chapters 3.5** and **3.6**), which in turn would mediate transcription of Vg genes (Wyatt *et al.*,1996; Belles, 2004; see **Chapter 3.5**). Signature response elements for the binding of the putative JH transcription factors could be found within the regulatory regions of these genes, in the same way as those for the ecdysteroid receptor (EcR) and related proteins.

Analysis of the *jhp21* gene of *L. migratoria* revealed the partially palindromic, 13-nucleotide motif AGGTTCGAG$^{A}/_{T}$CCT that is found in three copies from the transcription start point (Zhang and Wyatt, 1996). This motif is suggestive of a hormone response element, given that it is similar to the consensus ecdysteroid response element (see **Chapter 3.5**), as defined by Jiang *et al.* (2000). Furthermore, this nucleotide motif is very similar to the canonical sequence IR-1 (AGGTCAATGACCT), a

consensus-inverted repeat with a single nucleotide spacer that is recognized by the ecdysteroid receptor and that confers JH responsiveness upon genes in mammalian cells that contain farnesoid x receptor (FXR), a member of the nuclear receptor superfamily (Forman *et al.*, 1995).

3.9.2.3.3. Molecular mechanisms in juvenile hormone action Hypothesized JH specific DNA motifs require functional characterization, either by demonstrated activity in gene regulation or by binding to a known JH receptor or a transcription factor involved in JH response. Significant progress in this regard has been made on the 13-nucleotide motif AGGTTCGAG$^{A}/_{T}$CCT from the upstream region of the *jhp21* gene in *L. migratoria*. A cell-free transcription system was developed that uses nuclear proteins extracted from locust fat body, and transcription is measured with reporter constructs containing a short DNA sequence, lacking G in the transcribed strand, fused to the promoter sequence of interest (Zhang *et al.*, 1996). When transcription is carried out with the transcription terminator *O*-methyl-GTP and not GTP, only the DNA sequence lacking G is transcribed, and the transcript can be resolved by gel electrophoresis. With nuclear extracts from untreated adult female *L. migratoria*, constructs that include the promoter region of the vitellogenin or *jhp21* genes are transcribed, as is also the nonspecific promoter of the adenovirus major late antigen (*AdML*), which is used as a positive control. However, extracts from precocene treated females, while still transcribing *AdML*, fail to transcribe from the *jhp21* promoter (Zhang *et al.*, 1996). After transcription was found to be specific for the reproductive female fat body, truncated constructs of the promoter region of the *jhp21* gene were used to show that the DNA between nucleotides −1056 and −1200 from the transcription start site strongly enhanced transcription. In synthetic constructs, the incorporation of two tandem copies of the 15 nucleotide element GAGGTTCGAGACCTC (found at −1152) stimulated transcription as strongly as the native 145 nucleotide sequence, whereas the 15 nucleotide element, mutated at four positions and inserted into two copies, was inactive (Zhang *et al.*, 1996). These results suggested that the sequence GAGGTTCGAGACCTC might be a JH response element. Tests of nuclear extracts for specific protein binding to the putative JH response element with the electrophoretic mobility shift assay demonstrated the occurrence of a specific DNA binding protein in extracts from JH exposed fat body, whereas it was found to be lacking in JH deprived tissue (Zhang *et al.*, 1996). Furthermore, Zhou *et al.* (2002) have reported that this binding shows a preference for the inverted repeat GAGGTTC in the left half-site and that it is abolished by phosphorylation catalyzed by a protein kinase C present in the nuclear extracts. These results further support the identification of a putative JH response element that is bound by a transcription factor brought to an active state by JH. It is still uncertain whether the binding protein may be the nuclear JH receptor, a dimerization partner of the receptor, or another protein factor involved in the transcriptional process (Wyatt, 1997).

A putative JH response element has been identified in the JH esterase gene (*Cfjhe*) from the spruce budworm, *C. fumiferana* (Kethidi *et al.*, 2004). This 30 bp region contains two conserved hormone response element half-sites separated by a 4 nucleotide spacer similar to the direct repeat 4 (DR-4). The response element designated as JHRE is located between −604 and −574 of the *Cfjhe* promoter and is sufficient to support JH I induction. At the same time, the same region is responsive for 20E mediated repression of this gene. When a luciferase reporter was placed under the control of JHRE, a minimal promoter was induced by JH I in a dose and time dependent manner in a cell transfection assay. Moreover, the gel-retardation assay revealed the presence of a JHRE binding protein in the nuclear extract isolated from JH I treated CF-203 cells. The results described in the following sections suggest the hypothesis that JH acts via a nuclear receptor mode of action at the transcriptional level.

3.9.2.3.4. Is there a juvenile hormone nuclear receptor? Early experiments studying farnesol related molecules as possible ligands for the retinoid x receptor (RXR)–FXR mammalian receptor complex showed that JH III activated this complex (although it did not activate FXR or RXR alone), whereas methoprene did not (Forman *et al.*, 1995). Curiously enough, an independent study reported that RXR alone could be activated by methoprene acid but not by JH III (Harmon *et al.*, 1995). Although the physiological significance of these experiments remained unclear, attention has been drawn to *Ultraspiracle* (USP) (insect ortholog of RXR and obligatory dimerization partner for the EcR) as a possible candidate for a JH receptor.

Work along this line was published by Jones and Sharp (1997), who reported that JH at micromolar concentrations binds to *D. melanogaster* USP, modifying its conformation and inducing USP dependent transcription, whereas yeast two-hybrid assays indicated that JH could promote USP homodimerization (see **Chapters 3.5** and **3.7**). In response to ligand binding, *D. melanogaster* USP undergoes

conformational change to form a multihelix hydrophobic groove for recruitment of transcriptional coactivators (Jones and Jones, 2000). In addition, JH III binds to the ligand binding pocket of USP, and application of JH III to cells activates a transfected reporter construct containing a JH esterase core promoter and a DR12 hormone response element. The DR12 element confers enhanced transcriptional JH responsiveness, and it binds with specificity to recombinant USP (Jones *et al.*, 2001; Xu *et al.*, 2002).

In more primitive insects, USP has a ligand binding domain closer to vertebrate RXR than to dipteran or lepidopteran USP (Bonneton *et al.*, 2003). This has raised the question of whether an RXR ortholog of primitive insects might bind JH with higher affinity than USPs of higher insects. This possibility has been explored in *L. migratoria*, in which two isoforms of RXR are known: LmRXR-L is more closely related to vertebrate RXR and LmRXR-S has a deletion of 66 nucleotides in the ligand binding domain (Hayward *et al.*, 2003). Both LmRXR-S and LmRXR-L formed heterodimers with locust LmEcR *in vitro*, which bound the active ecdysteroid ponasterone A, as expected. However, neither LmRXR isoform alone nor LmRXR heterodimerizing with LmEcR bound JH III at nanomolar concentrations (Hayward *et al.*, 2003). Parallel work in *B. germanica* has led to cloning of the orthologs BgRXR-L and BgRXR-S. Interestingly, despite the highly fluctuating levels of circulating JH III, mRNA levels of both BgRXR-L and BgRXR-S isoforms in the fat body remain constant throughout the vitellogenic cycle (Maestro, Martin and Belles, unpublished data).

If either insect USP or RXR is a JH receptor, it behaves in a very different way to other members of the steroid/thyroid hormone nuclear receptor family, especially since its binding of JH is not saturable and is of relatively low affinity. One possibility is that USP or RXR form a heterodimer with a nuclear receptor other than EcR and that this complex might play the role of a JH receptor. Another possibility is inspired by promiscuous nuclear receptors like RXR, PPAR, or PXR of vertebrates (Harmon *et al.*, 1995; Zomer *et al.*, 2000; Watkins *et al.*, 2001), which are activated by a variety of effectors and have K_d values in the micromolar range. These nuclear receptors have been considered as chemical sensors rather than canonical specific receptors (Watkins *et al.*, 2001), a concept that could be extended to the interaction of JHs with the RXR/USP of insects.

The *Methoprene resistant* (*Met*) gene of *Drosophila* may also be a useful model for gaining insight into the molecular action of JH. Mutant *D. melanogaster* resistant to methoprene were generated in the 1980s, and these *Met* mutants were also highly resistant to JH III, JH bisexpoxide (JHB_3), and other JHAs, but not to conventional insecticides. Biochemical work later showed the presence of an 85 kDa protein in various tissues of wild-type *D. melanogaster* that bound specifically to JH III with high affinity ($K_d = 6.7$ nM), whereas in *Met* mutants the affinity of this protein for the hormone was lower by six-fold. In addition, JH III stimulation of protein synthesis in male accessory glands was clearly less pronounced in *Met* mutants than in wild-type flies, which suggested that *Met* is involved in the mechanism of action of JH (Shemshedini and Wilson, 1990; Shemshedini *et al.*, 1990; see **Chapter 3.7**). Sequencing of the corresponding gene showed that *Met* is a member of the bHLH-PAS family of proteins that behave as transcriptional regulators (Ashok *et al.*, 1998). These proteins show a basic helix–loop–helix domain (bHLH), which is characteristic of a number of transcription factors, and a PAS domain, named after the first members of the family: the products of the genes *period* (*Per*) (see **Chapter 4.11**) and *single-minded* (*Sim*) from *D. melanogaster*, and the aryl hydrocarbon receptor (AhR) and the Ah receptor nuclear translocator (ARNT) from vertebrates. Interestingly, *Per* and *Sim* have dimerization partners but no known ligands. AhR and ARNT form a functional dimer in the presence of a ligand, which upregulates a series of genes, the products of which metabolize foreign chemicals. Although *Met* mutants show no serious problems during embryonic and larval development or during metamorphosis (Wilson and Ashok, 1998), it is tempting to consider that the *Met* product could be involved in the transcription of JH-target genes, either by being the JH receptor or a transcription factor of the cascade triggered by JH.

These results also support the hypothesis that *Met* could be a receptor coactivator for the JH receptor, playing a role similar to that reported for the steroid hormone coactivator-1, the disruption of which results in hormone resistance in mice (Xu *et al.*, 1998). In this context, it is worth noting that the steroid hormone coactivator-1 also belongs to the bHLH-PAS family and that it and *Met* have the LXXLL motif, which in vertebrate transcriptional activators is needed for binding to nuclear receptors.

The identification of JH response elements in JH responsive genes might be the most promising approach to the characterization of the JH receptor. This strategy is being followed for the *jhp21* gene in *L. migratoria* and for the *cfihe* gene in

C. fumiferana, with the encouraging results described above. Also promising has been the discovery of JH responsive genes in *D. melanogaster*, such as *JhI-1*, *JhI-21*, *JhI-26*, and *minidiscs* (*mnd*) (Dubrovsky, 2002), whose promoter regions may provide the needed JH response elements more easily, given the genetic, conceptual, and technical background available in studies of this fly. Along this line, promoter regions of the *JhI-26* and *mnd* genes, which are directly inducible by JH, have already been tested for their ability to confer JH inducibility on heterologous reporter genes transfected to S2 cells. Constructs prepared with a 3 kb fragment from a region upstream of either the *JhI-26* or the *mnd* transcription initiation site cloned in front of the *hsp70-lacZ* reporter gene have served for the first experiments. After transfection and methoprene induction, the *JhI-26* promoter insertion conferred between a 6- and 15-fold increase in the 8-galactosidase activity, whereas that of *mnd* conferred between a three- and fourfold increase (Dubrovsky, 2002). The results suggest that these 3 kb promoter fragments contain JH response elements, and thus further study of shorter fragments should lead to the determination of their role. In the meantime, *in silico* studies on the promoter fragments used in the transfections have revealed several motifs showing a significant similarity with the estrogen response element (ERE), suggesting that they can be considered putative response elements (Dubrovsky, 2002).

3.9.2.4. Molecular Mechanisms of Ecdysteroid Action in Vitellogenesis

The early 20E-inducible gene *E75* has been implicated in the JH signaling pathway (Dubrovsky *et al.*, 2004). JH induces the E75A orphan nuclear receptor in *D. melanogaster*. The induction by JH is rapid and does not require protein synthesis, indicating direct action of JH on the *E75* gene. *E75A* mRNA is reduced in ovaries of *apterous*^{4} *Drosophila* mutant adults defective in JH secretion. However, *E75A* mRNA expression can be rescued by topical JH analog application. Furthermore, ectopic expression of E75A is sufficient to perform several functions of the JH signaling pathway. Ectopic E75 can downregulate its own transcription and potentiate the JH inducibility in the JH gene, *JhI-21*. In the presence of JH, E75A can also repress 20E activation of early genes including *Broad*. The occurrence of a putative response element for E75 (the motif TGACCAAATT) (Belles, 2004) in the promoter region of the Vg gene of *B. germanica*, led to clone the three isoforms of this transcription factor, BgE75A, BgE75B, and BgE75C, in this cockroach. The pattern of expression of the three isoforms and results of RNAi experiments suggest that JH enhances E75 induction by 20E and that E75 is involved in the modulation of the cycles of oocyte maturation in *B. germanica* (D. Mañé, D. Martin and X. Belles, unpublished).

In contrast to JH, the molecular mechanisms governing 20E regulation of reproductive events have been elucidated in detail for the yellow fever mosquito, *A. aegypti*, and the fruit fly, *D. melanogaster* (see **Chapters 3.5** and **3.6**). Owing to variations in their reproductive biology, studies of these insects often cover different events in female reproductive endocrinology, thus providing complementary information.

3.9.2.4.1. Genetic regulation of ecdysteroid response Ashburner *et al.* (1974) proposed a hierarchical model for the genetic regulation of polytene chromosome puffing by 20E (see **Chapter 3.5**). According to this model, the binding of 20E to its cognate receptor triggers the expression of a small set of early genes, which in turn activate expression of a large set of late genes and which, meanwhile, repress their own transcription. Subsequent genetic and molecular biological studies have revealed events underlying this ecdysteroid regulatory hierarchy in *D. melanogaster* (Thummel, 1996, 2002; Bender, 2003). Responses to ecdysteroids are mediated by the EcR complex, which consists of two members of the nuclear receptor gene family, EcR and USP, the latter being the retinoid X receptor homolog (Koelle *et al.*, 1991; Yao *et al.*, 1992, 1993). Upon binding 20E, this heterodimer recognizes a sequence specific DNA motif, ecdysteroid response element (EcRE), and directly induces the transcription of a small set of so-called early genes. These early genes encode mainly transcription factors, including E74, E75, and Broad-Complex (BR-C), of the Ets, nuclear receptor, and zinc-finger type, respectively, that transfer and amplify the hormonal signal through the regulation of numerous late ecdysone-responsive genes that specify stage and tissue specific effects of 20E (Thummel, 1996, 2002). The *E74*, *E75*, and *BR-C* early genes each encode a family of transcription factors by use of multiple promoters and alternative splicing. Biochemical and mutational analysis has shown that each of these genes acts as a 20E induced transcription factor and is required for the hormonal response in target tissues (Thummel, 2002; Bender, 2003).

3.9.2.4.2. Ecdysteroid receptor (EcR) The diversity of cellular responses to 20E may require particular combinations of EcR isoforms. Indeed,

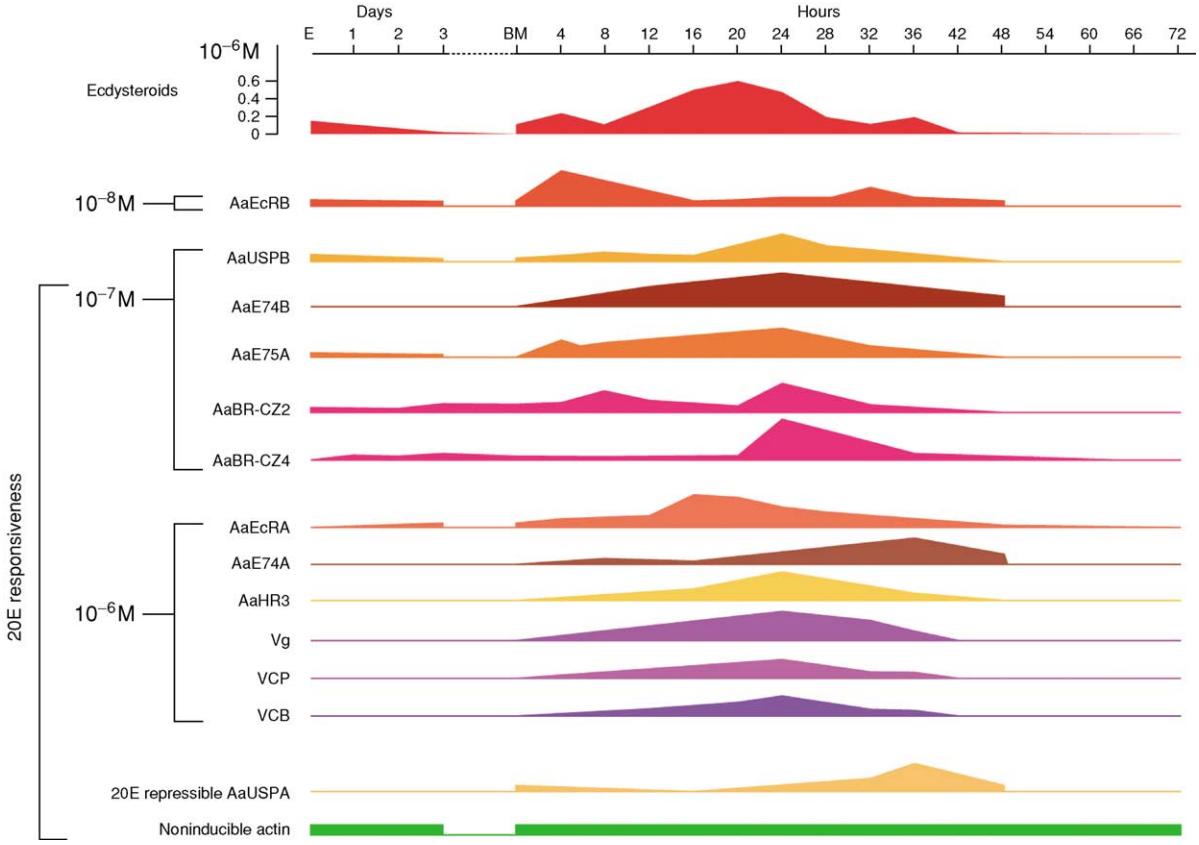

Figure 9 Expression profiles and hormone responsiveness of the key factors of the 20-hydroxyecdysone (20E) regulatory hierarchy and their target genes, yolk protein precursors (*YPP*), during vitellogenesis in *Aedes aegypti*. Hormone titers of 20E in *A. aegypti* females (Hagedorn *et al.*, 1975; Shapiro *et al.*, 1986). Transcript profiles and sensitivities of the transcription factors to 20E (AaEcRA and AaEcRB, AaUSPA and AaUSPB, AaE74A and AaE74B, and AaHR3) and the late genes (*Vg*, *VCP,* and *VCB*) were determined by Northern or RT-PCR analyses. Adapted from Cho and Raikhel (1992; *Vg*), Cho *et al.* (1991; *VCP*), Cho *et al.* (1999; *VCB*), Wang *et al.* (2002; AaEcRA and AaEcRB), Wang *et al.* (2000b; AaUSPB and the 20E repressible AaUSPA), Sun *et al.* (2002; AaE74A and AaE74B), Pierceall *et al.* (1999; AaE75A), Kapitskaya *et al.* (2000; AaHR3), and L. Chen, J. Zhu, and A.S. Raikhel (unpublished data; BR-C isoforms Z2 and Z4). E, eclosion; BM, blood meal. (Modified with permission from Sun, G.Q., Zhu, J.S., Raikhel, A.S., **2004**. The early gene E74B isoform is a transcriptional activator of the ecdysteriod regulatory hierarchy in mosquito vitellogenesis. *Mol. Cell. Endocrinol. 218*, 95–105.)

expression of specific *D. melanogaster* EcR isoforms could be correlated with patterns of responses of particular cell or tissue types to 20E (Talbot *et al.*, 1993). Genetic studies have confirmed that different EcR proteins are functionally distinct, with the greatest difference being between the EcR-A and EcR-B isoforms and the greatest overlap being between the EcR-B1 and EcR-B2 functions (Bender *et al.*, 1997; Schubiger *et al.*, 1998; Lee *et al.*, 2000; see **Chapter 3.5**).

Cloning of the *A. aegypti* EcR isoforms, AaEcR-A and AaEcR-B, has facilitated evaluation of their expression during mosquito vitellogenesis. Significantly, transcripts of both isoforms exhibit different patterns of expression after a blood meal triggers fat body vitellogenesis in female mosquitos (**Figure 9**). The AaEcR-B transcript level rose sharply by 4 h PBM, which coincides with the small ecdysteroid peak, and then declined and reached its lowest level at 16–24 h PBM. In contrast, the AaEcR-A transcript peaked at 16–20 h PBM, coinciding with the large ecdysteroid peak (Cho *et al.*, 1995; Wang *et al.*, 2002). Both isoform mRNAs were transcribed in a cycloheximide independent manner, suggesting that they are direct targets of 20E. However, AaEcR-A transcription requires the continuous presence of 20E, while the AaEcR-B mRNA level rose for 4 h and then declined under the same conditions. These results indicate that the mosquito EcR isoforms play distinct physiological functions during vitellogenesis in the mosquito fat body.

3.9.2.4.3. USP isoforms Like the EcR isoforms, two USP cDNA isoforms (AaUSP-A and AaUSP-B) were cloned from *A. aegpyti* (Kapitskaya *et al.*, 1996). They differ only at the N-terminus, thus

indicating their origin from the same gene, which recently has been confirmed by cloning the *USP* gene from *A. aegypti* (Wang, Zhu, and Raikhel, unpublished data). The isoform specific expression patterns in the *Aedes* fat body during vitellogenesis suggest that the two mosquito USP isoforms may carry out distinct functions. The level of AaUSP-B mRNA correlates with the high titer of ecdysteroid at 16–20 h PBM (Wang *et al.*, 2000b). Intriguingly, USP-B is activated with 20E, whereas USP-A is inhibited by the hormone (**Figure 9**) (Wang *et al.*, 2000b). AaEcR heterodimerizes with either mosquito USP isoform; the AaEcR-USP-B heterodimer displays stronger DNA binding and transactivation activities than the AaEcR-USP-A heterodimer (Wang *et al.*, 2000b).

3.9.2.4.4. Ecdysteroid response elements (EcREs) The canonical steroid receptor binding site is a DNA element with an AGGTCA consensus sequence that binds one or two receptor molecules as monomers or as homodimers and/or heterodimers (Tsai and O'Malley, 1994). The binding sites can be arranged as single half sites or as inverted, direct, or everted repeats. Although DNA binding by EcR-USP is highly sequence specific, a surprising variety of sequences have been identified as ecdysteroid response elements in *D. melanogaster* (see **Chapter 3.5**), suggesting that variability of EcREs provides yet another level of specificity in 20E gene regulation (Riddihough and Pelham, 1987; Cherbas *et al.*, 1991; Antoniewski *et al.*, 1994, 1995, 1996; D'Avino *et al.*, 1995; Horner *et al.*, 1995; Lehmann and Korge, 1995).

The DNA binding properties of the AaEcR-AaUSP heterodimer have been systematically analyzed with respect to the effects of nucleotide sequence, orientation, and spacing between half sites in natural *D. melanogaster* and synthetic EcREs (Wang *et al.*, 1998). AaEcR-AaUSP exhibits a broad binding specificity, forming complexes with inverted repeats (IRs) and direct repeats (DRs) of the nuclear receptor response element half-site consensus sequence AGGTCA separated by spacers of varying length. A single nucleotide spacer was optimal for both imperfect (IR^{hsp}-1) and perfect (IR^{per}-1) inverted repeats. Spacer length was less important in DRs of AGGTCA (DR-0 to DR-5). Although 4 bp was optimal, DR-3 and DR-5 bound AaEcR-AaUSP almost as efficiently as DR-4. Furthermore, AaEcR-AaUSP also bound DRs separated by 11–13 nucleotide spacers. Cotransfection assays utilizing CV-1 cells have demonstrated that the mosquito EcR-USP heterodimer is capable of transactivating reporter constructs containing either IR-1 or DR-4. The levels of transactivation are correlated with the respective binding affinities of the response elements (IR^{per}-1 > DR-4 > IR^{hsp}-1). Taken together, these analyses predict broad variability in the EcREs of mosquito ecdysteroid responsive genes (Wang *et al.*, 1998). These analyses enabled the identification of a functionally active EcRE that binds the heterodimer EcR-USP in the *A. aegypti Vg* gene. A direct repeat with a 1 bp spacer (DR-1) with the sequence AGGCCAaTGGTCG is the major part of the EcRE in the *Vg* gene (Martin *et al.*, 2001a).

3.9.2.4.5. Ecdysteroid early response genes The *A. aegypti E75* homolog consists of three overlapping transcription units encoding E75A, E75B, and E75C isoforms (Pierceall *et al.*, 1999), which are members of the nuclear receptor family. As in *D. melanogaster*, these E75 transcripts arise by alternative promoter usage and splicing of transcription unit-specific 5′ exons onto a set of shared downstream exons. All three transcripts are induced at the onset of vitellogenesis by a blood meal and are highly expressed in the mosquito ovary and fat body, which suggests that they are involved in the regulation of oogenesis and vitellogenesis, respectively. Furthermore, *in vitro* fat body experiments have demonstrated that AaE75 isoforms are induced by 20E. In contrast to the yolk protein transcripts, which show only a small gradual increase during the first 4 h PBM, E75 transcripts exhibit a sharp rise immediately after the onset of vitellogenesis, reaching a peak at 3–4 h PBM, when the hormone titer shows a first peak. After falling from their initial peak, the level of mosquito E75 transcripts gradually increase again, reaching the second peak at 18–24 h PBM, which coincides with the accumulation of yolk protein RNA in the fat body (**Figure 9**). E75 isoform transcription is appropriately 10 times more sensitive to 20E than is the *Vg* gene (Pierceall *et al.*, 1999).

Two isoforms of the homolog to the *D. melanogaster* transcription factor E74, which share a common C-terminal Ets DNA binding domain, yet have unique N-terminal sequences, are present in the mosquito. They exhibit a high level of similarity to DmE74 isoforms A and B and show structural features typical for members of the Ets transcription factor superfamily (Sun *et al.*, 2002). Furthermore, both mosquito E74 isoforms bind to a *D. melanogaster* E74 binding site with the consensus motif C/AGGAA. E74B mRNA reaches its peak at 24 h PBM and drops sharply thereafter, correlating with peak expression of the *Vg* gene. The AaE74B transcript is induced by a blood meal activated hormonal cascade in fat body and peaks at 24 h PBM, the peak of

vitellogenesis (**Figure 9**). However, unlike E75 transcripts, E74B does not have an early peak at 3–4 h. AaE74A is activated at the termination of vitellogenesis and exhibits a peak at 36 h PBM in the fat body and 48 h PBM in the ovary. The AaE74A and AaE74B isoforms most likely play different roles in regulation of vitellogenesis in mosquitos, as an activator and a repressor of YPP gene expression, respectively. Both AaE74 isoform mRNAs are induced by 20E in fat body *in vitro* and display superinduction when cycloheximide is applied together with 20E (Sun *et al.*, 2004). While both E74 isoforms are capable of binding to the E74 consensus sequence, only E74B induces a reporter gene expression through the consensus E74 binding site in cell transfection assays. Furthermore, a transient transfection assay using the *Vg* promoter region containing putative E74 binding sites has shown that E74B functions as an activator, whereas E74A serves as a repressor (Sun *et al.*, 2004).

The BR-C plays a key role in the genetic control of 20E responses. The BR-C encodes a family of DNA binding proteins that share a common (core) N-terminus fused by alternative splicing to one of four pairs of C2H2-type zinc finger domains, Z1, Z2, Z3, and Z4. All four BR-C isoforms are present in *A. aegypti* (Chen, Zhu, and Raikhel, unpublished data). In female fat body, expression of Z1, Z2, and Z4 is induced immediately after a blood meal. Both Z1 and Z4 transcripts exhibit a peak at 24 h PBM. The Z2 transcript exhibits a sharp increase after onset of vitellogenesis, reaching initial peak at 8 h PBM. After an initial decline, the level of Z2 rises again at 24 h PBM (**Figure 9**). The expression of BR-C Z1, Z2, and Z4 in the fat body is attenuated between 24 h and 36 h PBM. Z3 transcript expression in the fat body is very low throughout the vitellogenic cycle. The overall level of Z4 has been found to be much higher than those of the other isoforms. In the fat body *in vitro* experiments, the mRNA levels of all four isoforms were rapidly induced by 20E and increased in a dose dependent manner. Functional analysis using RNAi experiments has suggested that Z1 and Z4 serve as repressors and that Z2 serves as an activator of *Vg* gene expression. Remarkably, RNAi knockdown of Z1 or Z4 extends *Vg* expression to 36 h PBM, which suggests that these factors regulate hormone dependent timing of this gene transcription (Chen, Zhu, and Raikhel, unpublished data).

Analyses of 20E sensitivity by transcripts of several key mediators and their expression profiles in vitellogenic fat body of the mosquito have revealed potential roles in the 20E gene regulatory hierarchy cascade, thus providing a road map for future studies (Pierceall *et al.*, 1999; Kapitskaya *et al.*, 1998; Li *et al.*, 2000; Wang *et al.*, 2000b, 2002; Sun *et al.*, 2002, 2004) (**Figure 9**). At the top of the 20E cascade, AaEcRB, the dominant isoform for initiating mosquito vitellogenesis *in vivo*, exhibits high sensitivity to 20E at 10^{-8} M, and it is expressed predominantly during the first hours after blood meal activation of vitellogenesis (Wang *et al.*, 2002) (**Figure 9**). Its obligatory partner AaUSP-B, early gene products AaE74B and AaE75A, and BR-C isoforms constitute the group with the second highest sensitivity to 20E at 10^{-7} M (Pierceall *et al.*, 1999; Wang *et al.*, 2000; Sun *et al.*, 2004; Chen, Zhu, and Raikhel, unpublished data). AaEcR-A, AaE74A, and the early late gene *AaHR3* form the lowest 20E sensitivity group, being maximally activated at 10^{-6} M, which is similar to the target genes *Vg, VCP*, and *VCB* (**Figure 9**).

3.9.2.4.6. Hormonal enhancers in the regulatory region of mosquito yolk protein genes Transcriptional activation of hormonally controlled genes in specific tissues depends on interactions between sequence specific transcription factors and enhancer/promoter elements of these genes. Analysis of the 5′-upstream regulatory region of the mosquito *Vg* gene has revealed putative binding sites for EcR-USP and the early genes, E74, E75, and BR-C, which indicate this gene is regulated through a combination of direct and indirect hierarchies (**Figure 10**). Analyses of *D. melanogaster* and *A. aegypti* transformations, as well as DNA binding assays, have identified *cis*-regulatory sites in the *Vg* gene for stage and fat body specific activation via a blood meal triggered cascade (Kokoza *et al.*, 2001). Three regulatory regions in the 2.1 kb, 5′ region of the *Vg* gene are required for its blood meal activation and high-level expression (**Figure 11**). The proximal region, adjacent to the basal transcription start site, contains binding sites for several transcription factors that direct tissue and stage specific expression: EcR/USP, GATA transcription factor (GATA), CAAT binding protein (C/EBP), and hepatocyte nuclear factor 3/forkhead transcription factor (HNF3/fkh). It appears that a combinatorial action of these transcription factors brings about fat body specific expression. EcR/USP acts as a timer, allowing the gene to be turned on, but the level of expression driven through this response element is low. The median region contains the sites for early gene factors E74 and E75, and transgenic studies have shown that this region is required for a stage specific hormonal enhancement of the *Vg* gene expression

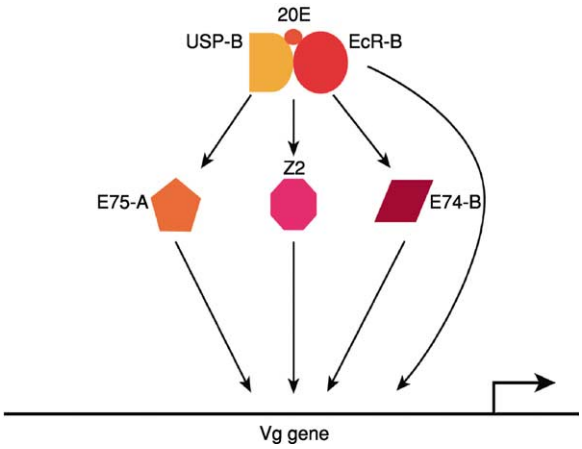

Figure 10 Direct and indirect regulation of vitellogenin (*Vg*) gene by 20-hydroxyecdysone in the mosquito fat body. The activated ecdysteroid receptor, consisting of EcR-B/USP-B binds to the EcRE response element in the regulatory region of the *Vg* gene. This binding is required to permit the *Vg* gene expression. After binding 20E, the EcR-USP heterodimer also activates early genes, *E74, E75*, and *BR-C*. The products of these genes E74-B, E75-A, and BR-C Z2 act as powerful activators of the *Vg* gene. (Based on Pierceall, W.E., Li, C., Biran, A., Miura, K., Raikhel, A.S., *et al.*, **1999**. E75 expression in mosquito ovary and fat body suggests reiterative use of ecdysone-regulated hierarchies in development and reproduction. *Mol. Cell. Endocrinol. 150*, 73–89; Kokoza, V.A., Martin, D., Mienaltowski, M.J., Ahmed, A., Morton, C.M., *et al.*, **2001**. Transcriptional regulation of the mosquito vitellogenin gene via a blood meal-triggered cascade. *Gene 274*, 47–65; Martin, D., Wang, S.F., Raikhel, A.S., **2001b**. The vitellogenin gene of the mosquito *Aedes aegypti* is a direct target of ecdysteroid receptor. *Mol. Cell. Endocrinol. 173*, 75–86; Sun, G.Q., Zhu, J.S., Li, C., Tu, Z.J., Raikhel, A.S., **2002**. Two isoforms of the early E74 gene, an Ets transcription factor homologue, are implicated in the ecdysteroid hierarchy governing vitellogenesis of the mosquito, *Aedes aegypti. Mol. Cell. Endocrinol. 190*, 147–157; L. Chen, J. Zhu, and A.S. Raikhel, unpublished data.)

(Kokoza *et al.*, 2001). Furthermore, *in vitro* cell transfection experiments have demonstrated that the ecdysteroid receptor and E74B act synergistically to bring about a high-level, 20E activated *Vg* gene expression (Sun and Raikhel, unpublished data). Finally, the distal portion is characterized by multiple response elements for a GATA transcription factor (Martin *et al.*, 2001b). In transgenic experiments using both *A. aegypti* and *D. melanoagaster*, this GATA- rich region is required for high expression levels characteristic to the *Vg* gene (**Figure 11**).

Recent analyses of the *A. aegypti Vg* promoter region have identified many binding sites for the BR-C transcription factors: one for Z1, two for Z2, three for Z3, and seven for Z4. When BR-C isoform expression vectors were introduced into Kc cells together with EcR and USP expression vectors in the presence of 20E, Z1 and Z4 repressed the activation mediated by EcR-USP, while Z2 enhanced this activation. The role of Z3 was negligible. When the Z1 binding site was mutated, luciferase activity driven by the *Vg* promoter was no longer inhibited by the overexpression of Z1. After removal of Z2 binding sites in the *Vg* promoter, Z2 lost the capability to increase luciferase activity. However, because there are seven binding sites of BR-C Z4 on the *Vg* promoter, the repression of *Vg* promoter by Z4 could not be completely released after mutation of all the Z4 binding sites. These experiments support the RNAi test performed *in vivo* and suggest that BR-C Z1 and Z4 serve as repressors while Z2 is an activator of the *Vg* gene expression. Z4 may not directly bind to the *Vg* promoter (Chen, Zhu, and Raikhel, unpublished data).

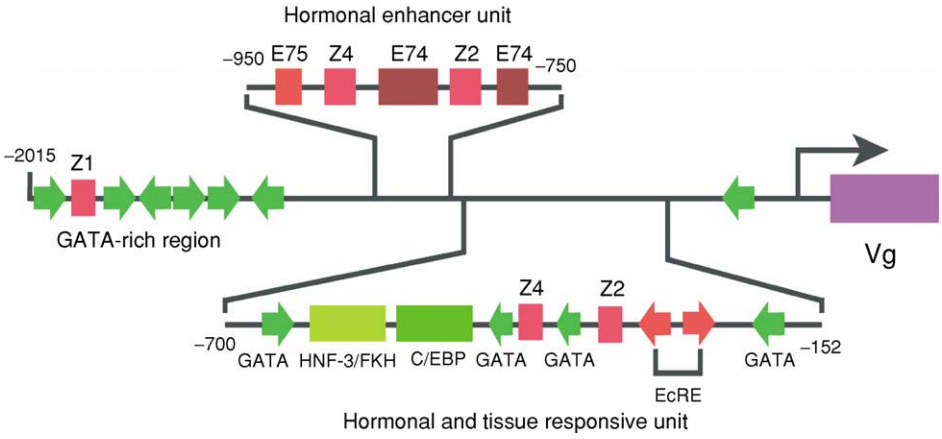

Figure 11 Schematic illustration of the regulatory regions of the *Aedes aegypti Vg* gene. Numbers refer to nucleotide positions relative to the transcription start site. Binding sites for hormonal transcription factors are depicted in red and those for tissue specific factors in green. C/EBP, response element of C/EBP transcription factor; EcRE, ecdysteroid response element; E74 and E75, response elements for respective early gene product of the ecdysone hierarchy; GATA, response element for GATA transcription factor; HNF3/fkh, response element for HNF3/forkhead factor; Vg (purple), coding region of the *Vg* gene; Z1, Z2, and Z4 are binding sites for corresponding Broad-Complex isoforms. (Modified with permission from Kokoza, V.A., Martin, D., Mienaltowski, M.J., Ahmed, A., Morton, C.M., *et al.*, **2001**. Transcriptional regulation of the mosquito vitellogenin gene via a blood meal-triggered cascade. *Gene 274*, 47–65.)

3.9.2.4.7. Competence to 20E response Female mosquitos, such as *A. aegypti*, require a previtellogenic preparatory period to attain the capability for host seeking behavior and blood feeding. During this time, the fat body becomes competent for massive yolk protein synthesis and secretion, and the ovary for accumulation of YPs. In the fat body, this process is manifested in the development of the endoplasmic reticulum and Golgi complexes, proliferation of ribosomes, and increase in cell ploidy (Dittmann *et al.*, 1989; Raikhel and Lea, 1990). JH III titers rise tenfold over the first 2 days after emergence and then slowly decline over the next 5 days. Blood ingestion causes an immediate decline of JH, which falls to its lowest level at 24 h PBM (Shapiro *et al.*, 1986). Activation of fat body nucleoli for ribosomal RNA production and ribosomal production is blocked by removal of the CA in newly eclosed adult females, but it can be restored by either implantation of CA or topical application of JH III to allatectomized females. These developmental events most likely are controlled by JH from the CA (Raikhel and Lea, 1990, 1991). The exposure of a newly emerged female mosquito to JH III is essential for the fat body to become responsive to 20E (Flanagan *et al.*, 1977; Li *et al.*, 2000; Zhu *et al.*, 2003a).

To dissect the molecular mechanism governing the acquisition of competence for vitellogenesis in the *A. aegypti* fat body, the mosquito homolog of the ecdysteroid response competence factor, βFTZ-F1, in *D. melanogaster* has been cloned (Li *et al.*, 2000). During metamorphosis in *D. melanogaster*, the stage specificity of the genetic response to 20E is set up by βFTZ-F1, an orphan nuclear receptor. Ectopic *βFTZ-F1* expression leads to elevated transcription levels for 20E-inducible *BR-C* and the early genes *E74* and *E75*. *βFTZ-F1* mutants pupate normally in response to the late larval 20E pulse but display defects in stage specific responses to the subsequent 20E pulse in prepupae (Lam *et al.*, 1997; White *et al.*, 1997). Mosquito *βFTZ-F1* is transcribed highly in the late pupa and in the adult female fat body during pre- and postvitellogenic periods, when ecdysteroid titers are low, and the transcripts nearly disappear in midvitellogenesis, when ecdysteroid titers are high. Each rise in the level of *βFTZ-F1* transcripts is preceded by a high expression of another nuclear receptor (HR3) that coincides with the 20E peaks (Kapitskaya *et al.*, 2000; Li *et al.*, 2000). This observation is consistent with the role of *HR3* in *D. melanogaster*, which facilitates induction of *βFTZ-F1* in mid-prepupa.

Although the genetic tools are limited for the *A. aegypti*, experiments with the fat body *in vitro* lend support at the functional level to the hypothesis that FTZ-F1 serves as a competence factor for the ecdysteroid response initiating vitellogenesis. In these experiments, FTZ-F1 transcription is inhibited by 20E and is superactivated by its withdrawal (Li *et al.*, 2000). Electrophoretic mobility-shift assay analysis of nuclear extracts from *A. aegypti* fat body demonstrated that the onset of ecdysone response competence in this tissue is correlated with the appearance of the functional FTZ-F1 protein at the end of previtellogenic development (Li *et al.*, 2000). Western blot analysis using antibodies to *A. aegypti* βFTZ-F1 show that the βFTZ-F1 protein is not detectable in the fat body nuclear extracts of newly emerged and 1-day-old mosquitos but is abundant at 3–5 days posteclosion (PE), and then not dectected shortly after blood feeding (Zhu *et al.*, 2003a). Furthermore, when fat body from females was isolated at 6 h PE and incubated for 18 h in medium with JH III, the nuclei contained βFTZ-F1 protein, in contrast to controls incubated with acetone (Zhu *et al.*, 2003a). Taken together, these findings indicate that JH III is quite likely the crucial factor modulating production of βFTZ-F1 protein in the fat body through posttranscriptional regulation of its gene in the previtellogenic stage (**Figure 12**).

To confirm that *βFTZ-F1* may encode a competence factor in the fat body of female *A. aegypti*, it was silenced by RNAi, which involves the introduction of homologous double-stranded RNA (dsRNA) in order to target specific mRNAs for degradation (Zhu *et al.*, 2003a). Compared with naïve females, the mRNA and protein levels of *βFTZ-F1* declined substantially in females treated with *βFTZ-F1* dsRNA but not in those treated with control dsRNA, which indicates that *βFTZ-F1* was selectively inhibited by RNAi. Expression of the *Vg* gene was dramatically diminished after blood feeding in the *βFTZ-F1* dsRNA treated mosquitos. These results suggest that *A. aegypti* βFTZ-F1 is essential for the stage specific 20E response in fat body during vitellogenesis, which is reminiscent of the role βFTZ-F1 plays in *D. melanogaster* during the prepupa to pupa transition. Functional analysis of *βFTZ-F1* by the RNAi technique suggests that βFTZ-F1 is in fact a competence factor that defines the stage specific 20E response during vitellogenesis in the mosquito (Zhu *et al.*, 2003a).

Despite extensive studies, the mode of JH action is still not well understood. During previtellogenic development in female mosquitos, JH controls the synthesis of βFTZ-F1 protein, but this posttranscriptional control of gene expression can occur at one or more levels: pre-mRNA splicing pattern, mRNA

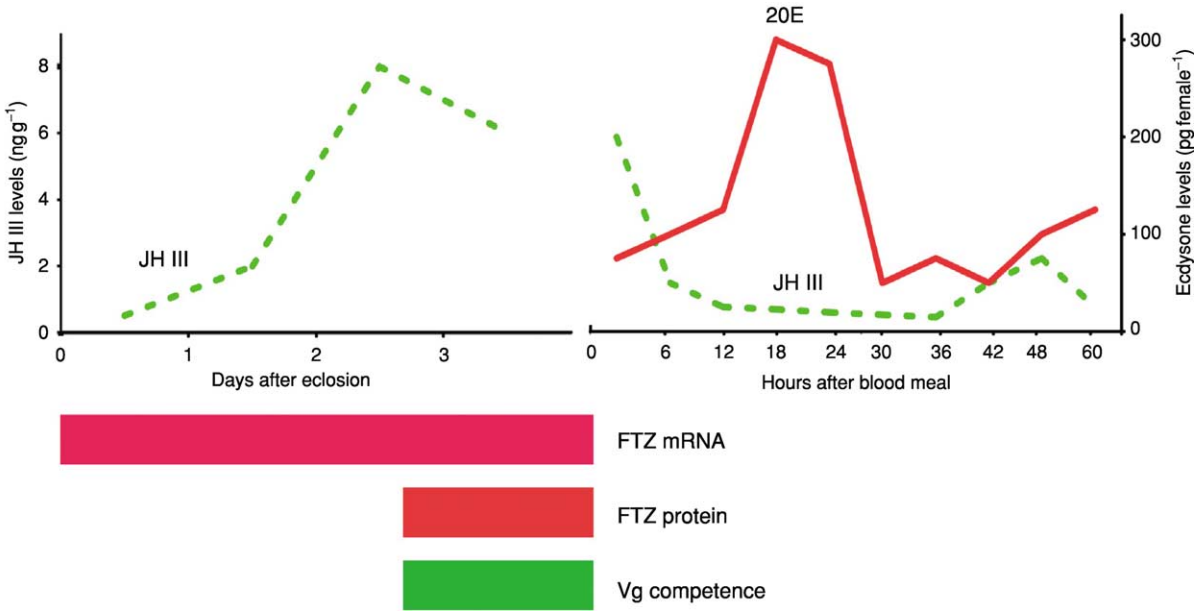

Figure 12 βFTZ-F1, the orphan nuclear factor (see **Chapter 3.5**), is implicated as a competence factor for stage specific response to ecdysteroid during vitellogenesis in the *Aedes aegypti* fat body. The *AaFTZ-F1* mRNA is present at late pupal and previtellogenic stages in newly eclosed females; however, the appearance of active AaFTZ-F1 factor coincides with the onset of competence for 20E response (Li *et al.*, 2000; Zhu *et al.*, 2003a). (Reprinted with permission from Raikhel, A.S., Kokoza, V.A., Zhu, J.S., Martin, D., Wang, S.F., *et al.*, **2002**. Molecular biology of mosquito vitellogenesis: from basic studies to genetic engineering of antipathogen immunity. *Insect Biochem. Mol. Biol. 32*, 1275–1286; © Elsevier.)

stability, mRNA transport, or translation rate. Although the mechanism underlying this regulation remains obscure, these findings represent another step towards understanding the mode of JH action.

3.9.2.4.8. Regulation of vitellogenesis cyclicity by ecdysteroid mediated signaling through heterodimerization with the RXR homolog Ultraspiracle

In many insects, vitellogenesis and egg maturation arecyclic and, as a consequence, these insects produce batches of eggs, unlike others that lay eggs continuously. Generally, the mode of egg production – continuous or cyclic – depends upon feeding and lifestyle adaptations. Cyclic egg production occurs in all insect genera irrespective of the type of hormonal control. In insects, in which reproduction is governed by JH, these mechanisms are poorly understood at the molecular level. However, for insects with cyclic egg production that depends primarily on ecdysteroids, recent studies of the mosquito, *A. aegypti*, have shed some light on these mechanisms.

Zhu *et al.* (2000, 2003b) and Miura *et al.* (2002) have demonstrated that during mosquito vitellogenesis, two nuclear receptors, HR38 and Svp, regulate the cyclicity of ecdysteroid mediated signaling via heterodimerization with USP. Both AaEcR and AaUSP proteins are abundant in nuclei of the previtellogenic female fat body at the state of arrest; however, the EcR-USP heterodimer capable of binding to the specific ecdysone response elements is barely detectable in these nuclei. Studies have shown that EcR is a primary target of 20E signaling modulation in target tissues at the state of arrest. A possible mechanism through which the formation of ecdysteroid receptor activity can be regulated is a competitive binding of other factors to either EcR or USP. Indeed, at this stage, AaUSP exists as a heterodimer with the orphan nuclear receptor, AHR38. This protein is a homolog of the *D. melanogaster* DHR38 and vertebrate NGFI-B/Nurr1 orphan receptors, and it acts as a repressor by disrupting EcR binding to DNA response elements and by interacting strongly with AaUSP. However, in the presence of 10^{-6} M of 20E, EcR efficiently displaces AHR38 and forms an active heterodimer with USP, as happens after a blood meal (**Figures 13** and **14**).

To regulate cyclicity in egg production, termination of Vg gene expression is needed in female mosquitos, so that egg maturation and deposition can be completed and the arrest stage restored until another blood meal can be obtained. To identify other negative regulators of the AaEcR/AaUSP mediated 20E response during vitellogenesis, a

mosquito homolog (AaSvp) of the vertebrate orphan nuclear receptor, chicken ovalbumin upstream promoter transcription factor (COUP-TF), and *D. melanogaster* Seven-up (Svp) has been cloned (Miura *et al.*, 2002). Coimmunoprecipitation experiments with nuclear extracts (Zhu *et al.*, 2003b) have clearly shown that in the female fat body AaSvp associates only with AaUSP. Furthermore, formation of AaSvp-AaUSP heterodimers occurs in a precise, timely manner at 30–33 h PBM, after the 20E titer declines to the previtellogenic level and when the expression of *YPP* genes, including *Vg*, is terminated (**Figure 13**). *In vitro* experiments have suggested that the declining titer of 20E could be a critical factor facilitating the formation of Svp-USP heterodimers (Zhu *et al.*, 2003a). Mosquito Svp, thus, represses the 20E mediated transactivation of vitellogenesis through its heterodimerization with USP (Zhu *et al.*, 2003b).

The modulation of vitellogenesis cyclicity appears to be regulated through alternative heterodimerization of the RXR homolog USP that blocks ecdysteroid mediated signaling (**Figure 14**). AaUSP exerts its functions by associating with distinct partners at different stages of vitellogenesis. During the arrest stage, AHR38 prevents the formation of the functional EcR complex by sequestering AaUSP, which blocks 20E dependent transactivation. After a blood meal, the AaEcR-AaUSP heterodimerization becomes dominant, and AaEcR-AaUSP binding to EcREs on the Vg gene permits its expression. When vitellogenesis proceeds to the termination stage, falling 20E titers shift AaUSP heterodimerization towards AaSvp, repressing USP-based hormone responses (**Figure 14**).

In summary, these studies clearly show that the cyclicity of vitellogenesis in the mosquito fat body is regulated through USP, which sequentially forms inactive or active heterodimers with either repressors (HR38 and Svp) or the activator (EcR) and directly affects ecdysteroid mediated signaling.

3.9.2.5. Molecular Endocrinology of Vitellogenesis in the Cyclorrhaphan Diptera

3.9.2.5.1. Vitellogenesis and hormones in *D. melanogaster* Like the mosquito, the fat body in fruit flies matures and differentiates at eclosion under the control of JH, but it immediately begins to synthesize YPs and secrete them into the hemolymph. Simultaneously, the uptake of YPs is initiated by developing oocytes that mature into eggs, which are fertilized and laid continuously. If the fly does not mate or has enough food, mature eggs are retained, and oocytes

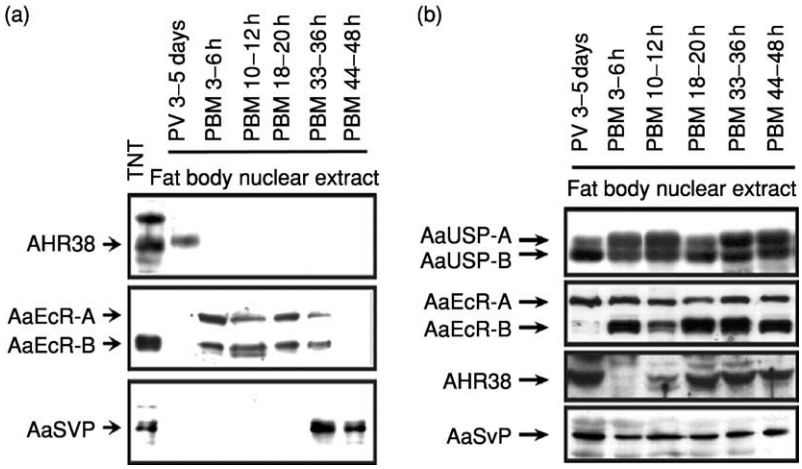

Figure 13 *Aedes aegypti Ultraspiracle* (AaUSP) interacts sequentially with a repressor AHR38, an activator AaEcR, and then with repressor AaSvp in the fat body during the vitellogenic cycle. Nuclear extracts were prepared from the fat bodies of 500 adult females for each time point. (a) Coimmunoprecipitation analysis. A protein aliquot equivalent to 100 mosquitos was incubated with anti-*Drosophila* USP monoclonal antibody. The resulting immune complexes were then precipitated by the addition of protein A-agarose beads. After extensive washing, immune complexes were dissociated and separated by means of SDS-PAGE followed by immunoblotting using rabbit anti-AHR38, rabbit anti-AaEcR, chicken anti-AaSvp antibodies, or the respective preimmune sera. *In vitro* translated proteins (AHR38, AaEcR-B, and AaSvp) were used as controls in Western blot analysis. (b) Western blot analysis of USP proteins during the vitellogenic cycle shows that two isoforms of USP are present throughout the vitellogenic cycle in fat body nuclei. A protein aliquot equivalent to 25 fat bodies was loaded in each lane for SDS-PAGE. Western blot analysis was performed using the anti-DmUSP monoclonal antibody. *In vitro* TNT expressed USP-A and USP-B proteins were used as positive controls. (Reproduced with permission from Zhu, J.S., Miura, K., Chen, L., Raikhel, A.S., **2003b**. Cyclicity of mosquito vitellogenic ecdysteroid-mediated signaling is modulated by alternative dimerization of the RXR homologue *Ultraspiracle. Proc. Natl Acad. Sci. USA 100*, 544–549.)

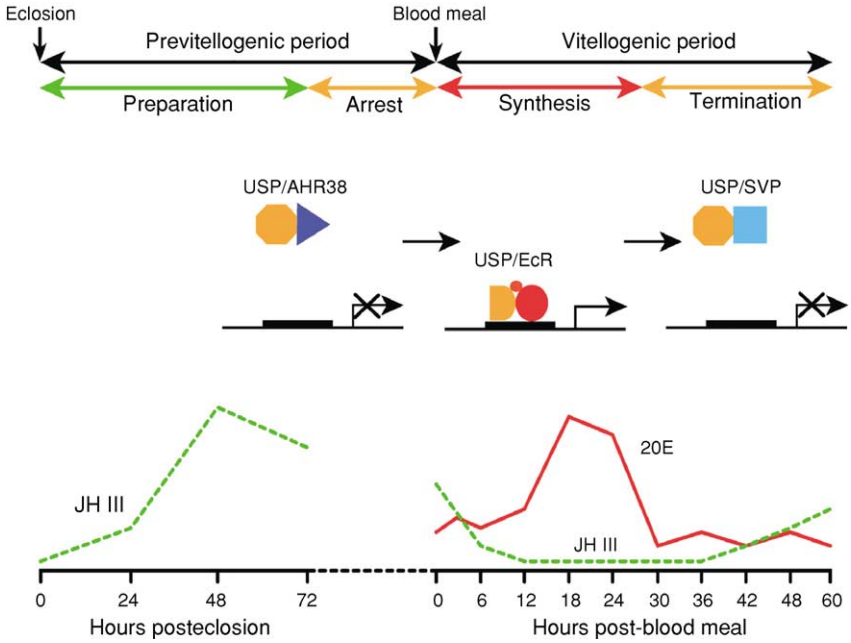

Figure 14 Heterodimerization of *Ultraspiracle* (USP), the obligatory partner of EcR, with repressor nuclear receptors during the first vitellogenic cycle in the mosquito *Aedes aegypti*. At the state of arrest, AaUSP was associated with AHR38, preventing activation of YPP production prior to blood feeding. After a blood meal, AaUSP heterodimerizes with AaEcR, and induces expression of YPP genes in the presence of an elevated 20E titer. Around 30–36 h PBM, AaSvp attaches to AaUSP, decelerating the massive protein synthesis. Top panel: stages of mosquito vitellogenic cycle; bottom panel: titers of juvenile hormone and ecdysteroids. (Modified with permission from Zhu, J.S., Miura, K., Chen, L., Raikhel, A.S., **2003b**. Cyclicity of mosquito vitellogenic ecdysteroid-mediated signaling is modulated by alternative dimerization of the RXR homologue *Ultraspiracle*. *Proc. Natl Acad. Sci. USA 100*, 544–549.)

arrest at a previtellogenic stage and do not enter vitellogenesis (Bownes, personal communication).

Despite the fact that in most cyclorrhaphan Diptera, including *D. melanogaster*, YPs are made in fat body and follicular epithelial cells surrounding the oocyte, the regulation of *yp* gene expression is different in these tissues (Bownes, personal communication). Ecdysteroids stimulate YP synthesis in the fat body of males and upregulate it in the fat body of females (Postlethwait *et al.*, 1980; Jowett and Postlethwait, 1980; Bownes, 1982; Bownes *et al.*, 1983). This is consistent with the finding that EcREs have been mapped to regions flanking the *yp* genes (Bownes *et al.*, 1996). JH also increases YP synthesis in the fat body of females (Bownes and Blair, 1986; Bownes *et al.*, 1987; Soller *et al.*, 1997), but it does not induce YP synthesis in males. No potential JH elements have been found in these genes. Walker *et al.* (1991) have shown that ecdysteroids increased CAT expression of *YP* promoter CAT fusion in the S3 cell line. JH had no effect on the same reporter construct. Thus, it is unlikely that JH plays any role in directly affecting transcription of *yp* genes in the fat body. JH may play a role in the regulation of yolk protein synthesis by follicle cells, as shown in studies of the *D. melanogaster* mutant ap^{56f} (Richard *et al.*, 1998, 2001). Female ap^{56f} produces low levels of JH and normal to elevated levels of ovarian ecdysteroids and are fertile but have delayed vitellogenesis. JH application to female ap^{56f} reversed the delay in vitellogenesis.

Many of the response genes in the ecdysteroid regulatory hierarchy, including *EcR/USP E74, E75*, and *BR-C*, that have been shown to be crucial for 20E regulation of *Vg* gene expression in the fat body of *A. aegypti* have not been studied in *D. melanogaster* with respect to YP synthesis in the fat body. This is mostly because in normal, mated, well-fed females the genes are constitutively active rather than regulated in response to a blood meal (Raikhel *et al.*, 2003; Bownes, personal communication).

3.9.2.5.2. Regulatory regions of yolk protein genes in *D. melanogaster* Relatively little is known about the *cis*-acting regions of the *yp* genes in any species of cyclorrhaphan Diptera. Several EcREs have been identified in the 5′ and 3′ regions and within the coding sequences of the *yp* genes of *D. melanogaster* (see **Figures 15 and 16**), and they appear to confer ecdysone inducible expression in males, when injected with 20E. These putative EcRE sequences are similar to those shown to bind the EcRE/USP heterodimer and lead to transcription of other genes in insect metamorphosis and the *Vg*

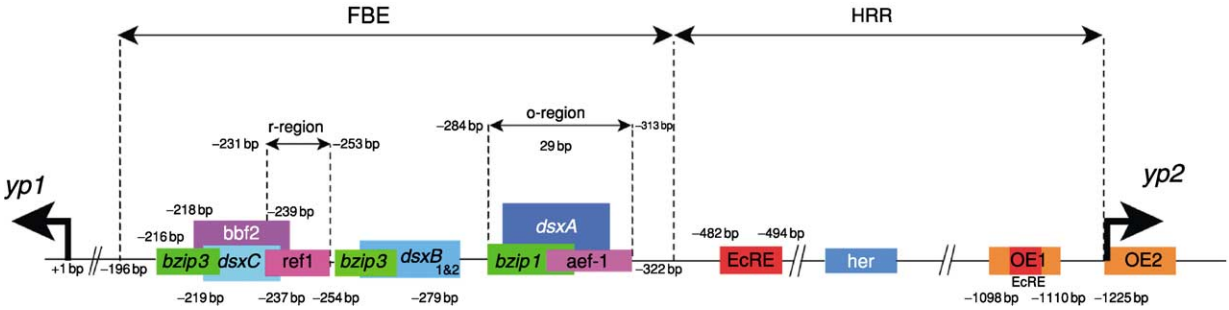

Figure 15 *Drosophila melanogaster yp* genes 1 and 2 are located in close proximity in opposite orientation and share a regulatory region called the intergenic enhancer region. The 1225 bp intergenic enhancer region of the *yp1* and *yp2* genes contains four enhancer regions, the fat body enhancer (FBE), the hermaphrodite response region (HRR), and two ovary enhancers (OE1 and OE2); aef-1, the adult enhancer factor-1; *bzip3*; C/EBP, CAAT enhancer binding protein; *dsx*, doublesex binding site; EcRE, ecdysone response element; her, binding site for the hermaphrodite protein. (Data kindly provided by M. Bownes.)

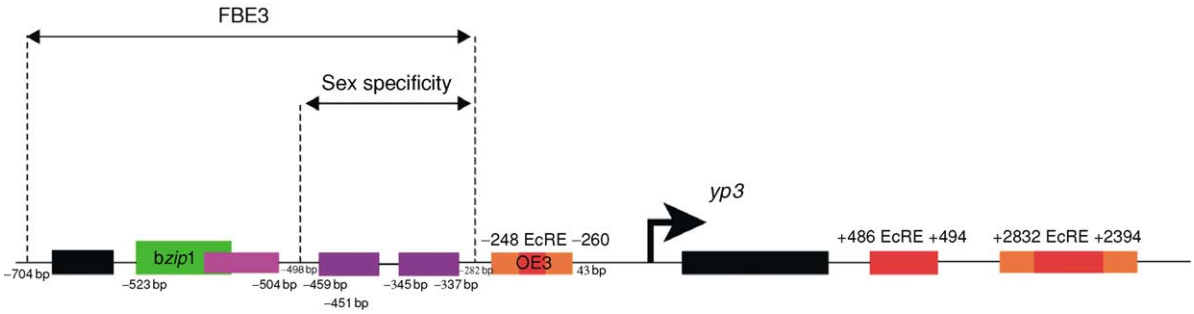

Figure 16 The *Drosophila yp3* gene is separated from two others on the X-chromosome and has a separate regulatory region, the upstream enhancer 3. aef-1, adult enhancer factor 1; bbf, binding site for the box-B binding factor; EcRE, ecdysone response element. (Data kindly provided by M. Bownes.)

genes in the mosquito (Bownes *et al.*, 1996). Although these EcREs have been mapped on the *yp* genes, it is not clear how much of the 20E inducibility of *yp* gene expression is a direct action through the binding of EcR and how much is due to downstream genes such as *BR-C, E74*, and *E75* (Bownes, personal communication).

Despite the fact that the hormonal and molecular control of *yp* gene transcription in *D. melanogaster* is poorly understood, the sex specific expression of these genes has been elucidated in detail (see **Chapters 1.5** and **1.7**). Doublesex (*dsx*) exerts the most important control of *yp* gene expression (Bownes and Nothiger, 1981; Coschigano and Wensink, 1993; Belote *et al.*, 1985). Alternate transcript splicing of *dsx* produces different proteins in male and female adults, Dsx^M and Dsx^F, respectively, that have similar DNA binding domains but different C-terminal extensions (Baker and Wolfner, 1988; Burtis and Baker, 1989; MacDougall *et al.*, 1995). The male and female proteins are essential for maintaining *yp* transcription in the female fat body and repressing it in males (Bownes and Nothiger, 1981; Belote *et al.*, 1985; Burtis *et al.*, 1991; Bownes, personal communication). In males, 20E injection overrides sex determination inhibition as shown by the transient expression of *yp* genes in the fat body of males (Bownes *et al.*, 1996; Bownes, personal communication). In females, *yp* gene expression in the ovarian follicle cells does not depend upon the sex determination pathway (Logan *et al.*, 1989; Logan and Wensink, 1990; Lossky and Wensink, 1995), and 20E is probably a key hormone regulating this expression in such cells (Bownes, 2004).

In *D. melanogaster*, *yp* genes 1 and 2 are located in close proximity in opposite orientation and share a regulatory region called the intergenic enhancer region (**Figure 15**). In females, expression of *yp1* and *yp2* occurs in fat body and follicle cells of developing egg chambers at stages 8–10 (Bownes, 2004). The 1225 bp intergenic enhancer region of these *yp* genes contains four enhancer regions: fat body enhancer (FBE), hermaphrodite response region (HRR), and two ovary enhancers (OE1 and OE2) (Logan *et al.*, 1989; Logan and Wensink,

1990; Garabedian *et al.*, 1985, 1986; Abrahamsen *et al.*, 1993; Lossky and Wensink, 1995). FBE drives expression of these genes in fat body, and HRR responds to the *hermaphrodite* gene that acts along with Dsx to control their sex specific expression (Li and Baker, 1998). OE1 and OE2 direct expression of these genes in follicle cells (Logan *et al.*, 1989; Logan and Wensink, 1990; Lossky and Wensink, 1995). With base pairing in respect to the *yp1* start site, several binding sites for activator or repressor molecules have been mapped, and binding has been demonstrated for some of these molecules (**Figure 16**). A putative binding site for *bzip3* overlaps a weak binding site for a Dsx, *dsxC*. Two overlapping binding sites for the mammalian CAAT enhancer binding protein (C/EBP) and the adult enhancer factor-1 (AEF-1) have been found in other fat body enhancers and the *Adh* gene of the mammalian liver to activate or repress transcription, respectively (Abel *et al.*, 1992). These two sites, in turn, overlap the strongest binding site for Dsx, *dsxA* (Bownes *et al.*, 1996). Further upstream, between −322 and −1225 bp, up to the *yp2* transcription start site, lies the HRR, which contains the binding site for the hermaphrodite protein, and OE1. A putative EcRE lies within OE1. A second EcRE is found between −482 and −494 bp of the HRR region. OE2 is located at the start site for *yp2* transcription (Logan *et al.*, 1989; Logan and Wensink, 1990; Lossky and Wensink, 1995; Bownes, ; Bownes, personal communication).

The *D. melanogaster yp3* gene is separated from two others on the X-chromosome and has a separate regulatory region, the upstream Enhancer 3 (Hutson and Bownes, 2003). The organization of the regulatory regions of this gene differs from those of *yp1* and *yp2*, resembling instead those in *Musca* and *Calliphora yp* genes (Hutson and Bownes, 2003). The 703 bp upstream enhancer sequence of the *yp3* transcription start site contains three regions that confer tissue specificity, sex specificity, and ovary specificity (**Figure 16**). The 419 bp fat body enhancer 3 (FBE3) is the furthest 5′ regulatory region spanning from −704 to −285 bp and controls expression of *yp3* in the female fat body (Hutson and Bownes, 2003). Most 5′ in the FBE3 is the binding site for *dsxA*. Downstream of the *dsxA* site is the overlapping binding site, also found in FBE1/2, for the activator molecule CAAT enhancer binding protein (C/EBP), and the repressor, adult enhancer factor 1 – AEF-1 (Falb and Maniatis, 1992a, 1992b). The region between −498 and −252 bp is essential for sex specificity. There are two binding sites for the box-B binding factor (BBF), a transcriptional activator (Abel *et al.*, 1992). A possible mechanism also involves a putative homolog of the unknown activator R that has also been found to be the final determinant needed for female specific fat body expression of *yp1* and *2* (Ronaldson and Bownes, 1995; Hutson and Bownes, 2003).

Hutson and Bownes (2003) have analyzed the regulation of *yp3* gene expression. JH increased stability of *yp3* transcripts, and 20E plays an important role in regulating transcription. Three putative EcREs have been mapped in FBE3 and *yp3* (Hutson and Bownes, 2003). Immediately 3′ to the FBE3 is the ovarian enhancer 3 (OE3) that is necessary for regulation of *yp3* transcription in the follicle cells of the developing egg chamber between stages 8 and 10. Within this region is an EcRE, and a second EcRE is located 3′ of the intronic sequence of *yp3*. The final EcRE is found within the second exon of *yp3*. 20E can override the negative effects of Dsx^M while maintaining the tissue specificity of *yp3* gene expression (Hutson and Bownes, 2003). Experiments using upstream, downstream, and coding sequences of *yp3* fused to reporter genes agree well with the locations of the putative EcREs, because there are ecdysteroid inducible sites upstream, downstream, and in the coding region (Hutson and Bownes, 2003).

3.9.2.6. Hormones and Ovarian Maturation

3.9.2.6.1. Previtellogenic development of the follicle Several aspects of ovarian previtellogenic development in insects are controlled by JH. Stimulation of previtellogenic growth of follicles by JH is best supported by past studies (Strong, 1965; Gwardz and Spielman, 1973; Tobe and Pratt, 1975; Tobe and Stay, 1977; Lanzrein *et al.*, 1978; Moobola and Cupp, 1978; Tobe and Langley, 1978; McCaffery and McCaffery, 1983). Differentiation of the follicular epithelium is another aspect of oocyte development that is regulated by JH (Davey and Hubner, 1974; Abu-Hakima and Davey, 1975; Elliot and Gillot, 1976; Koeppe and Wellman, 1980; Koeppe *et al.*, 1980). In *M. domestica*, JH is released soon after adult emergence, and it is essential for ovarian maturation, since without it ovaries remain immature (Adams, 1974, 1980). JH stimulates endopolyploidy in the nurse cells, primes the oocyte for growth, and potentially plays a role in follicle morphogenesis. Although, JH affects the previtellogenic stages of *M. domestica* oogenesis, it does not induce vitellogenesis. JH also is required for oocyte development in the mosquito *A. aegypti*, where it controls growth and differentiation of the follicular epithelium. These events are blocked by the removal of CA and restored by either implantation of CA or application of JH (Raikhel and Lea, 1991).

3.9.2.6.2. The role of 20E in ovarian development and egg maturation in Diptera Ecdysteroids are produced by the ovaries of many insects, including mosquitos and flies (Hagedorn *et al.*, 1975; Bownes *et al.*, 1984; Hagedorn, 1985). However, their role in ovarian development is not completely understood. In *D. melanogaster*, ecdysteroids can have an antagonistic effect on oocyte progression and lead to apoptosis of egg chambers. Ecdysteroid mutants, such as ecd^1 (Redfern and Bownes, 1982) and $l(3)3^{DTS}$ (Walker *et al.*, 1987), have defects in the progress of oogenesis. One of the early genes in the ecdysteroid regulatory hierarchy, *BR-C*, is crucial at several stages of oogenesis. It is first expressed in all follicle cells surrounding the oocyte prior to the control point (Deng and Bownes, 1997), and this early *BR-C* induction may be controlled by ecdysteroids. An exciting development is the isolation of the *D. melanogaster dare* gene that encodes adrenodoxin reductase, which is expressed in the ovary and plays a key role in ecdysteroid biosynthesis (Freeman *et al.*, 1999) (see **Chapter 3.3**). These authors postulated that ovarian expression of *dare* provides a maternal source of this enzyme to the developing embryo. Since it is first expressed just prior to activation of the *BR-C*, it is also possible that it could also support the synthesis of ecdysteroids, which would activate *BR-C* in nearby follicle cells at this stage of oogenesis. This scenario suggests the existence of an egg chamber autonomous timing event that regulates when and where ecdysteroids are available to control oocyte progression. *BR-C* expression in oogenesis is crucial for the regulation of endoreplication of polyploid follicle cells, specific amplification of the chorion genes, and later for positioning of the chorionic appendages (Deng and Bownes, 1997; Tzolovsky *et al.*, 1999).

Although *BR-C* is regulated by ecdysteroids in early oogenesis, the epidermal growth factor signaling pathway in the egg chamber is crucial for positioning the late *BR-C* expression pattern, which in turn positions the chorionic appendages (Deng *et al.*, 1999). Since the effects of 20E are mediated by EcR and USP, mutations in these genes in *D. melanogaster* affect oogenesis. EcR-A and Ec-R-B1 are expressed in the nurse cells and follicle cells throughout oogenesis (Deng, Mauchline, and Bownes, personal communication). A temperature sensitive mutant affecting these isoforms, EcR^{A483T}, reduced egg laying, induced abnormal egg clusters, and led to a loss of vitellogenic stages (Carney and Bender, 2000). Loss of EcR in germline clones leads to egg chamber arrest at around stage 6–7 of follicle development (previtellogenic stage). Carney and Bender (2000) proposed the existence of an ecdysone dependent checkpoint at this stage. Buszczak *et al.* (1999) have shown that the early ecdysone response genes, *E75* and *E74*, along with the *BR-C*, are crucial for egg chamber morphogenesis in mid-oogenesis. Germline clones of cells lacking *E75*, *dare*, or EcR functions results in the degeneration of these egg chambers at stage 8 (Buszczak *et al.*, 1999).

3.9.2.6.3. Regulation of patency by follicle cells The appearance of large intercellular spaces in the follicular epithelium during vitellogenesis, a phenomenon termed patency, has been reported in many insects, from the less modified species having panoistic ovaries, like *B. germanica* (**Figure 17**)

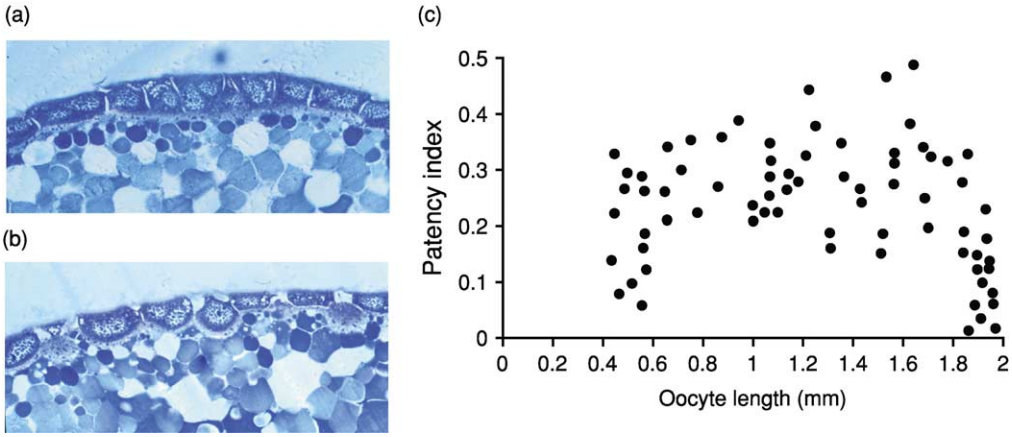

Figure 17 Patency of follicle cells in *Blattella germanica* during yolk protein uptake. (a, b) Sections at the equatorial zone in basal oocytes of (a) 0.68 mm length (3 days old) and (b) 1.52 mm length (5 days old). (c) Relation between patency index (PI) and basal oocyte length. PI = SE/SC, where SE is the surface of the intercellular spaces stained with Evans' blue, and SC the surface of the follicle cells. (Reprinted with permission from Pascual, N., Cerdà, X., Benito, B., Tomás, J., Piulachs, M.D., *et al.*, **1992**. Ovarian ecdysteroid levels and basal oocyte development during maturation in the cockroach *Blattella germanica* (L.). *J. Insect Physiol. 38*, 339–348, with permission from Elsevier.)

(Pascual *et al.*, 1992) to the most modified ones having polytrophic ovaries, like flies or mosquitos (Wyatt and Davey, 1996). Studies on *Rhodnius prolixus* by Davey and co-workers have suggested that patency is under JH control (Wyatt and Davey, 1996). Further studies in this bug have shown that JH increases the activity of Na^+/K^+ ATPase in membrane preparations from vitellogenic follicle cells incubated *in vitro*, and experiments with inhibitors and activators of protein kinase C indicated that this enzyme is involved in the activation of the ATPase (Sevala and Davey, 1989). Incubation of membrane preparations with JH I resulted in the phosphorylation of a 100 kDa protein, a process that was dependent on protein kinase C and was inhibited by ouabain. Based on these experiments, it was suggested that the 100 kDa protein could be the α-subunit of the ATPase (Sevala and Davey, 1993). According to this model, JH would act on the follicle cells via a protein kinase C dependent cascade to stimulate a membrane bound Na^+/K^+ ATPase (**Figure 18**). Then, changes in the ionic balance would cause water loss and shrinkage of the cells with the associated apparition of large intercellular spaces. An equivalent system seems to operate in the locust *L. migratoria* (Davey *et al.*, 1993) and the mealworm beetle, *Tenebrio molitor* (Webb *et al.*, 1997). The model proposed to explain these mechanisms acting in follicle cell patency (**Figure 18**) suggests the existence of a membrane receptor that binds JH. The first efforts to characterize such a receptor were carried out in *R. prolixus*. In this species, Ilenchuk and Davey (1985) have shown that JH I binds to membrane preparations of follicle cells with a K_d of 6.54 nM in a specific and saturable fashion. Neither JH II nor JH III exhibited biological activity, and they did not compete for the binding site (Ilenchuk and Davey, 1987). A 35 kDa protein in solubilized follicle cell membranes from *L. migratoria* was found to bind a photoaffinity analog of JH III, [^{3}H]EFDA (Sevala *et al.*, 1995). The membrane preparations bound [^{3}H]JH III with a K_d of 3.68 nM in a specific and saturable fashion, and the binding of EFDA was blocked when membranes were previously treated with JH III. Interestingly, EBDA, a photoaffinity analog of JH I, did not show any specific binding in equivalent assays; EHDA, a photoaffinity analog of JH II, bound specifically to a 35 kDa protein, and MDK, a photoaffinity analog of methoprene, bound specifically to a different 17 kDa protein (Sevala *et al.*, 1995). Although relatively small, these JH binding proteins may serve as membrane receptors; certainly the 35 kDa protein is within the size range of G protein-coupled receptors (see **Chapter 5.5**). Further investigations are required to elucidate the nature of these proteins.

Webb and Hurd (1995) have reported specific and saturable binding of JH III to microsomal preparations of vitellogenic follicle cells from the beetle, *T. molitor*. Results of Scatchard analysis indicated the occurrence of two sites of different affinity, one with a K_d of 10 nM and the other one of 400 nM. Both sites showed modest affinity for JH I. The specificity of JH binding by follicle cell membranes from the above species is intriguing, given the structural similarity of JH homologs: *R. prolixus* to JH I only, *L. migratoria* to JH III and II, and *T. molitor* to JH III and also JH I. Coincidently, JH III is found in *L. migratoria* and *T. molitor*, while that of *R. prolixus* is unknown.

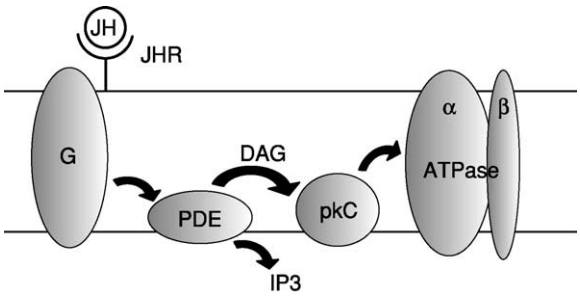

Figure 18 Model for the action of JH on the follicle cell membrane. The binding of JH to the JH receptor (JHR) on the outer surface of the membrane triggers a cascade involving a G protein, with which the receptor site is closely associated. Protein kinase C (pkC) is activated through phosphodiesterase (PDE) and diacylglycerol (DAG) and phosphorylates the α-subunit of the Na^+K^+ ATPase, thus activating the enzyme. (Modified from Wyatt, G.R., Davey K.G., **1996**. Cellular and molecular actions of juvenile hormone. II. Roles of juvenile hormone in adult insects. *Adv. Insect Physiol. 26*, 1–155.)

3.9.2.6.4. Development of oocyte endocytic complex and endocytosis When YPs reach the plasma membrane of oocytes (oolemma), they are internalized into the ooplasm through receptor mediated endocytosis, via coated pits and vesicles, and transferred to early endosomes in the cortical ooplasm. From early endosomes they are conveyed to yolk spheres, while receptors are recycled back to the oocyte surface (Raikhel and Dhadialla, 1992; Sappington and Raikhel, 1998a; Snigirevskaya and Raikhel, 2004; Raikhel *et al.*, 2002). In *D. melanogaster*, the endocytic pathway leading to the production of yolk spheres was visualized by Giorgi *et al.* (1993) with exposure to peroxidase *in vivo* or *in vitro*. The Golgi apparatus and the yolk spheres were then labeled by fixation with osmium zinc iodide (OZI). Starvation resulted in the OZI labeling being restricted to the Golgi apparatus and to an

extended tubular network, whereas feeding or treatment with JH caused the yolk spheres to become labeled with OZI and to incorporate peroxidase. Similar results were obtained in equivalent experiments with the mutant ap^4, which is defective in JH biosynthesis, or with vitellogenic follicular tissue incubated *in vitro* and treated or not with JH. The results of this study indicated that JH facilitates endocytic uptake by inducing the fusion of coated vesicles and tubules with the yolk spheres (Giorgi *et al*., 1993).

D. melanogaster ap^4 mutant flies produce low levels of JH and ovarian ecdysteroids and are infertile (Richard *et al*., 1998, 2001) This situation suggests that JH has a prominent role in regulating YP uptake by the ovary, given that the endocytic organelles are absent in ap^4 oocytes and YPs are present at normal levels in the hemolymph. The development of endocytic organelles can be restored in ap^4 flies by application of JH in a fashion similar to that of mosquito. In insects with continuous egg production such as *D. melanogaster*, it is not clear whether JH regulates the formation of endocytic organelles, stimulates the endocytosis itself or acts in both events, because these events are difficult to separate.

In insects with cyclic egg production, early developmental and vitellogenic events in egg chambers are separated at the arrest stage, which is broken by either food intake or other stimuli. Normally, these insects mature only a single egg per ovariole in each cycle. Raikhel and Lea (1985) have shown that during the JH dependent previtellogenic period of follicle development in the mosquito *A. aegypti*, a highly specialized endocytic complex consisting of microvilli, numerous coated vesicles, and endosomes appears in the oocyte cortex. The formation of this endocytic complex is controlled by JH III. It was blocked by CA ablation at eclosion, but restored by either CA implantation or the application of JH III. Analysis of several genes involved in receptor mediated endocytosis of YPs, e.g., clathrin heavy chain (Kokoza and Raikhel, 1997), Vg receptor (Sappington *et al*., 1996), and ovarian lipophorin receptor (Cheon *et al*., 2001), has revealed that their expression occurs very early in the previtellogenic development of mosquito ovaries, even before the formation of the primary follicle. Immunocytochemistry, however, shows that the Vg receptor is present as the development of the endocytic complex occurs in the oocyte cortex (Sappington *et al*., 1995). These findings suggest that JH likely acts at the posttranscriptional level on these genes in a manner similar to that in the fat body (Zhu *et al*., 2003a).

3.9.2.6.5. The ovary as an endocrine organ Many different cell types and tissues make up the insect ovary, and any of these cells or tissues may produce factors that affect itself (autocrine), neighboring cells (paracrine), or more distant cells (endocrine). The ovary is a primary source of ecdysteroids in female insects, as demonstrated in many different insect groups (e.g., Whiting *et al*., 1997; Marti *et al*., 2003), and autocrine, paracrine, and endocrine effects have been reported or ascribed to ovarian ecdysteroids in female insects (Hagedorn, 1985; Lanot *et al*., 1989). The follicle cells surrounding the oocyte are presumed to be the specific cell source, as first demonstrated for locusts (Kappler *et al*., 1986; 1988), although this has not been confirmed in higher orders of insects (e.g., Diptera). Ecdysteroids are sequestered by developing oocytes in all insects and are present in hemolymph to a lesser or greater degree depending on whether they are required for vitellogenesis. In general, ovarian ecdysteroidogenesis proceeds in dictyopteran females in parallel to vitellogenesis (Pascual *et al*., 1992; Romañá *et al*., 1995), but in female mosquitos and higher flies, this process is initiated by neurohormones (see Section 3.9.3.8.3) released in response to the ingestion of a blood or protein meal, respectively (Hagedorn *et al*., 1975; Trabalon *et al*., 1990).

For insects, the detailed and complete biosynthetic pathway for the conversion of cholesterol to ecdysteroids is not known, but it is believed to mimic vertebrate steroidogenesis in that precursor steroid molecules shuttle between the endoplasmic reticulum and the inner mitochondrial membrane during processing (Gilbert *et al*., 2002; see **Chapter 3.3**). Key proteins and enzymes involved in the ovarian ecdysteroid biosynthetic process have been identified by genetic analysis of *D. melanogaster*. Females with the *ecdysoneless* conditional mutation appear to lack the ability to transport an intermediate to the inner mitochondrial membrane for further processing at the restrictive temperature (Warren *et al*., 1996), but the protein responsible for the mutant phenotype has not yet been identified. Expression of the adrenodoxin reductase (*dare*) gene in ovaries of *D. melanogaster* females is required in the vitellogenic stage of oogenesis (Buszczak *et al*., 1999; Freeman *et al*., 1999). This mitochondrial enzyme mediates the transport of electrons from NADPH to adrenodoxin, which in turn donates them to the mitochondrial cytochrome P450 enzymes responsible for steroidogenesis. The *disembodied*, *shadow*, *phantom*, and *shade* genes encode cytochrome P450 enzymes involved in the final

hydroxylation steps of ecdysteroid biosynthesis (Gilbert *et al.*, 2002; Warren *et al.*, 2002; Petryk *et al.*, 2003) (see **Chapter 3.3** for details). These hydroxylases were localized within the adult ovary, and *disembodied* was expressed at the beginning of the vitellogenic stage of oocyte maturation (Chavez *et al.*, 2000). Ecdysone is the major ecdysteroid secreted by *A. aegypti* ovaries and is converted to the more active form of 20E by the ecdysone 20-monoxygenase in peripheral tissues, such as the fat body and ovaries (Hagedorn *et al.*, 1975; Borovsky *et al.*, 1986; Smith and Mitchell, 1986).

The ovary may be a source of JH and peptide or protein messengers. The ovaries of *A. aegypti* synthesize *in vitro* physiologically significant amounts of JH III and other JH-like compounds from radiolabeled farnesoic acid, methionine, and acetate (Borovsky *et al.*, 1994). Only one other study has investigated this phenomenon in a female insect, and under similar conditions, ovaries of *D. melanogaster* failed to produce JH (Richard *et al.*, 2001). For female Diptera, ovaries with mature eggs may be the source of peptide or protein factors shown to inhibit vitellogenesis directly or indirectly (see Sections 3.9.3.8.7 and 3.9.3.8.8). Unfortunately, hemolymph titers for such factors have not been profiled in these female insects during oogenesis; thus, their physiological function remains conjectural. Several different neuropeptides have been identified in neurons and neurosecretory cells associated with oviducts (see Section 3.9.2.8), and these peptides may have paracrine and endocrine effects when released at specific times during reproduction.

3.9.2.7. Hormone Action in Female Accessory Glands

3.9.2.7.1. The role of juvenile hormone in the colleterial glands Among the insect accessory sex glands and other organs associated with fertilization and oviposition, the colleterial glands of female cockroaches have been studied preferentially with regard to the endocrine basis of development and biosynthetic activity. The asymmetrical left and right colleterial glands form the ootheca, the hard egg case deposited by female cockroaches. The right gland produces an 8-glucosidase, whereas the left gland produces calcium oxalate, protocatechuic acid-8-glucoside, other 8-glucosides, a diphenol oxidase, and a group of structural proteins called oothecins. When the secretions of both glands mix, the glucosidase reacts with the 8-glucosides, and the resulting phenols are oxidized to quinones, which cross-link the oothecins through phenolic bridges (see Koeppe *et al.*, 1985). This gives the typical hard consistency of the ootheca.

The first studies demonstrating the key role of CA for accessory gland development in the cockroach *L. maderae* were reported by B. Scharrer in the 1940s. Subsequent research in other cockroach species, *P. americana* and *B. germanica*, involving allatectomy and hormonal treatment, have demonstrated that JH is essential for the production of oothecins and the other molecules in the left colleterial gland (**Figure 19**). JH does not influence production of 8-glucosidase in the right colleterial gland (see Koeppe *et al.*, 1985; Wyatt and Davey, 1996).

3.9.2.7.2. Oothecins – structural proteins regulated by juvenile hormone Oothecin synthesis in *P. americana* has been used as a model to investigate the effects of JH. Oothecins are composed of a 39 kDa protein, which is rich in valine and proline, and five smaller proteins (types A–E), which are rich in glycine and tyrosine. The sequence of a C type oothecin displays a number of similarities with chorion proteins of silk moths (Pau *et al.*, 1987). Expression studies in *P. americana* females have shown that the first appearance of oothecin mRNAs coincides

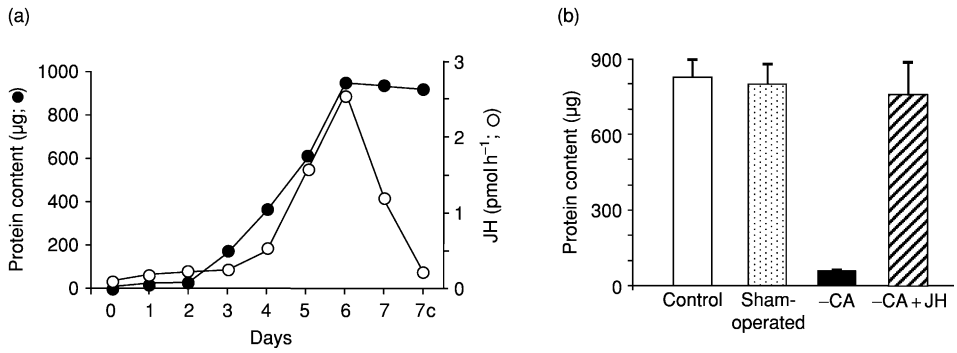

Figure 19 The increase of protein content in the female left colleterial gland of the *Blattella germanica* is paralleled by the increase in production of juvenile hormone (JH) by the corpora allata (a). Protein accumulation is regulated by JH, as shown by experiments of allatectomy (−CA) and subsequent treatment with 10 µg of JH III (−CA + JH) (b). (Based on data from Belles and Piulachs, 1983 and Danès and Piulachs, unpublished data.)

with rising rates of JH biosynthesis, whereas allatectomy abolishes their expression. No correlation was observed with the ecdysteroid concentration in the hemolymph, whereas ovariectomy, which depresses ecdysteroid titer, did not affect oothecin production (Pau *et al.*, 1987). Thus, ecdysteroids probably do not play a significant role in regulating oothecin expression. In a related study, 20E was shown to inhibit RNA synthesis *in vitro* in the left colleterial gland (Iris and Sin, 1988). This may be a pharmacological rather than physiological effect, given that the 20E dose ($1.2\,\mu g\,ml^{-1}$) was more than two orders of magnitude higher than maximal concentrations of ecdysteroids in female *P. americana* (Weaver *et al.*, 1984).

3.9.2.8. Oviposition

Oviposition by female insects is regulated by the central nervous system (CNS) in response to male accessory gland factors passed during fertilization (see **Chapter 1.5**), oocyte maturity, and physical site characteristics. Neurotransmitters and neuropeptides, acting as such, would have a direct action in activating or inhibiting this process, but JH and ecdysteroids may modulate nervous system regulation. Diverse neurotransmitters and neuropeptides have been localized in cells of the terminal ganglion that have axons extending to different oviduct regions in insects, and their effect on oviduct muscle contractions *in vitro* has been documented. Tachykinins (Kwok *et al.*, 1999), SchistoFLRFa (Kwok and Orchard, 2002), crustacean cardioactive peptide (Donini and Lange, 2002), proctolin and serotonin (Lange, 2002) stimulated such contractions, whereas octopamine and myosuppressin were inhibitory (HVFLRFa; Starratt *et al.*, 2000). The spermatheca also are innervated from the terminal ganglion, and octopamine and Arg-Phe amide peptides alter muscle contractions in these organs (Clark and Lange, 2003). Although allatostatin-A has no demonstrated effects on insect oviducts, it greatly increases in concentration in oocytes and oviduct epithelium and nerves of the female cockroach, *Diploptera punctata*, during vitellogenesis, possibly functioning as an anti-JH factor in ovaries (Woodhead *et al.*, 2003). In the cricket *A. domesticus*, ovipositor movements in a typical oviposition sequence are completely abolished in allatectomized females, whereas JH treatment restores that behavior (Strambi *et al.*, 1997). Oviposition by coleopteran females is inhibited by the application of 20E antagonists, largely due to their induction of abnormal egg maturation (hyperecdysonism) (Taïbi *et al.*, 2003).

Parturition hormone (PH) activity is present not only in the uterus of the tsetse fly *Glossina morsitans* but also in the oviducts of *Bombyx* and *Schistocerca*, as well as the ejaculatory duct of *S. gregaria* males (Zdarek *et al.*, 2000). Activity thus appears to be present in the reproductive ducts of diverse insect taxa. To determine whether any of the common insect neuropeptides are capable of mimicking the effect of PH, 35 identified neuropeptides and analogs were evaluated for PH activity. Modest PH activity was observed for only high doses of proctolin and a pyrokinin analog, thus suggesting that PH is unlikely to be closely related to any of the identified neuropeptides tested. While proctolin was highly effective in stimulating contractions of the *S. gregaria* oviduct, the extract from the tsetse fly uterus elicited only a weak response in this bioassay. PH activity, however, was effectively mimicked with an injection of 8 bromo-cyclic GMP, suggesting a potential role for this cyclic nucleotide in mediating the PH response. Neck-ligated, pregnant females were responsive to PH, other neuropeptides and cyclic nucleotides, whereas in intact females, the brain presumably negates the effects of the exogenous compounds.

3.9.2.9. Peptide Hormones Involved in Female Reproduction

As described above, JH and ecdysteroids are considered to be the primary hormones affecting female reproduction by acting separately or coordinately to stimulate oogenesis and vitellogenesis, depending on the insect group or species. Much is known about specific neuropeptides that either stimulate or inhibit JH or ecdysteroid synthesis in insects, but little evidence points to the direct effects of peptide hormones on physiological processes associated with egg maturation (reviews: Gade *et al.*, 1997; Klowden, 1997; De Loof *et al.*, 2001; Nässel, 2002; Taghert and Veenstra, 2003). In effect, neuropeptides regulating the secretion of JH and ecdysteroids could be considered the "master" hormones, because their secretion is directed by the CNS as an integrated response to external and internal cues, such as day length and nutrient stores (see **Chapter 3.2**).

Peptides acting as hormones or neurotransmitters have innumerable effects on behavior, ion and metabolite homeostasis, and locomotion, which are key to successful reproduction by insects (see **Chapter 3.10**). Only recently have comprehensive catalogs for peptide messenger genes been made available for *D. melanogaster* (Taghert and Veenstra, 2003) and *Anopheles gambiae* (Riehle *et al.*, 2002), thanks to their respective genome projects.

Approximately 40 different genes encoding peptide messengers are present in these dipterans, and many of these genes contain multiple copies of variant peptides. New peptide messenger genes await discovery in these insects, and future studies should elucidate the conservation of peptide messenger genes among insect orders. In addition, receptors for these peptides and elements of diverse signal transduction pathways have been revealed by bioinformatics in concert with gene expression and mutation for *D. melanogaster* (Taghert and Veenstra, 2003). Most importantly, elucidation of these pathways will lead to a greater understanding of how peptide messengers modulate biochemical processes and gene expression in insect cells.

3.9.2.9.1. Allatotropins The allatotropin (AT) family of structurally related peptides is so-named because the first peptide was isolated based on its stimulation of the CA *in vitro* to produce JH (see **Chapter 3.7**). In insects where vitellogenesis is JH dependent, this peptide is probably an important initiator of reproduction. The first peptide shown to have this activity was purified from head extracts of pharate adult *M. sexta* (Manse-AT; GFKNVEMMTARGFa; Kataoka *et al.*, 1989). Identical peptides and genes encoding related ones have been identified in other species of Lepidoptera (Taylor *et al.*, 1996; Oeh *et al.*, 2000; Truesdell *et al.*, 2000; Park *et al.*, 2002; Abdel-latief *et al.*, 2003). In *M. sexta*, three alternatively spliced mRNAs result from AT gene expression, which has been localized by *in situ* hybridization and immunocytochemistry to cells in the brain, frontal ganglion ventral ganglia, and midgut of different life stages (review: Elekonich and Horodyski, 2003). In *P. unipuncta*, expression of the AT gene was observed first in late pupae and continued during the adult stage (Truesdell *et al.*, 2000).

Related peptides and genes encoding such peptides have been identified in *L. migratoria* (Paemen *et al.*, 1991), a beetle (*L. decemlineata*; Spittaels *et al.*, 1996), two species of Diptera, *A. aegypti* (Veenstra and Costes, 1999) and *A. gambiae*, and in a few other invertebrates (Elekonich and Horodyski, 2003). Immunocytochemical studies with Manse-AT antisera have revealed the presence of AT-like peptides in cockroaches and two other dipterans, including *D. melanogaster* (Elekonich and Horodyski, 2003), but no ortholog AT gene has been identified in the *D. melanogaster* genome as yet (Taghert and Veenstra, 2003).

In vitro assays with adult CA are routinely used to investigate the pathway of JH biosynthesis. Manse-AT stimulates the CA to produce JH I, II, and III, but is limited by availability of farnesoic acid, a precursor (Elekonich and Horodyski, 2003). This AT also stimulates JH secretion *in vitro* by larval and adult CA of other lepidopteran species and the adult CA of the honeybee, *A. mellifera* (Rachinsky *et al.*, 2000) and blowfly, *Phormia regina* (Tu *et al.*, 2001). Similarly, JH secretion by the CA of older sugar fed female *A. aegypti* is increased *in vitro* by this species' AT or farnesoic acid alone, whereas both are required to activate JH synthesis in CA from newly eclosed females (Li *et al.*, 2003).

In adult insects, ATs have demonstrated effects on other processes associated with reproduction. *L. migratoria* AT may play a role in oviposition, as suggested by its myotropic activity on oviducts from locust and cockroach females, *Leucophaea maderae*, the basis for its isolation from male accessory glands (Paemen *et al.*, 1991). Immunocytochemistry showed that AT-immunoreactivity had a widespread and sexually dimorphic distribution in the nervous system of adult locusts and cockroaches (Nässel, 2002).

3.9.2.9.2. Allatostatins Three groups of neuropeptides in insects have been isolated based on their inhibition of JH secretion by CA *in vitro* and are known as allatostatins (ASTs). The first peptides with this bioactivity were isolated from the cockroach, *D. punctata* (Woodhead *et al.*, 1989) and are now termed the A- or cockroach-type of AST (AST-A). Typically, they are short peptides with the signature terminal sequence of F/YXFGLa, and identified AST-A cDNAs and genes encode a propeptide containing 13 or 14 peptides with slight sequence variations that are posttranscriptionally cleaved and processed (Belles *et al.*, 1999; Meyering-Vos *et al.*, 2001). The second group, known as the B- or cricket-type of AST (AST-B), is a family of peptides with a common sequence of $W(X)_6Wa$, e.g., GWQDLNGGWa and AWERFHGSWa, first identified in the cricket, *G. bimaculatus* and shown to inhibit JH biosynthesis *in vitro* in CA from virgin females (Lorenz *et al.*, 1995). Related peptides were isolated first from a locust, *L. migratoria*, based on their inhibition of oviduct contractions (Schoofs *et al.*, 1991). The third group or C-type of AST (AST-C) is represented by the 15 amino acid, non-amidated peptide (PEVRFRQCYFNPISCF), first discovered in *M. sexta* (Kramer *et al.*, 1991), based on its inhibition of JH synthesis by CA from adult moths.

All three AST types probably exist in insects, as indicated by the annotation of peptide messenger genes for *D. melanogaster* (Taghert and Veenstra, 2003) and *A. gambiae* (Riehle *et al.*, 2002), and other reports detail the isolation of related peptides

or characterization of encoding cDNAs or genes from a variety of insects (reviews: Bendena *et al.*, 1999; Stay, 2000). Numerous immunocytochemical studies have examined the distribution of AST types in a great variety of insects and even different life stages and organs (Nassel, 2002). Each AST type appears to have a unique distribution in the CNS, and the presence of an AST type in the CA is dependent on whether it affects JH secretion by the CA. Interestingly, AST-A is also present in midgut endocrine cells and hemocytes (Stay, 2000), thus suggesting an even greater endocrine repertory.

In general, it appears that one AST type inhibits JH secretion by CA in one or more life stages of a particular species, and the other types do not. The dipteran ASTs are exceptional in that none affect JH secretion by dipteran larval CA, but blowfly AST-A (callatostatin) inhibits JH biosynthesis by CA from cockroaches (Duve *et al.*, 1993). Notably, peptides from all three AST types have been shown to inhibit muscle contractions in a variety of organs from different insect groups. Receptors for ASTs have been identified in a cockroach (AST-A; Auerswald *et al.*, 2001), a silk moth (AST-A; Secher *et al.*, 2001) and *D. melanogaster*, after candidate G protein-coupled receptors (GPCR) were expressed in cell systems and shown to bind AST-A (two different GPCRs; Larsen *et al.*, 2001), AST-B (Johnson *et al.*, 2003), and AST-C (Kreienkamp *et al.*, 2002).

In addition, ASTs have demonstrated effects on female reproduction. An AST-A inhibited vitellogenin release *in vitro* by fat body of cockroach females, *B. germanica* (Marin *et al.*, 1996). The inhibitory effect was counteracted by the addition of mevalonolactone, thus suggesting that the AST-A inhibited synthesis of mevalonate, and hence dolichol, and therefore impairing the glycosylation and export of vitellogenin from the fat body. *In vitro* ecdysteroid biosynthesis by ovaries from a cricket, *G. bimaculatus*, was inhibited by an AST-B (Lorenz *et al.*, 1997), the same action as prothoracicostatic peptides in the AST-B family have on larval prothoracic glands from the silk moth, *B. mori* (Hua *et al.*, 1999; **Chapter 3.2**). Surprisingly, there are only a few reports on the *in vivo* effects of ASTs in female insects. After showing that bioactive AST-As were circulating in *D. punctata*, three synthetic AST-As were injected every 12 h for 3 days into mated females and were found to significantly decrease oocyte length and *in vitro* JH synthesis by the CA in treated females (Woodhead *et al.*, 1993). Similar injections of cricket AST-A and AST-B into female crickets, *G. bimaculatus*, resulted in decreased body and ovary weight, eggs/ovary, and ovary ecdysteroid biosynthesis and increased vitellogenin titer in hemolymph. The peptides had little or no effect on JH biosynthesis; however, these trends were not statistically significant when compared to controls (Lorenz *et al.*, 1998).

3.9.2.9.3. Ovary ecdysteroidogenic hormone and neuroparsin Ovaries in female insects produce ecdysteroids, but only in Diptera do these hormones direct the increased gene expression required for vitellogenesis. In a series of classic endocrine studies on female mosquitos, the first "egg development neurosecretory hormone" or gonadotropin was described for insects (Lea, 1972). Almost 30 years later, this hormone was isolated from the heads of female *A. aegypti*, structurally characterized as an 86 residue peptide, and renamed "ovary ecdysteroidogenic hormone" (OEH), based on its direct stimulation of ecdysteroid synthesis by ovaries *in vitro* (Brown *et al.*, 1998). Immunocytochemistry with OEH antiserum stained clusters of medial neurosecretory cells in brains, as well as other cells in the ventral ganglia and midguts of larvae and both sexes of *A. gambiae* and *A. aegypti* (Brown and Cao, 2001). Cloning of the OEH cDNA revealed the OEH prohormone and led to the bacterial expression of a recombinant OEH that was shown to have the same bioactivity *in vitro* and stimulated vitellogenesis and subsequent yolk deposition; this is considered to be an indirect effect of increased ovarian ecdysteroidogenesis (Brown *et al.*, 1998). An ortholog OEH gene has been identified in another mosquito, *A. gambiae*, but not in *D. melanogaster* (Riehle *et al.*, 2002).

In Diptera, activation of ovarian ecdysteroidogenesis by OEH or other brain factors may occur through either an insulin signaling pathway (see below) or a G protein-coupled receptor (GPCR)/cAMP pathway (Shapiro, 1983). Factors with OEH activity have been extracted from brains of higher flies (Adams and Li, 1998), and in the blowfly, *Phormia regina*, ovarian steroidogenesis is stimulated *in vitro* by cAMP analogs and preceded by a peak in ovarian cAMP levels after a protein meal (Manière *et al.*, 2000). This study also showed that crude brain extracts, as well as separate extracts of the medial neurosecretory region and the rest of the brain, stimulated ovarian ecdysteroidogenesis *in vitro* but, surprisingly, the medial neurosecretory extracts did not elicit an increase in ovarian cAMP levels, whereas the crude brain and nonneurosecretory region extracts did. These results offer additional support for the existence of ecdysteroidogenic brain factors in female flies that act through different signaling pathways.

Mosquito OEHs are closely related to the neuroparsins first isolated from the corpora cardiaca of

L. migratoria (Girardie *et al.*, 1987). Experiments showed that the purified native A and B forms (83 and 78 residues, respectively) administered to adult females inhibited oocyte growth, whereas injection of neuroparsin antibody alone had the opposite effect (Girardie *et al.*, 1987). These are considered to be anti-JH effects, since JH is required for vitellogenesis in locusts, and ecdysteroids have no known role, but the mechanism for these effects remains unknown. Neuroparsins also elevated trehalose and lipid levels in hemolymph of male locusts (Moreau *et al.*, 1988) and act as antidiuretics on rectal pads (Girardie and Fournier, 1993).

As revealed by the cloning of *L. migratoria* neuroparsin cDNA, both neuroparsin forms are processed from a prohormone (Langueux *et al.*, 1992). Other neuroparsins have been isolated and identified in the locust, *Schistocerca gregaria*, and have a similar bioactivity (Girardie *et al.*, 1998a). More recently, four different neuroparsin transcripts/cDNAs have been identified in *S. gregaria* (Janssen *et al.*, 2001; Claeys *et al.*, 2003); two of the transcripts were present only in brains of all life stages, whereas the other two transcripts were in the CNS, fat body, and male accessory glands and testis. Northern blot analyses showed that levels of all four transcripts changed throughout the life stages, with a significant increase in the two widely distributed ones preceding the sexual activity of males and females. Peptides for three of the transcripts have yet to be isolated, thus their role in reproduction is unknown.

Locust neuroparsins and mosquito OEHs constitute a peptide family, as substantiated by an analysis of nucleotide and protein sequence databases that revealed neuroparsin related peptides in the honeybee and diverse arthropod and mollusk species (Claeys *et al.*, 2003). This analysis further suggested that conserved features of these peptides are shared by insulin-like growth factor binding proteins, which modulate growth factor activity, in vertebrates.

3.9.2.9.4. Ovary maturing parsin A neurohormone was isolated from female locusts, *L. migratoria*, based on its stimulation of vitellogenesis and oocyte growth, and was thus designated "ovary maturating parsin" (OMP; Girardie *et al.*, 1991). The peptide of 65 residues occurs in two isoforms, differing only at one residue position. With immunocytochemistry, OMP was localized in brain neurosecretory cells and the corpora cardiaca of locusts in all stages (Richard *et al.*, 1994). In a later study, sets of female *L. migratoria* were injected daily with different amounts of OMP over 10 days after eclosion and found to have significantly increased hemolymph titer of ecdysteroids and oocyte length over controls (Girardie *et al.*, 1998b). OMP has been identified in another locust, *S. gregaria*, and exists as isoforms: two long forms corresponding to those of *L. migratoria* OMP and two shorter forms found only in mature adults (Girardie *et al.*, 1998b). Injection of mixed OMPs in female *S. gregaria* over 12 days similarly affected ecdysteroid and vitellogenin levels in hemolymph and stimulated oocyte growth, relative to controls (Girardie *et al.*, 1998b). Although locust OMPs are considered ecdysteroidogenic and gonadotrophic peptides *in vivo*, no direct effects have been reported for OMP on isolated locust ovaries or fat body, thus, the mode of action needs further study. OMPs are known to exist in acridian Orthoptera, but not in other insects (Richard *et al.*, 1994), as indicated by the failure to identify related peptides in the *D. melanogaster* and *A. gambiae* genome databases.

3.9.2.9.5. Short neuropeptide F, neuropeptide F, and head peptides The sequences of a great diversity of insect neuropeptides end in Arg-Phe-NH_2, and three such peptides of relevance were isolated from the Colorado potato beetle, *L. decemlineata*, (ARGPQLRLRFa and APSLRLRFa; Cerstiaens *et al.*, 1999) and the locust, *S. gregaria* (YSQVARPRFa; Schoofs *et al.*, 2001). In a subsequent experiment, multiple injections of the beetle peptides into virgin female locusts, *L. migratoria*, significantly increased oocyte growth relative to controls (Cerstiaens *et al.*, 1999). Injection of the *S. gregaria* peptide stimulated ovarian growth in female *S. gregaria*, presumably due to the increased vitellogenin levels in the peptide treated females (Schoofs *et al.*, 2001). It has not been determined whether these effects are specific to vitellogenesis or protein synthesis in general.

The two beetle peptides, along with peptides identified in other insect and arthropod species, belong to a "short neuropeptide F" (sNPF) family, as clearly indicated by ortholog genes in *D. melanogaster* (Taghert and Veenstra, 2003) and *A. gambiae* (Riehle *et al.*, 2002) that encode related short peptides ending in RLRFa or RLRWa. A functional GPCR for the *D. melanogaster* sNPFs has been characterized and its tissue expression characterized (Mertens *et al.*, 2002; Feng *et al.*, 2003), but no other bioactivities are known for insect sNPFs. The "head peptides" first identified in *A. aegypti* and later shown to inhibit host seeking behavior in nonoogenic females (Brown *et al.*, 1994) are not members of the sNPF family, as indicated by their terminus, KTRFa, and the structure of the *Aedes* head peptide cDNA (Stracker *et al.*, 2002).

Surprisingly, analysis of the *D. melanogaster* and *A. gambiae* genome databases revealed no ortholog "head peptide" genes (Riehle *et al.*, 2002).

The sequence of the *S. gregaria* peptide (probably a fragment) is more similar to the C-terminus of longer peptides (36 residues almost invariably) in the "neuropeptide F/Y" family of invertebrate and vertebrate peptides (Stanek *et al.*, 2002). A functional GPCR for the *D. melanogaster* NPF has been characterized (Garczynski *et al.*, 2002), and in *D. melanogaster*, NPF controls foraging and social behavior by larvae (Wu *et al.*, 2003), but no bioactivities are known for adults.

3.9.2.9.6. Insulin-like peptides and the insulin signaling pathway Insulin-like peptides (ILPs) are known for only a few insect species: *L. migratoria*, three species of Lepidoptera, and *D. melanogaster* (review: Claeys *et al.*, 2002). In all but the locust, multiple ILP genes have been identified, with *B. mori* having an astonishing 32 ILP genes (also known as "bombyxins") and *D. melanogaster* and *A. gambiae*, each with 7 ILP genes (Krieger *et al.*, 2004). In insects, clusters of neurosecretory cells in the medial, dorsal region of brains are immunostained by insulin and ILP antisera in different life stages and species (Cao and Brown, 2001; Rulifson *et al.*, 2002). The gut and reproductive tract may be another source of ILPs as reported for a few insect species (Montuenga *et al.*, 1989; Iwami *et al.*, 1996); these organs are the primary sources of insulin and related peptides in vertebrates. The presence of ILPs in hemolymph of larvae and adult silk moths has been established, and the ILP titer is much higher in males after eclosion (Satake *et al.*, 1999).

As characterized for vertebrates and invertebrates, insulin and related peptides act primarily through a receptor tyrosine kinase and an insulin receptor substrate (IRS), phosphatidylinositol 3-kinase (PI3K)/protein kinase B/Akt signaling pathway (Oldham and Hafen, 2003). For insects, the expression and function of proteins comprising this pathway have been characterized in detail only for *D. melanogaster* (Garofalo, 2002), and ortholog genes of the proteins were identified in *A. gambiae* (Riehle *et al.*, 2002). An ever-increasing number of studies show that this pathway is a nexus for the transcriptional and translational regulation of growth and longevity in *D. melanogaster* and other animals (Oldham and Hafen, 2003; Tatar *et al.*, 2003).

Perturbations in any gene encoding ILPs or proteins in this pathway result in multiple and dramatic effects on not only embryonic and postembryonic development, but also oogenesis and vitellogenesis in female *D. melanogaster*. Females with an ILP gene mutation had at most a single vitellogenic oocyte in each ovariole and thus a much reduced fecundity, in comparison to wild-type females (Ikeya *et al.*, 2002). Mutations in the gene encoding the insulin receptor (IR) result in sterile females due to reduced ovariole development and yolk deposition (Chen *et al.*, 1996). Females with an IR mutation are longer lived but deficient in JH synthesis by the CA and ovarian ecdysteroid production (Tatar *et al.*, 2001; Tu *et al.*, 2002). Treatment of these mutant flies with a JH analog initiates vitellogenesis and restores normal life expectancy. Vitellogenesis is blocked in females with a homozygous mutation in the IRS gene (Drummond-Barbosa and Spradling, 2001), yet another step in this key pathway.

There are only a few studies of the insulin signaling pathway in other insects. In the mosquito *A. aegypti*, the expression pattern and phosphorylation states of the IR and PKB/Akt in ovaries were characterized through previtellogenic arrest and a gonotrophic cycle after a blood meal (Riehle and Brown, 2002, 2003). Bovine insulin stimulates ecdysteroidogenesis by *A. aegypti* ovaries directly in a dose-dependent manner *in vitro*, whereas specific inhibitors of tyrosine kinase activity and PI3K inhibit ecdysteroid production (Riehle and Brown, 1999). In Lepidoptera, a putative IR was identified in tissues from *M. sexta* larvae with an IR antiserum (Smith *et al.*, 1997), and ovarian cells from three species showed high-affinity binding to a silk moth ILP, presumably to ovarian IRs recognized by an IR antiserum (Fullbright *et al.*, 1997).

Recently, the panoply of insulin-like peptide regulation in *D. melanogaster*, in part, has been shown to act through forkhead transcription factors and is nutrition dependent (Junger *et al.*, 2003). The possibility also exists that insect ILPs may activate MAP kinases and G protein-coupled receptor/cAMP pathways (see **Chapter 3.2**), as shown for insulin related peptides in vertebrates (Hsu *et al.*, 2002; Oldham and Hafen, 2003), and even elict cross-talk between signaling pathways. Conceptually, this activation of multiple pathways by ILPs has the potential to exhibit as profound an effect on the expression of genes required for reproduction in insects, as do JH and ecdysteroids.

3.9.2.9.7. Ovary peptides that block vitellogenesis Unrelated peptides have been isolated from mature ovaries of dipteran species based on their ability to block egg maturation. The first such peptide (YDPAPPPPPP) was extracted from the ovaries of the mosquito, *A. aegypti*, and shown to inhibit yolk deposition in blood-fed females (Borovsky,

2003). Later, the direct action of this peptide was determined to be inhibition of proteolytic enzyme biosynthesis (see **Chapter 4.7**) in the midgut (see **Chapter 4.5**), and thus is known as a "trypsin modulating oostatic factor" (TMOF). With a TMOF antiserum, this peptide was localized to the follicular epithelium surrounding oocytes only after 24 h PBM and was not present in other tissues (Borovsky *et al.*, 1994). This localization is supported by the characterization of a gene encoding a vitelline membrane protein, which contains TMOF sequences (Lin *et al.*, 1993), and this protein is secreted by follicular epithelium cells.

A different peptide (NPTNLH) with this same activity was purified from ovarian extracts of the flesh fly *Neobellieria bullata* (Bylemans *et al.*, 1994; Borovsky *et al.*, 1996). Injection of the hexapeptide inhibited trypsin synthesis by the midgut of liver-fed flesh fly females, resulting in a reduction of circulating vitellogenin and oocyte growth. Immunoassays determined that *N. bullata* TMOF-staining was localized exclusively over yolk granules in oocytes and that a 75 kDa precursor protein was present in ovary extracts (Bylemans *et al.*, 1996). A more recent study has shown that this peptide is probably a substrate for an angiotensin converting enzyme circulating in the hemolymph of female flies, and feeding of an inhibitor of this enzyme enhanced hemolymph vitellogenin levels (Vandingenen *et al.*, 2001). A second peptide (SIVPLGLPVPIGPIVVGPR) with a similar effect was also purified from ovarian extracts of this fly (Bylemans *et al.*, 1995) and named "colloostatin," given its sequence similarity to collagen. When administered *in vivo*, the peptide inhibits yolk uptake by previtellogenic oocytes and reduces the levels of circulating vitellogenin, but it does not inhibit trypsin biosynthesis in the gut.

3.9.2.9.8. Termination of vitellogenesis A few studies have shown that peptide hormones are involved in the transition from vitellogenesis to chorionogenesis so that egg maturation and ultimately oviposition can occur. Oostatic factors originating from ovaries are known for insects, and these factors may play a role in this transition through distinctly different actions, including inhibition of ovary ecdysteroidogenesis (Kelly *et al.*, 1986; Adams and Li, 1998), than that reported for TMOF and other such "oostatic" factors. In dipteran females, termination of ovary ecdysteroidogenesis indirectly may end vitellogenesis, and recent studies indicate that ovarian steroidogenesis is inhibited through signaling pathways that use Ca^{2+} or cGMP as intermediates. *In vitro* ecdysteroidogenesis by vitellogenic ovaries from the blowfly, *P. regina*, was inhibited by Ca^{2+} ionophores or thapsigargin, whereas inhibitors of Ca^{2+}-calmodulin phosphodiesterases increased ecdysteroidogenesis (Manière *et al.*, 2002). Analogs of cGMP inhibited *in vitro* steroid biosynthesis in vitellogenic ovaries from *P. regina*, thus correlating with the peak levels of cGMP detected in ovaries at the termination of vitellogenesis (Manière *et al.*, 2003). Notably, paracrine or autocrine factors, such as nitric oxide, were implicated in this inhibition, and not brain factors, thus pointing to an even greater degree of complexity in the regulation of insect reproduction.

Th adipokinetic hormone (AKH) family has key roles in regulating lipid and carbohydrate metabolism in all life stages of diverse insects (see **Chapter 3.10**). These peptides also are known to inhibit protein and vitellogenin synthesis and RNA synthesis in female fat body and circulate in the hemolymph of ovipositing locust females (Moshitzky and Appelbaum, 1990; Glinka *et al.*, 1995; Kodrik and Goldsworthy, 1995). AKH regulation occurs through a GPCR, now identified in *D. melanogaster* and *B. mori* (Staubli *et al.*, 2002), and a signaling pathway with cAMP and Ca^{2+} intermediates (Gäde *et al.*, 1997).

A GPCR/cAMP signaling pathway is involved in the termination of vitellogenesis in mosquitos (Dittmer *et al.*, 2003). Characterization of the gene encoding a cAMP response-element binding protein in the mosquito, *A. aegypti* (AaCREB) revealed signature domains for this family of transcription factors, and this gene was constitutively expressed in female fat body, where YPs are synthesized. Elicitors of the cAMP signal transduction pathway attenuated ecdysteroid stimulated YP gene expression by fat body *in vitro*. In cell transfection assays, AaCREB served as a potent repressor of transcription, and analysis of electrophoretic mobility shift assays detected CREB specific band-shift complexes in nuclear extracts from vitellogenic fat bodies at 24 h and 36 h post-blood meal, when YP gene expression reaches its peak then terminates. Examination of the regulatory regions of Vg and vitellogenic carboxypeptidase revealed putative CREB response elements, which bound *in vitro* expressed AaCREB and offers further support for its termination of YP gene expression in the fat body of this mosquito.

3.9.3. Hormones and Male Reproduction

3.9.3.1. Spermatogenesis

Insect spermatogenesis can be divided into three main steps: (1) mitotic proliferation of spermatogonia, leading to spermatocytes; (2) meiosis of

spermatocytes, giving the spermiocytes; and (3) spermiogenesis of spermiocytes, leading to spermatozoa. As for female reproduction, ecdysteroids, JHs, and peptides have demonstrated effects on male reproductive processes.

3.9.3.1.1. Ecdysteroids and early spermatogenesis In the early steps of spermatogenesis, involving mitoses and meioses, the stimulatory role of ecdysteroids has been demonstrated in many species of Orthoptera, Hemiptera, Lepidoptera, and Diptera (Hagedorn, 1985). In *R. prolixus*, for example, mitotic division of spermatogonia takes place in the last larval instar in the absence of JH and in the presence of high levels of ecdysteroids. In addition, it has been shown experimentally that 20E increases the rate of mitosis in the spermatogonial cells (Dumser, 1980). Further corroboration of the importance of ecdysteroids in early spermatogenesis has been provided by Jacob (1992) who reported that fragments of testes from the rhinoceros beetle, *Oryctes rhinoceros*, incubated *in vitro* require ecdysteroids and the testis sheath to complete the mitotic and meiotic processes.

The stimulatory function of ecdysteroids in early stages of spermatogenesis gives physiological sense to the discovery of ecdysteroid production in the testes of the lepidopteran *Heliothis virescens* (Loeb *et al.*, 1982). These authors observed that the testis sheath of tobacco budworm larvae, when incubated *in vitro*, secreted ecdysteroids into the culture medium. Testes ecdysteroid production has been demonstrated for other Lepidoptera belonging to the genera *Lymantria*, *Ostrinia*, *Mamestra*, *Leucania*, and *Spodoptera* (see Loeb *et al.*, 2001 and references therein).

3.9.3.1.2. The role of juvenile hormone The role of JH in spermatogenesis is less clear. Early reports suggested that JHs antagonize the stimulatory effects of ecdysteroids, especially in early stages of spermatogenesis (see Dumser, 1980; Koeppe *et al.*, 1985; Wyatt and Davey, 1996). However, other contributions have shown that JH seems to have a stimulatory effect on late spermatogenesis, especially in diapausing species when JH simultaneously accelerates spermiogenesis and interrupts the diapause (Koeppe *et al.*, 1985). For example, administration of a JHA to diapausing adult leafhoppers, *Draeculacephala crassicornis*, did not influence mitosis of spermatogonia but promoted the formation of spermatozoa (Reissig and Kamm, 1974). Results obtained in the beetle, *Oryctes rhinoceros*, in addition to showing that ecdysteroids are necessary in early spermatogenesis (see above), also demonstrated that spermiogenesis took place *in vitro* only if an active CA pair was present in the incubation medium (Jacob, 1992). The stimulatory role of JH on spermiogenesis may be related to polyamine synthesis, especially putrescine, spermidine, and spermine, given that JH promotes the production of these compounds, at least in fat body and neural tissue of crickets (Cayre *et al.*, 1995).

Another effect of JH related to sperm physiology has been discovered by Dean and Meola (1997) in the cat flea, *Ctenocephalides felis*. These authors have shown that sperm transfer into the epididymis is stimulated when the fleas are exposed to a filter paper treated with JH III or JHAs. As the concentration of the JHA or the exposure time increases, the percentage of fleas that transfer sperm also increases.

3.9.3.1.3. Peptides and proteins involved in spermatogenesis In relation to ecdysteroid biosynthesis in the testes, Wagner *et al.* (1997) have identified a 21 amino acid peptide (ISDFDEYEPLNDADNNEVLDF) in brain extracts of the gypsy moth, *L. dispar*, that has ecdysteroidogenic properties. Given that the peptide induces the synthesis of ecdysteroids specifically in the testes (induction experiments using prothoracic glands were unsuccessful), it has been called "testes ecdysiotropin."

Other peptide or protein factors are postulated to be directly involved in the regulation of spermatogenesis. As early as 1953, C. Williams reported the occurrence of a macromolecular factor in the hemolymph of *Hyalophora cecropia* that stimulated spermatogenesis in spermatocysts incubated *in vitro*. In 1971, Kambysellis and Williams showed that 20E allowed the entry of this factor into the testis (see Hagedorn, 1985), but since then, no such factor has been isolated. More recently, spermatid differentiation in *Drosophila* was shown to require a peptidyl peptidase ortholog of the mammalian angiotensin converting enzyme (ACE) (Hurst *et al.*, 2003), and ACE mRNA is found mainly in large primary spermatocytes, whereas it is not detectable in cyst cells.

3.9.3.2. Male Accessory Gland Function

The male accessory glands of insects generally are of mesodermal origin and exhibit a wide morphological diversity, from single pairs of histologically and morphologically identical tubules to multi-paired heterogeneous tubules, with multiple forms and contents (Happ, 1992). Development of male accessory glands is regulated by ecdysteroids (review: Happ, 1992), as with many other developmental processes. This section will focus on the endocrine regulation of the biosynthetic and secretory activity of the

glands. The secretions produced by male accessory glands have a wide diversity of functions (see **Chapter 1.5**), and some of these secretions, when transferred to the female through copulation, may affect the process of vitellogenesis, as described below.

3.9.3.2.1. Products of the male accessory glands The male accessory glands synthesize and secrete a complex mixture of proteins, carbohydrates, lipids, and amino acids (Chen, 1984; see **Chapter 1.5**) that are transferred to the female during copulation. The primary function of the accessory gland products is to facilitate sperm transfer to the female. For example, these glands produce the structural proteins needed for spermatophore formation, and these spermatophore proteins may serve as nutrient resources for the female. In addition, many secretory products of the accessory glands cause physiological and behavioral changes in the mated female. These changes may be sperm related (sperm protection, storage and activation, competition with the sperm of previous males) or may alter the behavior and reproductive physiology of the mated female. The most prominent behavioral effects are the reduction of attractiveness to males and induction of refractoriness. The effects on reproductive physiology include enhancement of oocyte growth and induction of ovulation and oviposition (Chen, 1984; Gillott, 2003).

In recent years, research has been focused on Drosophilid dipterans, and more than 75 accessory gland proteins have been molecularly characterized in *D. melanogaster* (Swanson *et al.*, 2001; **Chapter 1.5**). Of these proteins, the so-called sex peptide has been the most thoroughly studied compound (see **Chapter 1.5**). The sex peptide of *D. melanogaster* is synthesized as a 55 amino acid precursor, and the processed peptide depresses sexual receptivity and enhances oviposition. Ortholog peptides have been identified in other *Drosophila* species (Chen, 1996; Wolfner, 1997). In *D. melanogaster*, sex peptide stimulates the biosynthesis of JH *in vitro* by the CA of the adult female (Moshitzky *et al.*, 1996). This activity is possibly related to the results of Soller *et al.* (1999), which showed that the same peptide stimulates vitellogenic oocyte progression in *D. melanogaster* and that a JHA mimics such an effect. Interestingly, the sex peptide of *D. melanogaster* stimulates JH synthesis in *Helicoverpa armigera* (Fan *et al.*, 1999), which suggests that this Noctuid moth may have ortholog peptides with these allatotropic properties. Male accessory glands are an important source of JHs and related metabolites (see Section 3.9.3.2.2). These JHs can be transferred to females through copulation and enhance oocyte growth, as shown for certain lepidopterans in which JH regulates vitellogenesis (Shirk *et al.*, 1980; Park *et al.*, 1998).

3.9.3.2.2. Regulation of male accessory gland function by juvenile hormone In the 1940s, Wigglesworth carried out the first studies showing the importance of CA in male accessory gland function for *Rhodnius*. In the cockroach, *B. germanica*, JH influences the growth not only of the accessory glands (Piulachs *et al.*, 1992) (**Figure 20**) but also of the conglobate gland (Vilaplana *et al.*, 1996). Further research on other insect species, mainly orthopterans, dictyopterans, lepidopterans, and dipterans, has demonstrated that allatectomy depresses to a greater or lesser degree the accumulation of gland secretions, whereas JH treatment restores the usual secretion levels (Gillott, 1996; Wyatt and Davey, 1996). In a number of species, it has been reported that the pattern of secretion accumulation in the accessory glands is parallel to that of JH concentration in the hemolymph or to that of JH synthetic rates by the CA (Wyatt and Davey, 1996). Incubation of the male accessory glands from *D. melanogaster* with nanomolar concentrations of JH induced a nearly threefold stimulation of protein synthesis (Yamamoto *et al.*, 1988). Since many accessory gland proteins in *D. melanogaster* have been characterized at the molecular level (see above and **Chapter 1.5**), monitoring expression of these genes in response to JH has become easier. For example, Cho *et al.* (2000) have shown that the protein Mst57Dc is highly expressed after eclosion, when the titer of JH III peaks, and that in the JH deficient mutant *ap56f*, the levels of Mst57Dc mRNA are about 60% of those of the wild-type.

Subsequent studies have monitored the effect of JH on the pattern of proteins, or even on particular proteins, present in accessory glands for species of Hemiptera, Orthoptera, and Dictyoptera (Wyatt and Davey, 1996). For example, allatectomy differentially affects the accumulation of various proteins present in the glands, whereas treatment with JH affects the protein pattern in the grasshopper, *Melanoplus sanguinipes* (Gillott, 1996), and in *B. germanica* (Belles and Piulachs, 1992). In *M. sanguinipes*, treatment with JH III induces the accumulation of most proteins but depresses the accumulation of two (Gillott, 1996). A later study of *M. sanguinipes* showed that JH acts directly on the accessory glands to promote synthesis of a specific protein, LHPI, which constitutes more than 50% of the protein content (Gillott and Gaines, 1992). Juvenile hormone could not stimulate LHPI synthesis in glands from allatectomized males, unless there was an

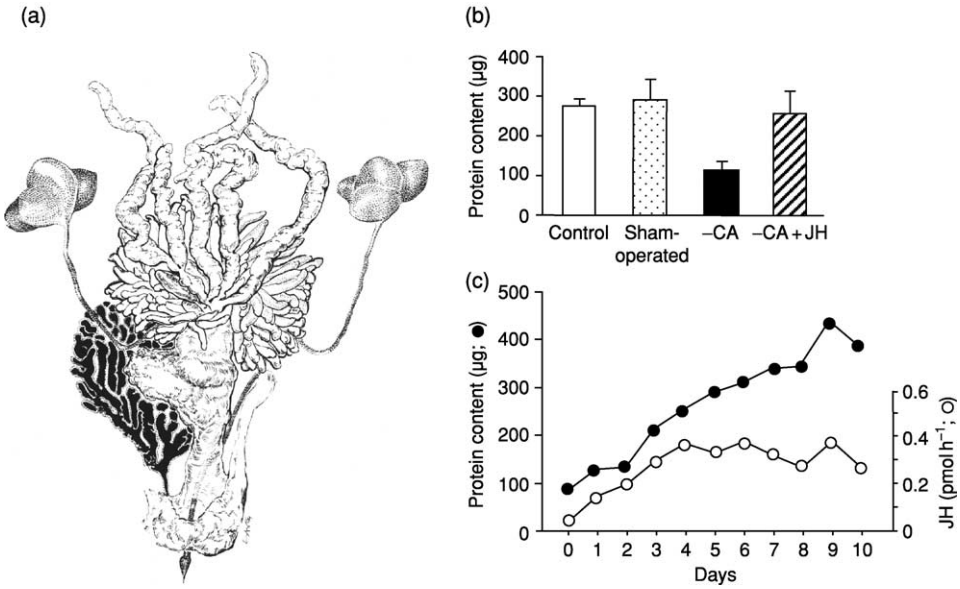

Figure 20 Protein accumulation in the male accessory glands of *Blattella germanica* (a) is regulated by juvenile hormone (JH), as shown by allatectomy experiments (−CA) and subsequent treatment with 10 µg of JH III (−CA + JH) (b). In addition, the patterns of increase in protein contents in the first days of adult life, and that of JH production by the corpora allata incubated *in vitro* are approximately parallel (c). (Based on data from Piulachs, M.D., Maestro, J.L., Belles, X., **1992**. Juvenile hormone production and accessory reproductive gland development during sexual maturation of male *Blattella germanica* (L.) Dictyoptera, Blattellidae). *Comp. Biochem. Physiol. 102A*, 477–480; and Belles and Piulachs, unpublished data.

overnight exposure to the hormone. Cycloheximide temporally abolishes the stimulatory effect of JH on LHPI synthesis in the glands of male *M. sanguinipes* (Ismail *et al.*, 1995), thus suggesting that the action of JH is mediated by protein factors involved in the transcription of LHPI. A similar priming effect of JH on protein synthesis in the accessory glands of male *L. migratoria* has been reported (Braun and Wyatt, 1995). It is worth noting that the protein Met of *D. melanogaster*, which may be involved in the action of JH action, is localized in the accessory glands and ejaculatory duct cells of adult males (Pursley *et al.*, 2000).

3.9.3.2.3. Production of juvenile hormone by the accessory glands As mentioned above, JH is a product of the accessory gland. In fact, the first active extracts of JH were obtained by Williams in the 1950s from the abdomen of the adult male of the moth, *Hyalophora cecropia*, and these glands are still known to be the most copious, natural source. It was later shown that the CA of the cecropia moth produce the acids of JH I and II and that these are converted into the corresponding JHs by the male accessory glands (Shirk *et al.*, 1983). Production of JH in the accessory glands has been reported in males of *Heliothis* as well (Park *et al.*, 1998).

JH production by male accessory glands may enable transfer of JH to females during copulation to directly enhance vitellogenesis and oocyte growth or indirectly to stimulate the female's CA. As shown for *H. virescens*, mating has an allatotropic effect, and JH measurements in males and females before and after copulation suggest the transfer of JH to females by the male (Park *et al.*, 1998). Also, production or sequestration of JH by male accessory glands may be another mechanism to regulate JH hemolymph titer or biosynthesis by CA.

3.9.3.2.4. Other hormones regulating male accessory glands Hormones other than JH also may regulate the biosynthetic activity of male accessory glands. In *R. prolixus* males, removal of certain brain neurosecretory cells impairs protein accumulation in the accessory glands, whereas treatment with JH I only partially restores protein accumulation; a peptide extracted from *R. prolixus* brains stimulates protein synthesis by isolated accessory glands (Wyatt and Davey, 1996). As these results indicate, a neuropeptide and JH are required for accessory gland function. Cerebral neurosecretion and JH are involved in male accessory gland secretory activity in another blood-sucking bug, *Panstrongylus megistus* (Regis *et al.*, 1987). In *M. sanguinipes*, cardioallatectomy inhibits the accumulation of protein LHPI to a greater degree than that provoked by allatectomy alone (Cheeseman and Gillott, 1988), which suggests again the

participation of a neurosecretory factor in the regulation of protein secretion by the male accessory glands.

Finally, 20E appears to stimulate total protein synthesis in the male accessory glands of the male lepidopterans, *S. littoralis* and *Chilo partellus* (Gillott, 1996). More detailed studies of *M. sanguinipes* have shown that 20E promotes protein accumulation in the accessory glands incubated *in vitro* (Ismail and Gillott, 1995) and that these glands incubated *in vitro* are able to produce ecdysteroids (Gillott and Ismail, 1995).

3.9.4. Future Directions

Our understanding of how JH, ecdysteroids, and peptide hormones control various aspects of insect reproduction has advanced but also broadened greatly over the last two decades. Of particular importance is the elucidation of genetic expression heirarchies regulating ecdysteroid action at the cellular level and hormonal networks controlling reproduction at the organismal level. Despite the fact that the vitellogenic action of JH is one of the hallmarks of reproduction in most insects, its precise mechanism of action remains poorly understood. Elucidating this mechanism represents the most challenging task for future research. Genetic ecdysteroid networks governing insect development and metamorphosis have been studied in detail. Our understanding of similar networks occurring in reproduction is still limited. Future studies should take advantage of techniques for gene knockout and functional genomics to gain further insight into the regulation of female and male reproduction. Likewise, these techniques offer many advantages for research on peptide hormones involved in insect reproduction.

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