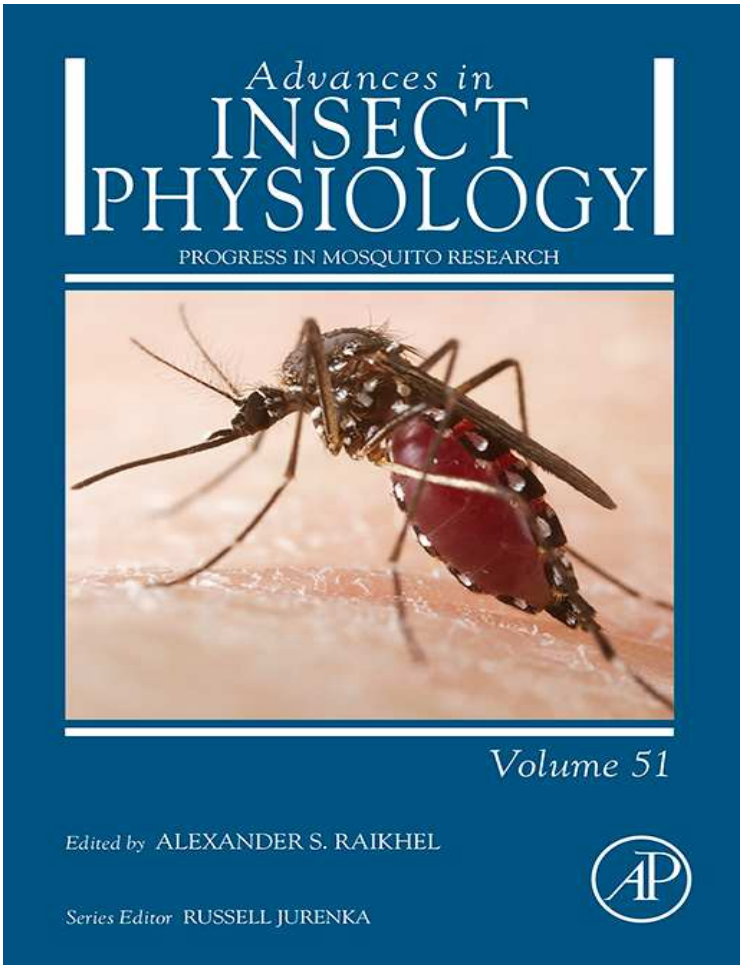

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CHAPTER FIVE

Regulation of Reproductive Processes in Female Mosquitoes

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Contents

Abstract

Females of the vector mosquito species require vertebrate blood for egg development and consequently transmit numerous disease pathogens of humans and animals. Previous studies have determined that a steroid hormone, 20-hydroxyecdysone (20E), is the major hormone regulating egg maturation in female mosquitoes. Its action has been particularly well studied in the fat body, a metabolic tissue functionally

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analogous to the vertebrate liver. This chapter discusses the regulation of ecdysteroid synthesis and the molecular basis of 20E action in reproducing female mosquitoes. Research has also revealed that in addition to 20E, a number of other regulators, such as insulin-like peptides and amino acids, are involved in directing sequential gene expression and the progression of a reproductive cycle. The role of these factors has been reviewed in detail. The concluding section describes recent studies related to differential expression of genes and their temporal regulation, which have been possible due to the availability of mosquito genomes and new and improved molecular techniques.

1. INTRODUCTION

Reproduction in haematophagous insects, like mosquitoes, is linked to blood intake. Mosquitoes such as *Aedes aegypti* and *Anopheles gambiae* are the most important disease vectors. They are anautogenous with an absolute requirement for blood feeding. The females develop and lay a batch of eggs only when they acquire a large enough blood meal. Therefore, reproduction in female mosquitoes is cyclic and serves as a foundation for pathogen transmission. The female mosquito reproductive cycle can be divided into two distinct phases, previtellogenic (posteclosion in the first cycle, PE) and vitellogenic (postblood meal, PBM), during which female mosquitoes undergo dramatic shifts in feeding, alternating between consumption of sugars (nectar) and proteins (blood), respectively. This biphasic reproductive cycle is regulated mainly by alternating peaks of juvenile hormone (JH) and 20-hydroxyecdysone (20E) (Fig. 1). Female mosquitoes undergo a JH-controlled PE phase during which their reproductive tissues mature for subsequent vitellogenesis. At about 72 h PE, females enter the state of reproductive arrest, while seeking a host. A blood meal triggers the onset of a series of swift and synchronized reproductive events, required for 20E-controlled vitellogenesis and the completion of egg development and oviposition within a 72-h period. Following the fall of ecdysteroid titre, vitellogenesis is terminated and the reproductive tissues go through a preparatory stage for the next gonadotrophic cycle. Understanding the regulatory mechanisms governing egg developmental cycles is of paramount importance for exploration of novel control approaches and has been a focus of researchers for decades.

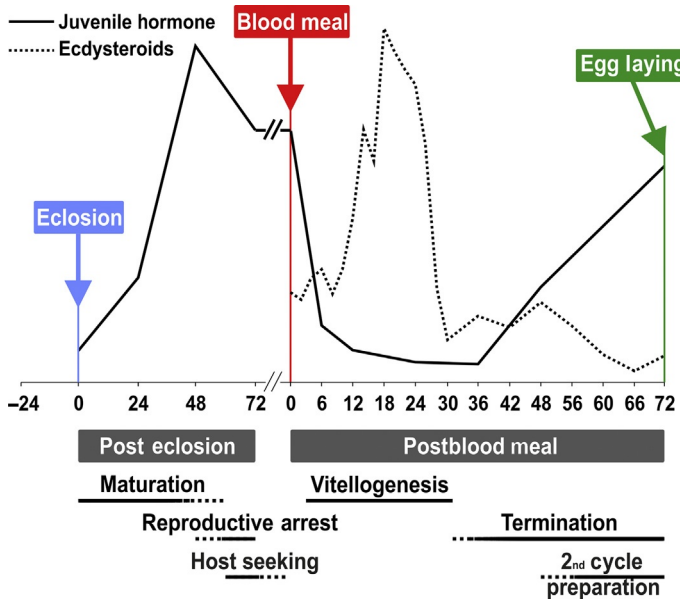

Fig. 1 A schematic diagram of events during the first reproductive cycle of the *Aedes aegypti* female. After eclosion, a rise in juvenile hormone titre promotes the growth of primary ovarian follicles and overall maturation of the female. Around 72 h posteclosion (PE), a female actively seeks a host for a blood meal. Ingestion of a blood meal shuts down JH synthesis in the corpora allata and concurrently triggers synthesis of ecdysteroids in the ovary. Ecdysteroids are the main regulator of vitellogenesis in the fat body. The titre rises at 12 h postblood meal (PBM), peaks at 18 h PBM and sharply declines at 30 h PBM. Low ecdysteroid level is a signal for the cessation of vitellogenesis and start of the terminal phase of the reproductive cycle when the eggs develop fully and are ready to be laid around 72 h PBM. JH titre increases again around 36 h PBM to prepare the female for the next reproductive cycle. JH and ecdysteroid titres are from Shapiro et al. (1986) and Hagedorn et al. (1975), respectively. See chapter "The Role of Juvenile Hormone in Mosquito Development and Reproduction" by Zhu and Noriega for details on JH synthesis.

2. EARLY STUDIES OF ENDOCRINE REGULATION OF MOSQUITO REPRODUCTION

Ever since the classic studies of the blood-sucking bug *Rhodnius prolixus* pioneered by the insect physiologist Sir Vincent Wigglesworth (Wigglesworth, 1947, 1951, 1957), researchers have been searching for factors regulating mosquito reproduction. The role of endocrine signalling in

the link between ovarian development and blood feeding in the mosquito *Anopheles maculipennis* was first recognized by Detinova (1945). Studies of the yellow fever mosquito *Ae. aegypti* revealed that a factor from the mosquito head was required for normal egg development (Clements, 1956; Gillet, 1956, 1957; Larsen and Bodenstein, 1959). In a series of elegant microsurgical experiments, Lea (1967) showed that the source of this factor is brain medial neurosecretory cells (NSCs); removal of these cells led to arrested egg maturation, which was restored by reimplantation of the cells. Lea (1972) also demonstrated that an endocrine factor from the cells is stored in the corpus cardiacum and is released only after a mosquito takes a blood meal.

The source of JH is the corpora allata (CA), a paired gland that resides in the mosquito prothorax. Removal of this gland (allatectomy) from the *Ae. aegypti* female at eclosion arrested egg development (Lea, 1969). However, if allatectomy was performed at 72 h PE, it did not prevent egg maturation, indicating the importance of the CA for the PE phase. Gwadz and Spielman (1973) demonstrated that the CA is responsible for the PE growth of primary ovarian follicles; reimplantation of the CA restored follicular development previously arrested by allatectomy at eclosion. Hagedorn et al. (1977) further established that ovarian growth is controlled by JH. This hormone has also been implicated in the development of ovarian responsiveness to a brain factor (Shapiro and Hagedorn, 1982).

Early studies also established the role of ecdysteroids in mosquito reproduction. Injection of ecdysteroids into female *Ae. aegypti* stimulated egg development, suggesting involvement of this hormone in female reproduction (Spielman et al., 1971). In a breakthrough study, Hagedorn and coworkers found that the ovaries of female *Ae. aegypti* secrete ecdysone (E) after a blood meal (Hagedorn et al., 1975). E secretion reached its maximal level at 16 h PBM and rapidly declined thereafter. Hagedorn et al. (1979) further showed that a brain extract stimulates E production by follicle cells of the ovary. The significance of this discovery has been highlighted by numerous follow-up studies demonstrating that female reproduction in all Diptera and some Lepidoptera species is controlled by ovary-derived ecdysteroids (reviewed in Brown et al., 2009).

Vitellogenesis is the central event of female reproduction. It involves production and secretion of yolk protein precursors (YPPs) such as vitellogenin (Vg) by the fat body and internalization of YPPs by developing oocytes through receptor-mediated endocytosis (reviewed in Raikhel, 2005). Hagedorn and Fallon (1973) established that Vg synthesis occurs in

the *Ae. aegypti* fat body and further observed that it is controlled by 20E at the transcriptional level (Fallon et al., 1974; Hagedorn et al., 1973, 1975). These early studies, collectively, laid a foundation for our understanding of the network of endocrine factors controlling sequential periods of the female mosquito reproductive cycle.

3. REGULATION OF ECDYSTEROIDOGENESIS IN THE OVARY

3.1 E Biosynthesis

The prothoracic glands (PGs) synthesize and release ecdysteroids during immature stages of insects; in female dipterans, however, the ovary is the major source of these hormones. Ecdysteroid biosynthesis is conserved in both of these tissues (reviewed in Brown et al., 2009; Rewitz et al., 2007). A unique feature of insect metabolism is the inability to synthesize cholesterol de novo; therefore, it must be acquired from food (reviewed in Brown et al., 2009). In adult mosquitoes, cholesterol is obtained directly from blood. Cholesterol delivery from the gut to the site of E biosynthesis by a lipid carrier protein, lipophorin, has been observed in lepidopteran insects (Gilbert et al., 2002; Jouni et al., 2002, 2003). Although the transport of cholesterol to the ovary has not been demonstrated in mosquitoes, the ovarian lipophorin receptor has already been characterized (Cheon et al., 2001). In the ecdysteroidogenic tissues, the first step is the conversion of cholesterol to 7-dehydrocholesterol, catalysed by 7,8-dehydrogenase (Fig. 2). This is followed by multiple biosynthetic steps mediated by P450 enzymes encoded by "Halloween genes" that are localized in the endoplasmic reticulum (ER) and mitochondria (reviewed in Gilbert, 2004; Niwa and Niwa, 2014). The product of ecdysteroid biosynthesis is a prohormone, E, which is secreted into the haemolymph and internalized by peripheral tissues. There, the final step in conversion of E to an active hormone, 20E, is mediated by the cytochrome P450 enzyme, CYP314A1 (Petryk et al., 2003). Several ecdysteroidogenic enzymes have been identified in ovaries of *Ae. aegypti* and *An. gambiae* females (Pondeville et al., 2013; Sieglaff et al., 2005). Their transcriptional upregulation positively correlates with the blood meal and the peak of ecdysteroids during the reproductive cycle, suggesting a link between the blood meal regulatory cascade and E biosynthesis in the ovaries (Sieglaff et al., 2005).

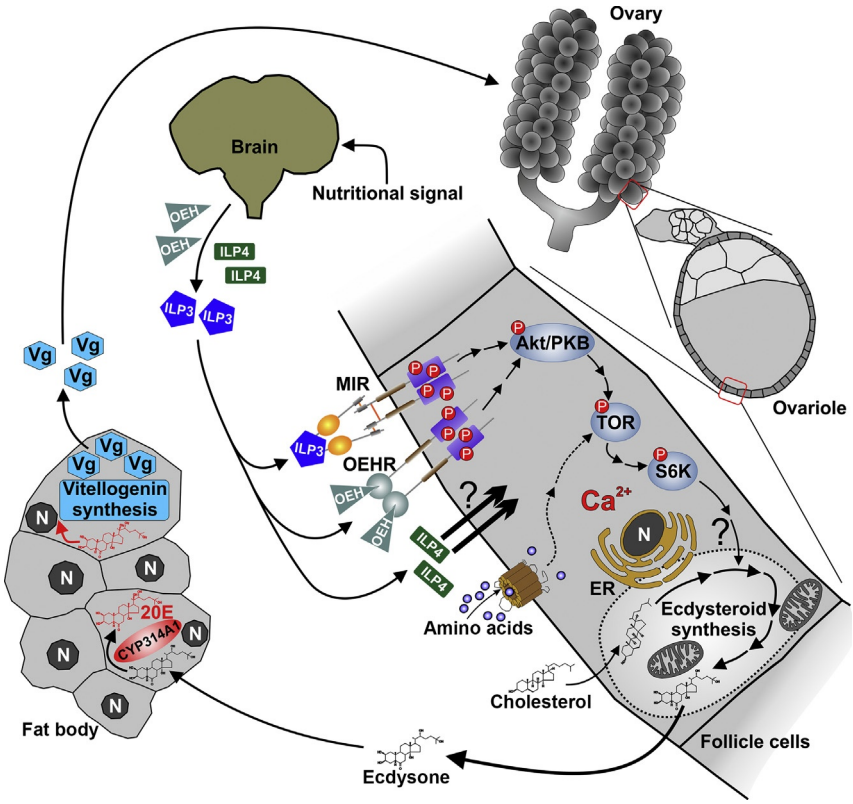

Fig. 2 Ecdysteroidogenesis in the mosquito ovary. Nutritional signals from the blood meal triggers release of neuropeptides, ovary ecdysteroidogenic hormone (OEH) and insulin-like peptides 3 and 4 (ILP3 and ILP4, respectively) from the brain of an *Aedes aegypti* female. These neuropeptides bind their corresponding receptors in the membrane of ovarian follicle cells: OEH binds ovary ecdysteroidogenic hormone receptor (OEHR), ILP3 binds mosquito insulin receptor (MIR) and ILP4 binds to an unknown receptor. After binding, OEHR and MIR are phosphorylated and along with amino acid signalling activates insulin and AA/TOR signalling pathways, manifested by phosphorylation of Akt/PKB, TOR and S6 kinases. Activation of these pathways drives the multistep synthesis of ecdysteroids from cholesterol in the endoplasmic reticulum (ER) and mitochondria of the follicle cells. Ca^{2+} flux seems to be an important factor for the ecdysteroidogenesis although its precise role remains unclear. Ecdysone secreted into the haemolymph is converted by the cytochrome P450 enzyme CYP314A1 to the active form of 20-hydroxyecdysone (20E) in the peripheral tissues. 20E triggers *vitellogenin* (Vg) expression in the fat body and, once released into the haemolymph, Vg is taken by the oocytes and packed in yolk granules. Abbreviations: *20E*, 20-hydroxyecdysone; *Akt/PKB*, Akt/protein kinase B; *ILP3*, insulin-like peptide 3; *ILP4*, insulin-like peptide 4; *MIR*, mosquito insulin receptor; *N*, Nucleus; *P*, phosphorylation; *OEH*, ovary ecdysteroidogenic hormone; *S6K*, p70 ribosomal S6 kinase; *TOR*, target of rapamycin; *Vg*, Vitellogenin.

3.2 Endocrine Control of E Biosynthesis in the Ovary

The neuropeptide prothoracicotropic hormone (PTTH) is a major regulator of E biosynthesis in the PG (reviewed in Rewitz et al., 2013). PTTH is expressed in the lateral NSCs of the brain and stored and released from a pair of neurohaemal organs—corpora cardiaca in most insects or ring gland in higher Diptera—in response to developmental stimuli (Rybczynski, 2005). Once released, it binds Torso, a receptor tyrosine kinase (RTK) in the membrane of PG cells, and signals via the mitogen-activated protein kinase pathway to induce ecdysteroid biosynthesis (Rewitz et al., 2009). Neither recombinant *Ae. aegypti* nor *Bombyx mori* PTTH activated ecdysteroid production in *Ae. aegypti* ovaries in vitro, indicating that PTTH is not a regulator of ovarian ecdysteroidogenesis in mosquitoes (McKinney et al., 2016).

3.2.1 Ovary Ecdysteroidogenic Hormone

As discussed earlier, Hagedorn et al. (1979) described gonadotropic activity in brain extracts of blood-fed mosquitoes that induces ecdysteroid synthesis in *Ae. aegypti* ovaries. Brown et al. (1998) isolated a neuroparsin-like factor, named ovary ecdysteroidogenic hormone (OEH), from mosquito head extracts and identified it as an ecdysteroidogenic gonadotropin with highly specific activity in bioassays with *Ae. aegypti* ovaries (see chapter "Mosquito Peptide Hormones: Diversity, Production, and Function" by Strand et al. for additional details). Blood meal-originated nutrients, particularly amino acids (AAs), trigger the release of OEH from the medial NSCs in the brain of *Ae. aegypti* females (Brown et al., 2008; Wen et al., 2010). Recombinant *Ae. aegypti* OEH and 20E were shown to restore egg maturation in decapitated, facultatively autogenous *Georgecraigius atropalpus* female mosquitoes without a blood meal (Gulia-Nuss et al., 2012).

OEH signals via the recently characterized RTK AAEL001915 (Fig. 2) (Dhara et al., 2013; Gulia-Nuss et al., 2012; Vogel et al., 2015). This receptor is referred to as the OEH receptor (OEHR) and is conserved in mosquitoes. Its expression peaks at 12 h PBM, preceding the ecdysteroid peak in *Ae. aegypti*. Vogel et al. (2015) demonstrated that OEHR depletion via RNA interference (RNAi) attenuated both E production in the ovary and subsequent Vg synthesis in the fat body.

3.2.2 Insulin-Like Peptides

Riehle and Brown (1999) showed that inhibition of the insulin pathway with specific inhibitors sequesters ecdysteroidogenesis in the *Ae. aegypti*

ovary. Later, it was demonstrated that two *Ae. aegypti* insulin-like peptides (ILPs), ILP3 and ILP4, exhibit gonadotrophic activity and stimulate ovaries to produce ecdysteroids (Fig. 2) (Brown et al., 2008; Wen et al., 2010). Like OEH, ILPs are produced by the medial NSCs in the brains of *Ae. aegypti* females (Brown et al., 2008; Wen et al., 2010). Gulia-Nuss et al. (2012) demonstrated that, in the same species, ILP3 stimulates serine protease activity in the midgut of blood-fed females, and thus contributes to the availability of AAs for Vg synthesis.

The mosquito insulin receptor (MIR) is present in both nurse and follicle cells in *Ae. aegypti* ovaries (Graf et al., 1997; Helbling and Graf, 1998; Riehle and Brown, 2002). MIR RNAi knockdown prevents ILP3, but not ILP4, from inducing ecdysteroid synthesis by the ovaries of *Ae. aegypti* (Brown et al., 2008; Wen et al., 2010). This suggests that, unlike ILP3, ILP4 signalling is independent of the MIR. Moreover, ILP3 binds specifically to MIR, while ILP4 binds to a 55-kDa membrane protein (Fig. 2) (Wen et al., 2010). However, functional insulin signalling is required for the gonadotropic activity of both ILP3 and ILP4.

It is also known that OEH and ILP3 signal through their respective receptors and, together with AAs, converge on activation of the insulin signalling and amino acid–Target of Rapamycin (AA/TOR) pathways to stimulate synthesis of ecdysteroids in *Ae. aegypti* ovaries (Dhara et al., 2013). This convergence is supported by independent activation of Akt/protein kinase B by ILP3 and OEH in the ovary. Furthermore, ILP3 and OEH with AAs costimulate phosphorylation of p70 S6 kinase (S6K) in the AA/TOR pathway (Fig. 2) (Dhara et al., 2013; Vogel et al., 2015). Recently, Yamanaka et al. (2015) characterized complex regulation of E release from the PG by calcium-stimulated, vesicle-mediated exocytosis in the ring gland of *Drosophila melanogaster*. Ca^{2+} signalling seems to play some role in the *Ae. aegypti* ovary as well, since it has been demonstrated that Ca^{2+} flux is likely contributing to, but not essential for, OEH and ILP3 activity (Fig. 2) (McKinney et al., 2016).

4. MOLECULAR BIOLOGY OF 20E ACTION IN FEMALE MOSQUITO REPRODUCTION

4.1 Studies of the 20E Regulatory Hierarchy

4.1.1 The Ecdysone Receptor

Since the early discoveries described earlier, significant progress has been achieved in understanding the action of 20E. We owe most of the present

knowledge to genetic studies in *D. melanogaster* (reviewed in Yamanaka et al., 2013). However, despite the wealth of information provided by *Drosophila* studies, solving the 20E regulation of reproductive events activated by a blood meal in female mosquitoes represents a unique problem.

Ashburner et al. (1974), observing formation of 20E-responsive chromosomal puffing in salivary glands of *Drosophila* larvae, had predicted the existence of the 20E regulatory hierarchy, in which this hormone acts through its receptor causing transcription of so-called early responsive genes. The protein products of these genes in turn serve as transcription factors that activate a larger number of effector genes (Ashburner, 1990; Ashburner et al., 1974). The molecular events underlying the Ashburner model have been subsequently worked out using rapidly developing genetic and molecular methods.

As Ashburner had predicted, 20E was found to act through its cognate receptor (Koelle et al., 1991). However, the 20E functional receptor was identified as a heterodimer, composed of two nuclear receptors—ecdysone receptor (EcR) and Ultraspiracle (USP) (Thomas et al., 1993; Yao et al., 1992, 1993). EcR and USP are orthologs of the vertebrate farnesoid X receptor and the retinoid X receptor, respectively (reviewed in King-Jones and Thummel, 2005). Both EcR and USP possess a conserved structure characteristic of members of the nuclear receptor superfamily, consisting of the DNA-binding (DBD) and ligand-binding (LBD) domains, as well as amino-terminal and carboxyl-terminal transactivation domains (Fig. 3A). EcR binds 20E as its ligand with high affinity; in contrast, unliganded USP interacts with EcR (reviewed in Hill et al., 2013). Crystallographic studies have shown that EcR LBD consists of 12 alpha helices and a beta-sheet forming a ligand-binding pocket fitting 20E. In contrast, USP LBD has a closed configuration with a pocket filled with lipid (Billas et al., 2003; Hill et al., 2013). Upon binding of 20E, the EcR–USP heterodimer interacts with the ecdysone response element (EcRE) consisting of two hexamers (AGGTCA) forming an inverted or direct palindrome separated by a variable number of nucleotides (Fig. 3B) (reviewed in King-Jones and Thummel, 2005).

The two isoforms of EcR, EcR-A and EcR-B, identified in the *Ae. aegypti* mosquito, are differentially expressed during the PBM phase of the female reproductive cycle (Wang et al., 2002). In the fat body, the highest EcR-A transcript abundance corresponds to the 20E peak at 18 h PBM, while the EcR-B transcript is maximally present at the termination stage, 36–42 h PBM (Mane-Padros et al., 2012). Unlike *D. melanogaster*, in which

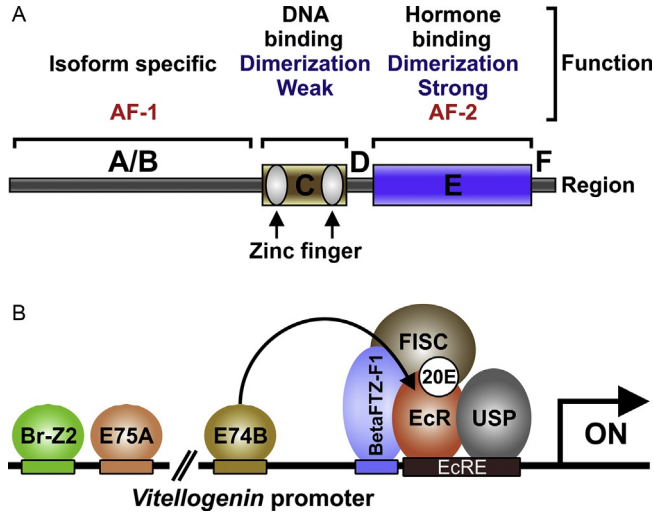

Fig. 3 Molecular mechanism of 20E action. (A) Schematic representation of nuclear receptor protein structure. Nuclear receptors share six conserved domains, A–F. The variable, N-terminal isoform-specific (A/B) domain containing the activation function 1 (AF-1) is responsible for relatively weak transactivation. The conserved DNA-binding domain (DBD) (C) with two zinc fingers specifically binds DNA sequences, called hormone response elements, in the gene promoter region and partially participates in receptor dimerization. The hinge-region (D) is variable in sequence and connects the DBD and the following ligand-binding domain (LBD). It also contributes to intracellular trafficking and subcellular distribution of the receptors. The moderately conserved LBD (E) covers a wide range of functions from ligand-binding and ligand-dependent transactivation through its activation function 2 (AF-2), receptor dimerization, to coactivator and corepressor binding. The last, C-terminal domain (F) is variable in sequence and flexible in structure. (B) Transactivation complex on the promoter of the *vitellogenin* (*Vg*) gene. The nuclear receptor, ecdysone receptor (EcR), binds 20E with high affinity, heterodimerizes with another nuclear receptor, ultraspiracle (USP) and the heterodimer binds ecdysone response elements (EcREs) in the *Vg* gene promoter in the fat body of the *Ae. aegypti* female. Nuclear receptor BetaFTZ-F1 binds the histone acetyltransferase p160/SRC (FISC) in a ligand-independent manner and the complex is recruited to the EcR/USP heterodimer in the presence of 20E. BetaFTZ-F1/FISC/EcR/USP complex upregulates *Vg* transcription which is further enhanced by the binding of Br-Z2, E75A and E74B transcription factors to the *Vg* promoter.

there is only a single USP, in *Ae. aegypti*, two USP isoforms, USP-A and USP-B, were found (Kapitskaya et al., 1996). Multiple USP isoforms were later identified in other insects (Jindra et al., 1997; Lan et al., 1999). Similar to EcR isoforms, *Ae. aegypti* USPs are expressed differentially, with USP-B being constitutively present during the PBM phase and USP-A exhibiting a sharp expression peak at the termination stage (Mane-Padros et al., 2012).

20E has opposite effects on USP isoform transcripts; it is required for sustaining a high level of USP-B expression but downregulates expression of USP-A (Wang et al., 2000). USP-B, which has been implicated as the primary heterodimerization partner for EcR during vitellogenesis, binds the EcREs with a much higher affinity than USP-A (Wang et al., 2000). However, the precise functions of EcR and USP isoforms in *Ae. aegypti* gene regulation remain to be determined.

4.1.2 The Early Genes of the 20E Genetic Hierarchy

Molecular and genetic studies in *D. melanogaster* confirmed the Ashburner model by identifying genes corresponding to 20E-responsive early chromatin puffs (King-Jones and Thummel, 2005; Yamanaka et al., 2013). The *E74* gene is located within the E74EF puff and consists of two transcription units, E74A and E74B (Fletcher et al., 1995). As a result, there are two isoforms of the early factor E74 that belong to the ETS family of transcription factors. E74 is involved in control of stage- and tissue-specific expression of 20E-regulated genes in *Drosophila* (Fletcher and Thummel, 1995). In *Ae. aegypti*, two E74 isoforms play different roles in vitellogenesis. E74B is induced after a blood meal and expressed during the peak of vitellogenesis, while E74A is expressed during the termination stage (Sun et al., 2002). There are several E74 binding sites on the *Vg* gene promoter (Sun et al., 2005). E74B activates expression of the *Vg* gene, whereas E74A has no effect (Sun et al., 2004). E74B serves as an enhancer, synergistically acting with EcR/USP (Fig. 3B) (Sun et al., 2005). A direct protein–protein interaction between EcR and E74B on the *Vg* promoter was demonstrated by means of two-hybrid assays and coimmunoprecipitation analysis. A dominant negative E74 mutant abolished Vg activation in a cell transactivation assay (Sun et al., 2005).

The nuclear receptor E75, an insect ortholog of the vertebrate Rev-erb, is another classic component of the 20E genetic hierarchy (Segraves and Hogness, 1990). In *Drosophila*, E75 is represented by several isoforms that play different roles in coordinating the 20E response (reviewed in Thummel, 1997). In *Ae. aegypti*, there are three E75 isoforms—E75A, E75B and E75C—that are activated by a blood-meal-regulated hormonal cascade (Pierceall et al., 1999). RNAi-mediated silencing of E75 isoforms has revealed their distinct roles in regulating the level and timing of expression of key genes involved in vitellogenesis (Cruz et al., 2012). RNAi depletion of E75A results in repression of *Vg* expression, whereas depletion of E75C results in sustained elevation of Vg levels (Cruz et al., 2012). Haem

serves as a ligand for E75, reversibly binding to its LBD (Reinking et al., 2005). During the process of blood digestion, mosquitoes accumulate haem. A combinatorial action of 20E and haem results in a very high level of *Vg* expression, providing an additional link between blood feeding and activation of vitellogenesis (Cruz et al., 2012). Thus, haem derived from a blood meal serves as a signalling molecule mediated by E75 and assures adequate nutrient availability.

Broad (Br) encodes a family of C2H2-type, zinc-finger DNA-binding transcription factors that are critical members of the 20E regulatory hierarchy and is required for expression of other early genes, E74 and E75 (Karim et al., 1993). Br plays a critical role in insect metamorphosis and serves as a pupal stage specifier (Bayer et al., 2003; Zhou and Riddiford, 2002). However, studies in adult mosquitoes have revealed that Br is essential for female reproduction (Chen et al., 2004; Zhu et al., 2007). Four Br isoforms have been identified in *Ae. aegypti*—Z1, Z2, Z3 and Z4—all of which play different roles in vitellogenesis. Expressions of Z1, Z2 and Z4 are stimulated after blood feeding and by 20E in vitro. The regulatory region of the *Vg* gene contains multiple binding sites for Br isoforms. Cell transfection and RNAi analyses showed that Z2 acts as an activator by directly binding to the *Vg* promoter, whereas Z1 and Z4 function as repressors (Chen et al., 2004; Zhu et al., 2007).

In *Ae. aegypti*, transcription of the *Vg* gene is controlled via blood-meal-triggered pathways, with 20E signalling being the central regulator. The *Vg* 5′-regulatory region contains several EcREs, providing evidence of direct control of this gene by EcR/USP (Kokoza et al., 2001; Martin et al., 2001b; Zhu et al., 2006). The authenticity of *Ae. aegypti* Vg EcREs has been confirmed using electrophoretic mobility shift and cell transfection assays, as well as by transgenesis (Kokoza et al., 2001; Martin et al., 2001a; Zhu et al., 2006). The presence of additional binding sites for early gene products of the 20E hierarchy in the *Vg* gene promoter indicates combinatorial control of its expression (Chen et al., 2004; Cruz et al., 2012; Sun et al., 2005).

4.2 Regulation of Developmental Transitions

Timing of 20E signalling during metamorphosis in *Drosophila* is controlled by a set of sequentially expressed nuclear receptors, eg, HR3 and betaFTZ-F1 (Broadus et al., 1999; Lam et al., 1999; White et al., 1997). HR3 is an orphan receptor that binds target genes as a monomer and serves as a developmental switch required for the prepupal–pupal transition (Lam et al.,

1999). HR3 activates the betaFTZ-F1 nuclear receptor that determines competence for stage-specific responses to a subsequent 20E pulse (Broadus et al., 1999). Surprisingly, this genetic signalling is reiteratively utilized during the reproductive cycle in female *Ae. aegypti*, demonstrating conservation of regulatory pathways (Kapitskaya et al., 2000; Li et al., 2000). HR3 is expressed in the late pupa and activates betaFTZ-F1 after the decrease of 20E titre, whereas betaFTZ-F1 continues to be expressed during the JH-controlled PE phase (Li et al., 2000).

Studies of *Ae. aegypti* have established the molecular basis of competency for 20E responsiveness (Fig. 3B). RNAi silencing of betaFTZ-F1 attenuated expression of early genes and the target *Vg* gene after either a blood meal or 20E treatment, providing proof that this nuclear receptor is the factor determining competence for the 20E response in females (Zhu et al., 2003). JH is critical for acquisition of responsiveness by reproductive organs to 20E during the PE phase because it has been implicated in the translational control of betaFTZ-F1 (Zhu et al., 2003). Translated betaFTZ-F1 protein binds the histone acetyltransferase p160/SRC (FISC) in a ligand-independent manner. In the presence of 20E, the betaFTZ-F1/FISC complex is recruited to the EcR/USP heterodimer, initiating loading of ECR/USP/FISC onto target gene promoters and leading to local histone acetylation and robust activation of 20E-responsive genes (Zhu et al., 2006).

A timely cessation of *YPP* gene expression is essential for transition to another gonadotrophic cycle. HR3 plays a central role in orchestrating developmental transitions during the termination stage (Mane-Padros et al., 2012) and is expressed again during the declining 20E titre at 24–36 h PBM, downregulating *Vg* expression. This negative effect of HR3 is a direct result of its binding to its cognate recognition site in the promoter of the *Vg* gene. HR3 is also essential for turning off TOR signalling and initiating programmed autophagy in the fat body (Bryant and Raikhel, 2011; Mane-Padros et al., 2012; Smykal and Raikhel, 2015). Similar to the pupal–adult transition, HR3 activates betaFTZ-F1, which in turn is critical for progression to the second gonadotrophic cycle (Mane-Padros et al., 2012).

5. AA/TOR AND INSULIN SIGNALLING IN MOSQUITO VITELLOGENESIS

Although ecdysteroids are major regulators of mosquito vitellogenesis (Hagedorn et al., 1975), other pathways contribute significantly to the precise regulation of female mosquito reproduction. The AA/TOR and insulin

pathways control many aspects of insect life, including nutrient-dependent growth during development, vitellogenesis and oocyte growth (reviewed in Badisco et al., 2013; Hansen et al., 2014; Okamoto and Yamanaka, 2015; Smykal and Raikhel, 2015). They serve as check points, ensuring that sufficient nutrients are available for the vitellogenic phase. The relative contribution of AA/TOR and insulin pathways in the nutritional regulation of vitellogenesis is not completely clear, although simultaneous inhibition of both pathways has been shown to block egg production, suggesting their synergistic action (Gulia-Nuss et al., 2011).

5.1 The AA/TOR Signalling

A blood meal obtained by a female mosquito serves as a major source of AAs and other nutrients for the upcoming reproductive cycle. Hansen et al. (2004) identified a critical role of the AA/TOR pathway in PBM regulation of vitellogenesis in the female *Ae. aegypti* fat body (Fig. 4). AAs are transported by specific transporters that belong to a group of transceptors, which are involved in both AA binding and transport (Boudko et al., 2015; Kriel et al., 2011). Multiple studies have shown that the RNAi silencing of some of the AA transporters/transceptors lowers TOR signalling and mosquito fertility in *Ae. aegypti* females (Attardo et al., 2006; Boudko et al., 2015; Carpenter et al., 2012; Hansen et al., 2011).

AAs signal by means of a nutrient-sensitive TORC1 (hereafter TOR) branch of the TOR signalling pathway (Kim and Guan, 2011; Loewith and Hall, 2011; Wullschleger et al., 2006). Most signals upstream of TOR converge on tuberous sclerosis complex 1 and 2 (TSC1/2), a heterodimeric negative regulator of Rheb (Ras homolog enriched in brain) GTPase (Inoki et al., 2003; Tee et al., 2003). Hansen et al. (2004) showed that, in contrast to TOR knockdown, RNAi of TSC2 upregulated Vg expression in the female *Ae. aegypti* fat body. Roy and Raikhel (2011) reported that Rheb, a cell membrane protein localized at the lysosome, activates TOR protein kinase activity in response to AAs in *Ae. aegypti* and controls egg development. Rag A/B and Rag C/D, Ras-related small GTP-binding protein GTPases, also participate in transmitting the AA signal to TOR by localizing TOR protein to the lysosome and enabling its activation by Rheb (Groenewoud and Zwartkruis, 2013; Yang et al., 2013). Other critical components of the TOR signalling pathway have been connected to egg development in mosquito females. Once activated by a nutritional signal, TOR kinase phosphorylates p70 S6 kinase (S6K) and

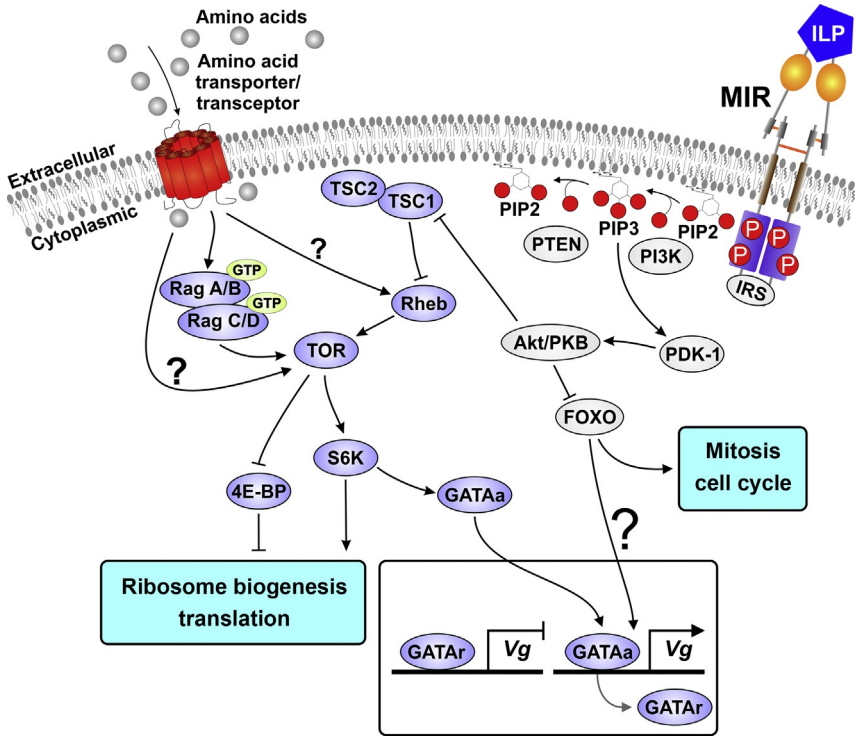

Fig. 4 Insulin and AA/TOR signalling pathways. Amino acids released during digestion of a blood meal are sensed and transported into the fat body cells of *Ae. aegypti* where they participate in activation of the target of rapamycin (TOR) kinase via Ras homolog enriched in brain (Rheb). Activated TOR kinase phosphorylates downstream proteins and thereby derepresses ribosome biogenesis and protein translation by inactivation of the translational inhibitor eukaryotic translation initiation factor 4E-binding protein (4E-BP) and by activation of p70 S6 kinase (S6K). Activated S6K elicits massive translation of GATAa transcription factor that replaces repressive GATAr in the *Vg* gene promoter and participates in *Vg* transcription. Insulin-like peptides (ILPs) bound to the mosquito insulin receptor (MIR) localized in the membrane of follicle cells results in its phosphorylation and activation. Activated MIR transduces its signalling through a series of phosphorylation events to activate phosphatidylinositol 3-kinase (PI3K). PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3), which activates Akt/protein kinase B (Akt/PKB). Active Akt/PKB can stimulate TOR signalling pathway through repression of the TOR-negative regulators, tuberous sclerosis complex 1 and 2 (TSC1/2). Forkhead box O (FOXO) transcription factor regulates cell cycle progression and mitosis, and Akt/PKB phosphorylation keeps FOXO from its translocation to the nucleus and likely repression of *Vg* transcription. However, the role of FOXO in Vg expression needs verification. Phosphatase and tensin homolog (PTEN) negatively regulates insulin signalling by dephosphorylation of the PIP3 to PIP2. Other abbreviations: *GTP*, guanosine-5′-triphosphate; *IRS*, insulin receptor substrate; *P*, phosphate/phosphorylation; *PDK*-1, 3-phosphoinositide-dependent protein kinase-1; *Rag A/B* and *Rag C/D*, Ras-related small GTP-binding protein GTPases; *Vg*, Vitellogenin.

eukaryotic translation initiation factor 4E binding protein (4E-BP), a repressor of cap-dependent mRNA translation (Hansen et al., 2005; Roy et al., 2007). Weng and Shiao (2015) suggested possible cross-talk between AA/TOR and Wnt signalling pathways, when silencing of Wnt membrane receptor Frizzled 2 decreased S6K phosphorylation and Vg expression.

Following TOR activation, phosphorylation of 4E-PB derepresses systemic translation inhibition and, together with translational activator S6K, permits both translation of GATA activator (GATAa) transcription factor in the *Ae. aegypti* fat body and *Vg* expression (Attardo et al., 2003; Park et al., 2006). GATAa replaces the GATA repressor in the *Vg* promoter, thus permitting expression (Attardo et al., 2003; Martin et al., 2001a; Park et al., 2006).

5.2 Insulin Signalling

In addition to the role in E biosynthesis described earlier, ILPs play a significant role in the nutritional regulation of vitellogenesis. ILPs are peptide hormones with three interchain disulfide bonds (Sajid et al., 2011) that are structurally and functionally analogous to vertebrate insulin (Luckhart and Riehle, 2007; Wu and Brown, 2006). In mosquitoes and other insects, the ILP and insulin-like growth factor signalling pathways play a major role in regulation of growth, development, lifespan and reproduction (Broughton et al., 2005; Giannakou and Partridge, 2007; Hyun, 2013; Okamoto and Yamanaka, 2015; Smykal and Raikhel, 2015; Taguchi and White, 2008; Wu and Brown, 2006).

Various numbers of ILPs have been identified in mosquito species: eight in *Ae. aegypti* (Riehle et al., 2006), seven in *An. gambiae* (Krieger et al., 2004), five in *Anopheles stephensi* (Marquez et al., 2011) and at least three in *Culex pipiens* (Sim and Denlinger, 2009). Brown et al. (2008) showed that injection of decapitated, blood-fed *Ae.* aegypti with ILP3-stimulated ovarian ecdysteroidogenesis and egg maturation. RNAi of ILP1 or insulin receptor expression in *C. pipiens* arrested ovarian development in the previtellogenic stage and mimicked a diapause-like state in nondiapausing females (Sim and Denlinger, 2008, 2011). Wen et al. (2010) demonstrated that although ILP3 and ILP4 likely signal via different receptors, they both stimulate ecdysteroidogenesis in the ovaries (see earlier). Interestingly, Kang et al. (2008) described activation of insulin signalling in the midgut by insulin and insulin-like growth factor ingested in a blood meal by female *An. stephensi*. ILPs activate phosphatidylinositol

3-kinase (PI3K), which phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). *Ae. aegypti* PI3K participates in dose-dependent ecdysteroid production by the ovaries and *Vg* expression in the fat body in vitro (Pri-Tal et al., 2008; Riehle and Brown, 1999; Roy et al., 2007). PIP3 activates the main effector Akt/protein kinase B and 3-phosphoinositide-dependent protein kinase-1 (PDK-1). Akt represses the negative regulators of TOR, TSC1/2 and permits TOR activation by phosphorylation (Loewith and Hall, 2011; Wullschleger et al., 2006). Akt expression has been detected in embryos, in fat bodies and in *Ae. aegypti* ovaries (Dhara et al., 2013; Riehle and Brown, 2003; Roy et al., 2007; Vogel et al., 2015). Bovine insulin can stimulate insulin signalling in the fat body of *Ae. aegypti* females, and insulin together with 20E triggers S6K phosphorylation and *Vg* expression in vitro, in the absence of AAs. RNAi of Akt or TOR expression blocks insulin- and 20E-mediated *Vg* expression (Hansen et al., 2004; Riehle and Brown, 1999; Roy et al., 2007).

In transgenic *Ae. aegypti* and *An. stephensi* females, fat body-specific overexpression of an active Akt increased *Vg* expression but not the number of eggs (Arik et al., 2015). Furthermore, fat body-targeted expression of active Akt in both mosquito species also activated the downstream signalling molecules Forkhead box O and S6 kinase, and increased mosquito survivorship PBM (Arik et al., 2015). Hansen et al. (2007) demonstrated that other members of the Forkhead box gene family seem to negatively affect *Vg* expression and egg production in *Ae. aegypti*, but the full understanding of their roles in mosquito reproduction needs further study. Phosphatase and tensin homolog (PTEN, a direct antagonist of PI3K) dephosphorylate PIP3 to PIP2 leading to inactivation of PI3K and shutdown of the insulin pathway. Arik et al. (2009) increased egg production in *Ae. aegypti* during the first reproductive cycle by knocking down PTEN splice variant 6 (PTEN6). Moretti et al. (2014) showed that knockdown or inhibition of another phosphatase, AAEL001919, an ortholog of protein-tyrosine phosphatase 1B (PTP1B), a negative regulator of the insulin pathway, decreased *Vg* expression and egg production in *Ae. aegypti*.

6. TEMPORAL COORDINATION OF GENE EXPRESSION IN MOSQUITO REPRODUCTION

Coordinated expression of regulatory and functional genes is essential for proper development and reproduction. Advances in genome sequencing, microarray and high-throughput RNA-seq techniques have profoundly

influenced our understanding of differential gene expression in various organisms. These approaches have been frequently used in recent years to study genome-wide changes in gene expression related to haematophagy and mosquito reproductive cycles, mostly in *An. gambiae* and *Ae. aegypti* (Akbari et al., 2013; Bonizzoni et al., 2011; Dana et al., 2005; Holt et al., 2002; Hou et al., 2015; Marinotti et al., 2006; Price et al., 2011; Ribeiro, 2003; Roy et al., 2015; Zhu et al., 2010; Zou et al., 2013).

The first genomic-scale study of haematophagy was performed by Holt et al. (2002). They identified differential abundance of 168 ESTs between sugar- and blood-fed (24 h PBM) *An. gambiae* females. An additional 267 genes, which were differentially expressed PBM, were found by Ribeiro (2003). Dana et al. (2005) identified 359 more gene products that were differentially expressed, by examining seven different time points within the first 48 h PBM. They also determined the temporal patterns of gene expression and suggested that gene transcription may be influenced by changes in JH and 20E titres. Marinotti et al. (2006) conducted a detailed microarray-based study of differential gene expression in *An. gambiae*. These authors demonstrated global changes in transcript accumulation during five different time points PBM, during a gonadotrophic cycle (3–96 h PBM). They reported that almost 7500 genes (50% of those probed) showed significant variation in transcript accumulation during the first 72 h after a blood meal. Marinotti et al. (2006) also reported differential expression of genes in ovaries and fat bodies by comparing gene expression at 24 h PBM to that in tissue from nonblood-fed females.

All of the earlier mentioned studies were done in *An. gambiae* and were related to differential expression of genes PBM. In another microarray-based study, Zhu et al. (2010) analysed the transcriptomic changes in newly emerged female *Ae. aegypti*, after topical application of exogenous JH, which is essential for reproductive maturation. They identified a group of JH target genes and paved the way for elucidating genetic interactions in the JH-controlled maturation phase.

Zou et al. (2013) later demonstrated that over 6000 genes were differentially regulated during 72 h of the JH-controlled PE phase in the fat body of *Ae. aegypti* females. These authors identified temporal patterns of gene expression in the PE fat body. They also confirmed that gene transcription in the PE phase is influenced by changes in JH titres, as suggested by Dana et al. (2005). The role of JH and its receptor Methoprene-tolerant in the up- and downregulation of gene expression at different time points within the fat body during this period was experimentally validated. The authors further

demonstrated that the functional identity of the gene clusters differs dramatically: genes belonging to metabolic processes, including carbohydrate and lipid metabolism, were upregulated during the first half of the PE phase, when the titre of JH is low; genes related to DNA replication, transcription and translation were highly expressed at a high titre of JH, late in the PE phase (Zou et al., 2013). They also reported that transcripts encoding ribosomal proteins represent a large portion of the fat body transcriptome in PE females, and these results concurred with those of Price et al. (2011).

Two studies seeking haematophagy-related differential expression of genes in *Ae. aegypti* examined changes in gene expression at 5 and 24 h PBM compared with that of nonblood-fed females. Bonizzoni et al. (2011) reported that ~6000 transcripts were differentially expressed between sugar- and blood-fed (5 h PBM) females on the basis of RNA-seq analysis. Price et al. (2011) summed up the results of 454-pyrosequencing by listing ~5000 genes that were differentially expressed in the fat body/abdomen tissue of female *Ae. aegypti*.

Organ- or tissue-specific transcriptome analyses have become more common in the last few years as they are particularly valuable for understanding differential gene expression. Akbari et al. (2013) provided a detailed investigation of ovary-specific genes transcribed over the gonadotrophic cycle in *Ae. aegypti*. While Hou et al. (2015) unravelled the expression dynamics of carbohydrate metabolism genes in the fat body using two separate time course microarray transcriptomes spanning the entire first gonadotrophic cycle, 0–6 h PE to 72 h PE, and 3–72 h PBM, respectively.

Roy et al. (2015) determined that about 7500 transcripts exhibited differential expression over nine time points during the 72-h PBM phase in the fat body of *Ae. aegypti* females. These numbers are similar to those from *An. gambiae*, reported by Marinotti et al. (2006). Roy et al. (2015) identified four major and sequential waves of gene expression over the 72 h PBM. Moreover, these results presented experimental evidence that AAs, 20E, HR3 and JH are the main regulators of differential regulation of the gene expression program during a blood-meal-activated gonadotrophic cycle. Each of these key regulators provides activating and repressive effects on one or more gene sets, thereby maintaining a strict temporal coordination of gene expression essential for sequential reproductive events (Roy et al., 2015). Taken together, these studies have contributed significantly to our understanding of temporal coordination of differential gene expression during mosquito gonadotrophic cycles at the genomic level.

7. CONCLUDING REMARKS

A clear understanding of the regulation of reproductive processes in female mosquitoes is of great importance as blood feeding and disease transmission are intrinsically linked to these processes. We have come a long way since the early studies of egg development, and now much more is known about the endocrine regulation of tissue-specific reproductive processes and their timing. To establish new and advanced control methods, unravelling of the molecular mechanisms behind these processes is of utmost importance. As a result, mosquito biologists have shifted their focus from studying 'what is happening' to 'how things are happening'. During the last decades, the perspective of studying mosquito reproduction from physiological and biochemical standpoints has moved towards molecular biology and genomics, yielding to new paradigms in the regulatory network controlling female mosquito reproduction. For example, studies employing molecular biology techniques coupled with bioinformatics tools have recently identified the roles of microRNAs involved in regulation of events related to blood feeding and reproduction (Bryant et al., 2010; Puthiyakunnon et al., 2013; Liu et al., 2014; Lucas et al., 2015a,b), thereby opening up an entirely new domain of research (see chapter "Functions of small RNAs in mosquitoes" by Hussain et al.).

The molecular biology of 20E action has been worked out in considerable detail. However, our current knowledge is based on studies of a limited number of genes. As a result, there are still important gaps in our understanding of the regulatory mechanisms of the mosquito reproductive cycle. Investigating the temporal regulation of gene expression in the vitellogenic fat body, Roy et al. (2015) postulated that in addition to activating some gene clusters, 20E differentially represses others. The intriguing question that remains to be investigated is the molecular mechanism of 20E repressive action. In this respect, it is important to elucidate which coregulators are involved in this pathway. Although previous studies have shown that AA/TOR and insulin pathways play important roles in controlling vitellogenesis, the relative contribution of each of these pathways remains unclear. Little is known about the complex networks of transcriptional factors and microRNAs responsible for differential regulation of gene expression during female reproduction. The role of posttranslational modifications and epigenetic events remains largely unexplored. Rapid development of novel molecular techniques and approaches and the exponential increase in genomics, metabolomics and bioinformatics studies should achieve a new level of understanding of the complex regulatory networks controlling female mosquito reproduction.

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