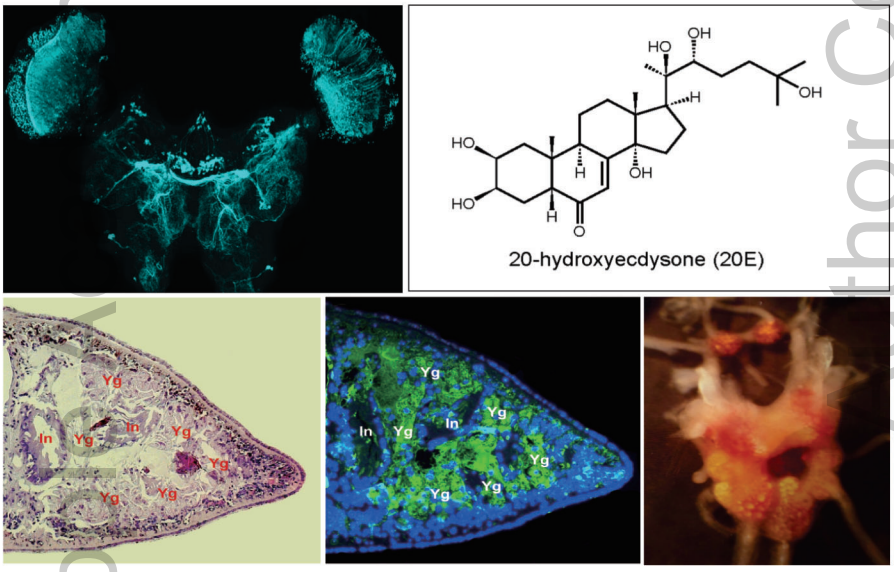
Advances in Invertebrate (Neuro)Endocrinology

A Collection of Reviews in the Post-Genomic Era

Volume 2 Arthropoda



Editors Saber Saleuddin | Angela Lange | Ian Orchard

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ADVANCES IN INVERTEBRATE (NEURO)ENDOCRINOLOGY

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VOLUME 2: Arthropoda

Edited by

Saber Saleuddin, PhD

Angela Lange, PhD

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Contents

CHAPTER 1

Juvenile Hormone Regulation and Action

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1.1 INTRODUCTION

Sesquiterpenoid hormone production in bilaterians shares a conserved mevalonate biosynthetic pathway, which originates from acetate and dates back to a common ancestor in the Ordovician (approximately 444–488 mya). The pathway diverged to generate various final products in different taxa, namely cholesterol in vertebrates, juvenile hormone (JH) in insects, and methyl farnesoate (MF) and farnesoic acid (FA) in crustaceans (Kenny et al., 2013; Cheong et al., 2015). The JHs are a family of insect acyclic sesquiterpenoids produced by the *corpora allata* (CA), a pair of endocrine glands connected to the brain (Tobe and Stay, 1985; Goodman and Cusson, 2012; Hiruma and Kaneko, 2013). The JHs are involved in the regulation of reproduction, metamorphosis, behavior, caste determination, diapause, stress response, and numerous polyphenisms (Nijhout, 1994; Riddiford, 1994; Wyatt and Davey, 1996; Hartfelder and Emlen, 2012; Jindra et al., 2013; Zhu and Noriega, 2016; Roy et al., 2018; Santos et al., 2019). It is likely that early in the evolution of insects, the original function of JH was to regulate adult female reproduction before it was co-opted as a hormone controlling metamorphosis (Tobe and Bendena, 1999).

The field of JH research thrives in the post-genomic era. In the last years, seminal progress has been made in our understanding of the regulation of JH titers and mode of action. We can highlight the identification of all the enzymes involved in JH synthesis, and the measurement of changes in transcripts, JH precursor metabolites, and enzymatic activities in the minute CA of insects (Nouzova et al., 2011; Rivera-Perez et al., 2014). The elusive intracellular JH receptor has been discovered (Charles et al., 2011; Jindra et al., 2013) and its mode of binding to JH and to JH-response DNA elements has been partially unveiled (Charles et al., 2011; Li et al., 2011, 2014; Kayukawa et al., 2012; Bittova et al., 2019). A JH signaling pathway that regulates metamorphosis has been supported with genetic evidence from diverse insect models (Konopova and Jindra, 2007, 2008; Minakuchi et al., 2009; Riddiford et al., 2010; Konopova et al., 2011; Ureña et al., 2014; Bellés and Santos, 2014; Jindra et al., 2015b; Daimon et al., 2015; Kayukawa et al., 2017). The repertoire of target genes downstream of the JH receptor has been explored (Zou et al., 2013; Saha et al., 2016). A new branch of plasma membrane-initiated JH signaling has been put forward (Liu et al., 2015). This chapter aims to provide an overview of general aspects on JH biosynthesis, transport, and degradation, as well as mechanisms of action and roles of JH in controlling development and reproduction in insects.

1.2 REGULATION OF JH TITERS

JH titers are regulated by the balance between biosynthesis and release of the hormone from the CA, as well as its degradation and clearance from the hemolymph by tissue uptake and excretion (Feyereisen, 1985; Goodman and Cusson, 2012). Numerous studies indicate that JH biosynthesis is a major regulator of JH titer; it is also widely accepted that JH is not stored in the CA and therefore the amount of JH released to the incubation medium or hemolymph represents the amount of JH synthesized (Feyereisen, 1985; Hernandez-Martinez et al., 2015).

1.2.1 JH SYNTHESIS

1.2.1.1 JH HOMOLOGUES

Eight different forms of JH have been identified, and at least one JH homolog has been detected in more than 100 insect species (Goodman and Cusson,

2012) (Figure 1.1). It is estimated that more than 2.5 million insect species inhabit the earth (Mora et al., 2011); therefore, it is conceivable that additional forms of JH could be discovered in the future. The first JH (JH I) was identified in the moth *Hyalophora cecropia* (Röller et al., 1967), and its structure was later established as a 2*E*, 6*E*, 10-*cis* isomer (Dahm et al., 1968), with a chiral center 10*R*, 11*S* (Meyer et al., 1971). Four additional JHs have been reported in Lepidoptera: JH 0, JH II, JH III and 4-methyl JH I (Meyer et al., 1968, 1971; Judy et al., 1973; Bergot et al., 1981) (Figure 1.1). JH III is the homolog found in the majority of insects (Goodman and Cusson, 2012; Rivera-Perez et al., 2014). The CA of *Drosophila melanogaster* and other brachyceran Diptera secrete a bis-epoxide JH III (JHB3) (Richard et al., 1989), as well as MF (Harshman et al., 2010; Wen et al., 2015) (Figure 1.1). The possible role of MF as a "JH" in insect preimaginal stages was a controversial issue that is just starting to be addressed (Wen et al., 2015; Jindra et al., 2015b). MF is abundant in the hemolymph of immature stages of several insects (Teal et al., 2014), including 4th instar larvae of *Aedes aegypti* mosquitoes (Hernandez-Martinez et al., 2015). MF is the immediate biosynthetic precursor of JH in mosquitoes, and therefore is very abundant in CA extracts (Rivera-Perez et al., 2014). On the other hand, MF is not released by the CA of adult *A. aegypti*, and it is undetectable in the hemolymph of adult females (Hernandez-Martinez et al., 2015). Another bis-epoxide form, JH III skipped bisepoxide (JHSB3) is present in some heteropterans such as *Plautia stali* (Kotaki et al., 2009, 2011) (Figure 1.1).

All these JH homologs share common structural features which might be necessary for a full biological activity; they contain a methyl ester (α, β-unsaturated) moiety group at the C1 position and an epoxide group at the C10-C11 position. JHB3 and JHSB3 have an additional epoxide group at C2-C3 and C6-C7, respectively. Hydroxylated JHs are generally considered products of the biological inactivation of JHs (Goodman and Cusson, 2012); though, in some insect species, they have been described as more active than the non-hydroxylated forms (Darrouzet et al., 1997).

JH has long been considered a target for the development of novel insecticides (Williams, 1967; Slama et al., 1974; Cusson et al., 2013). Different approaches have been used to identify natural products and to create synthetic compounds with anti-JH activities, such as inhibition of JH biosynthesis, increase in JH catabolism or interference of JH signaling. In addition, juvenoids, such as methoprene, are functional mimics of the endogenous JHs and true agonists of the intracellular JH receptor (Jindra et al., 2015b; Jindra and Bittova, 2019) that can prevent metamorphosis (Jindra et al., 2013) or interfere with normal reproduction (Staal, 1986; Cusson et al., 2013).

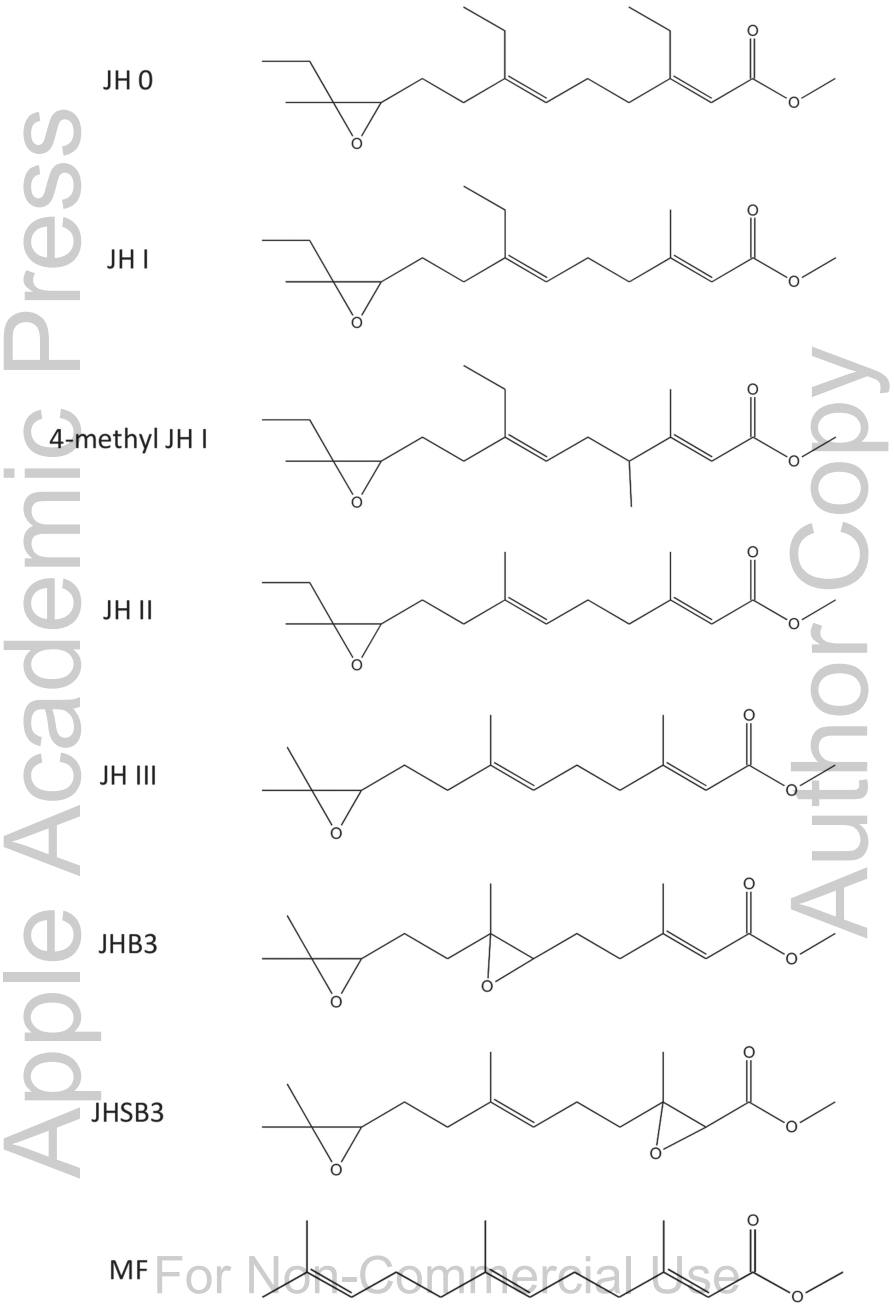

FIGURE 1.1 JH homologs.
Chemical structures of juvenile hormone homologs isolated from insects.

1.2.1.2 CORPORA ALLATA (CA) AND OTHER SYNTHETIC TISSUES

The principal endocrine organ responsible for JH synthesis is the CA, a pair of endocrine glands connected to the brain (Tobe and Stay, 1985; Hiruma and Kaneko, 2013). In larvae of higher Diptera the CA are in close association with another neuroendocrine organ, the *corpora cardiaca* (CC), and along with a third endocrine gland, the prothoracic gland (PG), are fused into the ring gland or "gland complex" (Burgess and Rempel, 1966). The size, shape, and composition of the gland complex changes as a fly or mosquito pupae transform into adults (Burgess and Rempel, 1966). The most important of these changes is the PG degeneration, a programmed cell death process (Martau and Rommer, 1998).

Innervation of the CA plays a key role in the regulation of CA activity (Tobe and Stay, 1985; Goodman and Cusson, 2012). In mosquitoes, stimulatory and inhibitory effects of brain factors have been described (Li et al., 2004; Hernandez-Martinez et al., 2007); separation of the CA from the brain (denervation) results in a remarkable activation of JH synthesis in early pupae (Areiza et al., 2015). In contrast, denervation prevents the 10-fold activation of JH synthesis that occurs 12 h after adult eclosion (Hernandez-Martinez et al., 2007). In sugar-fed and blood-fed females, denervation causes a significant increase in JH synthesis (Li et al., 2004). All these results indicate that stimulatory and inhibitory brain factors control CA activity.

It has been suggested that JH could be produced in organs other than the CA. Some insect males transfer JH, present in the accessory glands (AG), to females at mating (Shirk et al., 1980; Clifton et al., 2014), but there is no clear evidence if JH is synthesized de novo in the AG, or just sequestered there from the hemolymph. The AG of *H. cecropia* moths contains a JH acid (JHA) methyltransferase (JHAMT) that methylates JHA in the presence of S-adenosyl-L-methionine (SAM) (Peter et al., 1981). It has been recently suggested that the adult gut populations of intestinal stem cells and enteroblasts, are a new source of JHs in *D. melanogaster* (Rahman et al., 2017). This local and gut-specific JH activity is synthesized by and acts on the intestinal stem cell and enteroblast populations, regulating their survival and cellular growth through the JH receptor Gce and its partner Tai (see Section 1.3). Recently a lepidopteran betaentomopoxvirus has been reported to encode a JHAMT. The recombinant protein has a SAM-dependent methyltransferase activity (Takatsuka et al., 2017). The gene is expressed in virus-infected insect tissues, and the protein accumulates in the hemolymph. There, it transforms JHA into JH, thus inhibiting host metamorphosis. This

inhibition is advantageous for viral transmission in host insect populations via increased virus production and inhibition of pupation-associated behavior.

1.2.1.3 JH SYNTHESIS PATHWAY

The biosynthetic pathway of JH in the CA of insects includes 13 enzymatic reactions, and it is generally divided into early and late steps (Nouzova et al., 2011; Goodman and Cusson, 2012) (Figure 1.2). The early steps follow the mevalonate pathway (MVAP) to form farnesyl pyrophosphate (FPP) (Belléset al., 2005). First, three units of acetyl-CoA are condensed into mevalonate through three sequential steps involving the enzymes acetoacetyl-CoA thiolase (THIOL), HMG-CoA synthase (HMGS) and HMG-CoA reductase (HMGR) (Figure 1.2). Mevalonate is later converted to isopentenyl diphosphate (IPP) via three enzymatic reactions catalyzed by mevalonate kinase (MevK), phosphomevalonate kinase (P-MevK), and mevalonate diphosphate decarboxylase (PP-MevD), respectively (Nouzova et al., 2011). FPP synthase (FPPS), a short-chain prenyltransferase, generates FPP by completing two sequential couplings: first IPP and dimethylallyl pyrophosphate (DMAPP) condense in a head-to-tail manner to produce geranyl diphosphate (GPP). This type of head-to-tail condensation is repeated by the further reaction of GPP with IPP yielding FPP (Figure 1.2).

Insect FPP syntheses (FPPS) are typically active as homodimers (Bellés et al., 2005; Sen et al., 1996; 2006; 2007a; 2007b). In the mustard leaf beetle *Phaedon cochleariae* (Frick et al., 2013) and *A. aegypti* (Rivera-Perez et al., 2015), FPPSs possess an interesting product regulation mechanism; they change the chain length of their products depending on the cofactor present. The protein produces C_{10}-GPP in the presence of Co^{2+} or Mn^{2+}, while it yields the longer C_{15}-FPP in the presence of Mg^{2+}. That allows insects to supply precursors for different terpene pathways using a single enzyme. The production of DMAPP, the allylic isomer of IPP, is catalyzed by an IPP isomerase (IPPI). Insect IPPIs requires Mg^{2+} or Mn^{2+} for full catalytic activity (Diaz et al., 2012).

The enzymes of the MVAP are well conserved in eukaryotes. In insects, all the MVAP enzymes seem to be encoded by single-copy genes, and identification of predicted amino acid sequences was possible based on sequence homology (Noriega et al., 2006; Kinjoh et al., 2007; Nouzova et al., 2011). Nevertheless, biochemical characterization of purified or recombinant enzymes of the MVAP in insects is limited to HMGS (Sen et al., 2012),

HMGR (Martinez-Gonzalez, 1993; Buesa et al; 1994), MevK (Nyati et al., 2015) IPPI (Diaz et al., 2012) and FPPS (Bellés et al., 2005; Sen et al., 1996; 2006; 2007a; 2007b; Cusson et al., 2006; Rivera-Perez et al., 2015).

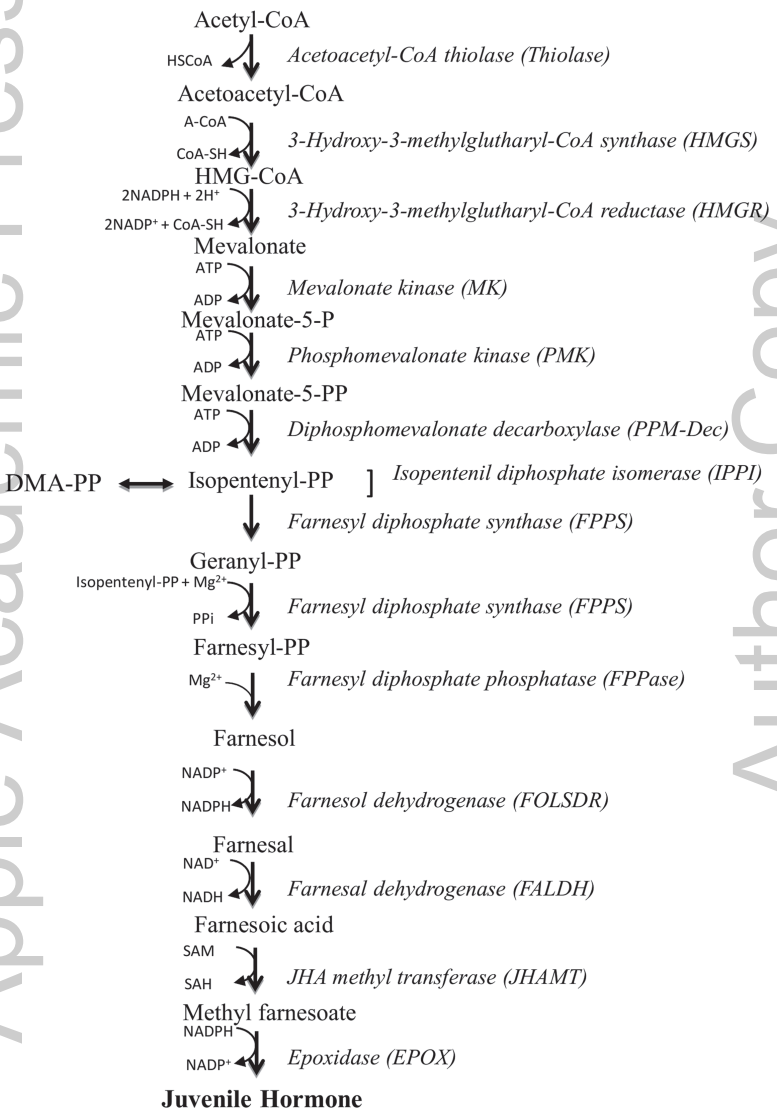

FIGURE 1.2 JH biosynthesis pathway.
Precursors are connected by arrows. Enzymes are shown in italics. Abbreviations for the enzymes are between the parenthesis. Cofactors are in smaller letters and connected to the pathway by arrows.

In the late steps comprising the JH-specific branch (Figure 1.2), conversion of FPP to farnesol (FOL) is catalyzed in *D. melanogaster* by a FPP phosphatase (FPPase or FPPP) (Cao et al., 2009), a member of the NagD haloalkanoic acid dehalogenase family (HAD), with orthologues in several insect species, including *A. aegypti* (Nyati et al., 2013). The mosquito FPPase (AaFPPase-1) is an Mg^{2+} dependent NagD HAD protein that efficiently hydrolyzes FPP and GPP, but not IPP (Nyati et al., 2013). Subsequently, FOL undergoes two sequential oxidation reactions that generate farnesol and FA (Figure 1.2). In mosquitoes, the first reaction is catalyzed by a short chain farnesol dehydrogenase (AaSDR-1), a member of the "classical" NADP-dependent cP2 SDR subfamily that presents broad substrate and tissue specificity (Mayoral et al., 2009b). Oxidation of FOL into FAL in mosquitoes is effected by an NAD^+-dependent aldehyde dehydrogenase class 3 (AaALDH3-1) showing tissue and developmental-stage-specific splice variants (Rivera-Perez et al., 2013). Homologs of farnesol and farnesol dehydrogenases having similar activities in the CA of other insects have not yet been described.

The order of the last two biosynthetic steps, methyl esterification and epoxidation (Figure 1.2), catalyzed by a JHAMT (Shinoda and Itoyama, 2003) and a P450 monooxygenase epoxidase (EPOX) (Helvig et al., 2004), differs among insects (Defelipe et al., 2011; Goodman and Cusson, 2012). In the Lepidoptera, epoxidation precedes esterification by JHAMT (Shinoda and Itoyama, 2003). In the Orthoptera, Dictyoptera, Coleoptera, and Diptera, epoxidation follows methylation (Defelipe et al., 2011). In all insect species studied, recombinant JHAMTs were able to methylate JHA and FA at similar rates (Shinoda and Itoyama, 2003; Minakuchi et al., 2008a; Niwa et al., 2008; Sheng et al., 2008; Mayoral et al., 2009a; Marchal et al., 2011). Homology modeling and docking simulations confirmed that JHAMT is a promiscuous enzyme capable to methylate FA and JHA (Defelipe et al., 2011). In contrast, epoxidases have narrow substrate specificity; while the EPOX from the cockroach *Diploptera punctata* efficiently epoxidizes MF and is unable to process FA (Helvig et al., 2004), *Bombyx mori* EPOX exhibits at least 18-fold higher activity for FA than for MF (Daimon et al., 2012). Therefore, the order of the methylation and epoxidation reactions may be primarily imposed by the epoxidase's substrate specificity (Defelipe et al., 2011). In the Lepidoptera, epoxidase has higher affinity than JHAMT for FA, so epoxidation precedes methylation, while in most other insects there is no epoxidation of FA, but esterification of FA to form MF, followed by epoxidation to JH III.

The late steps of JH biosynthesis were generally considered to be JH-specific (Goodman and Cusson, 2012), and the identification of these enzymes was hindered by the small size of the CA gland, making their isolation and biochemical characterization difficult. All the genes encoding these enzymes have now been identified in insects using molecular approaches that included EST sequencing (Helvig et al., 2004; Noriega et al., 2006), mRNA differential display (Shinoda and Itoyama, 2003) or homology to orthologue enzymes (Cao et al., 2009; Rivera-Perez et al., 2013). Identification of the three enzymes involved in the conversion of FPP to FA in mosquitoes has proven that the three proteins are encoded by families of paralogue genes with broad substrate specificity and expression in a wide number of tissues (Mayoral et al., 2009a, 2013; Nyati et al., 2013; Rivera-Perez et al., 2013). The presence of AaFPPase, AaSDR, and AaALDH3 isozymes with several isoforms capable of catalyzing each of the three enzymatic reactions in mosquitoes might have facilitated the evolution of more efficient substrate specificities, as well as a better tissue and developmental regulation. On the other hand, caution needs to be applied when trying to identify orthologues of these enzymes in other insect species, since not always the closest orthologue might play the same role in the CA.

On the contrary, the last two enzymes of the pathway (JHAMT and EPOX) are encoded by single genes in most insect species and are expressed predominantly in the CA (Shinoda and Itoyama, 2003; Nouzova et al., 2011). It is also noteworthy that EPOX genes appear to be insect-specific and have not been found in other arthropods. EPOX genes may be an evolutionary innovation that occurred in ancestral insects for the epoxidation of MF to JH (Daimon and Shinoda, 2013).

1.2.1.4 REGULATION OF JH SYNTHESIS

1. Long-Term and Short-Term Mechanisms of Allatoregulatory Activity

The CA activity is modulated by long-term (slow) and short-term (rapid) control mechanisms (Applebaum et al., 1991). Regulatory signals control the CA at least at three different levels (Unnithan et al., 1998): (1) Cytological/developmental responses are the gross morphological, microscopic or enzymatic changes that determine the overall physiological status of the glands and their maximal potential output. For example, changes in cell volume and cell number, which normally proceed in conjunction with

developmental changes, such as the transition to adult (Chiang et al., 1995). (2) Constitutive/long term responses, such as variations in enzyme levels during cycles of CA activity, are measured on a time scale of several hours to days. Examples of constitutive responses are the acquisition and loss of sensitivity to allatoregulatory peptides by the CA in *D. punctata* (Unnithan and Feyereisen, 1995) and *A. aegypti* (Li et al., 2003). (3) Dynamic/short term responses occur on a time scale of minutes or hours, and are measured easily *in vitro*, such as the inhibition of JH synthesis by allatostatins (AST) or the stimulation of JH synthesis by allatotropin (AT). These responses are normally reversible upon removal of the stimulus (Li et al., 2004).

2. Allatoregulatory Factors

A number of factors have been described that can stimulate (ATs) or inhibit (AST) CA activity (Weaver and Audsley, 2009). In different insect species and at different stages of development, these regulatory factors include three types of inhibitory AST, as well as several stimulatory compounds, such as AT, insulin-like peptides (ILP), ecdysis triggering hormone (ETH) and 20-hydroxyecdysone (20E).

Three families of AST have been described in insects: cockroach AST (YXFGL-amide or type-A), cricket AST (W2W9 or type-B) and Manduca sexta AST (PISCF or type-C) (Stay et al., 1994; Bendena et al., 1999; Audsley et al., 2008). Each of the three structurally unrelated types of AST (A, B, and C) are associated with a unique G-protein-coupled receptor (GPCR) family that includes vertebrate orthologues. The AST-A receptors are related to the vertebrate galanin receptors (Kreienkamp et al., 2002), the AST-B receptors to the bombesin receptors (Johnson et al., 2003), and the AST-C receptors show similarity to the somatostatin/opioid receptors (Kreienkamp et al., 2004; Mayoral et al., 2010). The AT receptor is also a GPCR and shows homology to the vertebrate orexin/hypocretin receptors (Yamanaka et al., 2008; Horodyski et al., 2011; Vuerinckx et al., 2011; Nouzova et al., 2012). Stimulatory and inhibitory effects of brain factors have been described in mosquitoes (Li et al., 2004; 2006). Allatostatin-C and AT are present in the brain of *A. aegypti* (Hernandez-Martinez et al., 2005); they both modulate JH synthesis *in vitro* (Li et al., 2004; 2006) and their receptors are expressed in the CA-CC complex (Mayoral et al., 2010; Nouzova et al., 2012).

The insulin/TOR signaling network is evolutionarily conserved in metazoans. It plays a central role in the transduction of nutritional signals that regulate cell growth and metabolism (Siddle, 2012; Howell and Manning,

2011). There are several studies describing that the insulin pathway modulates JH synthesis in insects. In *D. melanogaster,* specific silencing of the insulin receptor (InR) in the CA completely suppresses HMGR expression and renders a JH-deficient phenotype (Belgacem and Martin, 2007). In addition, *D. melanogaster* InR mutants have reduced JH synthesis (Tu et al., 2005). The insulin/TOR pathway has also been suggested as a link between nutritional signals and JH synthesis regulation in the CA of the cockroach *Blattella germanica* (Maestro et al., 2009; Abrisqueta et al., 2014), and FOXO knockdown using systemic RNAi *in vivo* in starved females elicited an increase of JH biosynthesis (Süren-Castillo et al., 2012). In *A. aegypti*, starvation decreases JH synthesis via a decrease in insulin signaling in the CA (Perez-Hedo et al., 2013). Starvation-induced upregulation of the insulin receptor, increased CA insulin sensitivity and "primed" the gland to respond rapidly to increases in insulin levels. During this response to starvation, the synthetic potential of the CA remained unaffected, and the gland rapidly and efficiently responded to insulin stimulation by increasing JH synthesis to rates similar to those of CA from non-starved females (Perez-Hedo et al., 2014).

Several additional factors modulate JH biosynthesis, including 20E, ETH, and Short Neuropeptide F (sNPF). The steroid hormone 20E controls molting and metamorphosis in insects (Yamanaka et al., 2013). During metamorphosis in mosquitoes, the increase of 20E titer provides temporal cues for the execution of a CA maturation program; 20E acts as a developmental signal that ensures proper reactivation of JH synthesis in the mosquito pupae (Areiza et al., 2015). 20E stimulates JH synthesis by increasing JHAMT activity, which catalyzes the conversion of FA into MF in the CA (Areiza et al., 2015). 20E also modulates JH synthesis in *B. mori* larvae (Gu and Chow, 1996; Kaneko et al., 2011), possibly by means of direct control on the expression of some of the JH biosynthetic enzymes (Hiruma and Kaneko, 2013).

ETH is a small C-terminally amidated peptide synthesized and secreted into the hemolymph by specialized endocrine cells called Inka cells; it plays a major role in regulating ecdysis (Adams et al., 2006; Zitnan et al., 2007). Yamanaka and collaborators (2008) reported very high expression of the ETH receptor (ETHR) in the CA of *B. mori*, leading them to suggest that ETH might have a role in the regulation of JH synthesis. In *A. aegypti* pupae the levels of ETHR transcripts in the CA rise in synchrony with 20E levels before ecdysis (Areiza et al., 2014). ETH acts as an allatotropic regulator of CA activity, ensuring the proper timing of JH synthesis in pharate adult

mosquitoes (Areiza et al., 2014). CA from late pupae stimulated with ETH show increases in JHAMT activity and JH synthesis. Inhibition of IP_3R-operated mobilization of endoplasmic reticulum Ca^{2+} stores prevented the ETH-dependent increases of JH biosynthesis and JHAMT activity in mosquitoes (Areiza et al., 2014). The role of ETH as an "AT" has been recently confirmed by two elegant studies in *D. melanogaster* (Meiselman et al., 2017; Lee et al., 2017). Specific knockdown of the ETHR in the CA led to an ETH signaling deficiency and sharply reduced JH levels. ETH induced calcium mobilization in the CA of *D. melanogaster* (Meiselman et al., 2017; Lee et al., 2017).

The sNPF modulates feeding, metabolism, reproduction, and stress responses in insects (Nässel and Wegener, 2011). The sNPF has been reported as an allatoregulatory peptide in *B. mori*; in the silk moth, the AT receptor is not expressed in the CA, but rather in the CC, specifically in a group of four cells that also express the sNPF (Yamanaka et al., 2008). According to the model proposed for *B. mori*, AT inhibits the release of sNPF, and this peptide inhibits JH synthesis; so AT exerts an indirect allatotropic effect by "derepression." This model has not been tested in additional insect species.

Little is known about the targets and mechanisms of action of allatoregulatory factors. In mosquitoes, AST-C exerts a strong, rapid, and reversible inhibition of JH synthesis that can be overridden by addition of any of the 13 JH precursors, indicating that the AST-C target is located before the entry of Acetyl-CoA into the JH biosynthetic pathway (Nouzova et al., 2015). Stimulation experiments using different sources of carbon (glucose, pyruvate, acetate, and citrate) revealed that AST-C acts after pyruvate is converted to citrate in the mitochondria (Nouzova et al., 2015). AST-C inhibits JH synthesis by blocking the citrate carrier (CIC) that transports citrate from the mitochondria to the cytosol, obstructing the production of cytoplasmic Acetyl-CoA that sustains JH synthesis in the CA of mosquitoes. *In vitro* inhibition of the CIC transporter mimics the effect of AST-C, and can be overridden by the addition of citrate or acetate (Nouzova et al., 2015). Similar results have been described in the inhibition of JH synthesis by AST-A in the cockroach (Huang et al., 2014).

In mosquitoes, the role of each of these endocrine regulators might be limited to particular periods of CA activity. Developmental modulators such as ETH and 20E play important roles during pupal maturation of the CA, and tend to modulate the activity of key enzymes like JHAMT (Areiza et al., 2014; 2015). Nutritional modulators like AST-C and insulin control the availability of precursors, such as cytoplasmic acetyl-CoA, without affecting

the synthetic potential of the CA (Nouzova et al., 2015). In the CC-CA of mosquitoes, we have detected the expression of receptors for many of these allatoregulatory factors, including ETHR A and B, the 20E receptor components EcR and Usp (both A and B), as well as receptors for ILP, AT, AST-C,-A and -B, and sNPF. It is possible that signals from all these modulators are integrated in the CA, which suggests that the regulation of JH synthesis is extremely complex (Zhu and Noriega, 2016).

3. Flux Control and JH Synthesis

The JH biosynthetic rate is influenced by a complex interplay of changes in precursor pools, enzyme levels and external modulators such as nutrients and allatoregulatory factors (Rivera-Perez et al., 2014; Zhu and Noriega, 2016). The identification of all the genes encoding the JH biosynthetic enzymes opened the door for more profound studies on the regulation of CA activity. Comprehensive studies on the expression of transcripts have been implemented in several holometabolan and hemimetabolan insects, including *B. mori* (Kinjoh et al., 2007; Ueda et al., 2009), *A. aegypti* (Nouzova et al., 2011; Rivera-Perez et al., 2014), *D. punctata* (Noriega et al., 2006; Huang et al., 2015) and *Schistocerca gregaria* (Marchal et al., 2011). The transcripts for most JH biosynthetic enzymes are highly enriched or exclusively expressed in the CA, and their expression is often coordinated with JH titers (Kinjoh et al., 2007; Rivera-Perez et al., 2014). In mosquitoes, the genes operating in the early and late steps of the pathway (MVAP and JH-branch) are transcriptionally co-regulated as a single unit, and catalytic activities for enzymes of the MVAP and JH-branch also change in a coordinated fashion in "active" and "inactive" CA (Rivera-Perez et al., 2014).

JH synthesis is controlled by the rate of flux of isoprenoids; therefore JH precursor pool concentrations and fluxes (which are flows into and out of pools) are critical variables in JH regulation (Nouzova et al., 2011). In mosquitoes, global fluctuations in the intermediate pool sizes in the MVAP and JH-branch are not functioning as a unit, but behave inversely, when MVAP precursors are high, JH-branch metabolites are low, and vice versa (Rivera-Perez et al., 2014). Principal component analysis (PCA) of the metabolic pools indicated that in reproductive female mosquitoes, at least four developmental switches alter JH synthesis by modulating the flux of isoprenoids at distinct points. Metabolic analysis established four distinct CA physiological conditions that were named: inactive, active, modulated, and suppressed CA, respectively (Rivera-Perez et al., 2014).

A recent study demonstrated the ability of two different quantitative approaches to describe and predict how changes in the individual metabolic reactions in the pathway affect JH synthesis (Martinez-Rincon et al., 2017). Generalized additive models (GAMs) described the association between changes in specific metabolite concentrations with changes in enzymatic activities and substrate concentrations. Changes in substrate concentrations explained 50% or more of the model deviances in 7 of the 13 metabolic steps analyzed. Addition of information on enzymatic activities usually improved the fitness of GAMs built solely based on substrate concentrations. In addition, a system of ordinary differential equations (ODE) was developed to describe the instantaneous changes in metabolites as a function of the levels of enzymatic catalytic activities. ODEs underscored that in the active CA, enzymatic activities were not limiting (Martinez-Rincon et al., 2017). Stimulation of JH synthesis with exogenous precursors has been reported for the CA of many insect species (Nouzova et al., 2015; Huang et al., 2015), and it seems that having an excess of enzymes is common in most insects studied. In *A. aegypti,* the individual addition of 200 μM of any of nine different precursors (ACoA, MVA, MevP, MevPP, FPP, FOL, FAL, FA, and MF) resulted in a stimulation of 2–3 fold of JH synthesis (Nouzova et al., 2015); confirming that enzymatic flux capacities were higher than the basal flux rates observed in controls.

1.2.2 JH TRANSPORT

JH is transported from the sites of synthesis to target tissues by hemolymph carriers named JH binding proteins (JHBP) (Goodman and Chang, 1985; Trowell, 1992; Prestwich et al., 1996; Goodman and Cusson, 2012). JHBPs are synthesized in the fat body (Rodriguez et al., 2002; Orth et al., 2003), and they protect JHs from non-specific hydrolysis by enzymes present in the hemolymph. Binding of JH to JHBPs regulates circulating JH concentration (Goodman et al., 1978; Touhara et al., 1993), which is thought to be crucial for effective signaling by the hormone (Suzuki et al., 2011). Four types of JHBPs have been characterized in different insect species (Trowell, 1992; Goodman and Cusson, 2012; Kim et al., 2017): (1) low molecular weight JHBPs similar to the Takeout proteins; (2) low molecular weight JHBPs related to the Odorant binding proteins (OBP); (3) lipophorin type high molecular weight JHBPs; and (4) hexameric high molecular weight JHBPs distinct from lipophorins. Examples of all four protein types are listed in Table 1.1.

TABLE 1.1 Characteristics of Selected[a] Insect Hemolymph JH Binding Proteins

Protein (type)	Species	Size (kDa)	JH Bound	Affinity[b] (nM)	Structure (PDB ID)	References
Hexamerin* (HMW)	*Locusta migratoria*	74[c]	10*R*-JH III	0.9	-	Braun and Wyatt, 1996
Hexamerin (HMW)	*Schistocerca gregaria*	77[c]	10*R, S*-JH III	19	-	Tawfik et al., 2006
Hexamerin (HMW)	*Gryllus bimaculatus*	81[c]	10*R, S*-JH III	28	-	Tawfik et al., 2006
Lipophorin (HMW)	*Blattella germanica*	670	10*R*-JH III	9.8	-	Sevala et al., 1997
hJHBP* (LMW)	*Manduca sexta*	32	10*R*, 11*S*-JH I 10*R*, 11*S*-JH II	90 110	- -	Prestwich et al., 1987
hJHBP* (LMW)	*Manduca sexta*	32	10*R*, 11*S*-JH I 10*R*, 11*S*-JH II	11 42	- -	Touhara et al., 1993
hJHBP (LMW)	*Manduca sexta*	ND	10*R*, 11*S*-JH I 10*R*, 11*S*-JH II 10*R, S*-JH III	0.7 0.7 1.9	- - -	Park et al., 1993
hJHBP* (LMW)	*Heliothis virescens*	32	JH I	40	-	Wojtasek and Prestwich, 1995
hJHBP (LMW)	*Galleria mellonella*	32	10*R*, 11*S*-JH I 10*R*, 11*S*-JH II 10*R*, 11*S*-JH III	85 72 470	- - -	Ozyhar and Kochman, 1987
hJHBP* (LMW)	*Galleria mellonella*	32	none	-	2RCK	Kolodziejczyk et al., 2008
hJHBP (LMW)	*Bombyx mori*	32	JH I JH II JH III	90 114 390	- - -	Kurata et al., 1994
hJHBP* (LMW)	*Bombyx mori*	32	JH III	450	-	Vermunt et al., 2001
hJHBP* (LMW)	*Bombyx mori*	32	none JH II JH III MPD[d]	- - - -	3AOT 3AOS 2RQF 3A1Z	Suzuki et al., 2011 Fujimoto et al., 2013

TABLE 1.1 *(Continued)*

Protein (type)	Species	Size (kDa)	JH Bound	Affinity[b] (nM)	Structure (PDB ID)	References
mJHBP* (LMW, OBP)	*Aedes aegypti*	35	10*R*-JH III 10*S*-JH III 10*R, S*-JH II 10*R*-JH III	23 63 25 -	- - - 5V13	Kim et al., 2017

[a] Earlier references to hemolymph JHBPs can be found in a previous review (Trowell, 1992).

[b] Determined from direct binding of a labeled JH (K_d) or from the competition (K_i) against a labeled JH ligand.

[c] Calculated size of the monomer without lipid component.

[d] Two molecules of an artificial ligand, 2-methyl-2,4-pentanediol, bound to both hJHBP pockets.

*Recombinant protein. ND not determined.

1.2.2.1 HIGH MOLECULAR WEIGHT JHBPS

High molecular weight binding proteins form complexes exceeding 300 kDa. The high molecular weight JHBPs are divided into two subgroups, the lipophorins, and the storage proteins or hexamerins, both displaying relatively high affinity for JH (Trowell, 1992) (Table 1.1). Lipophorins are multi-subunit hemolymph proteins that carry dietary lipids, pheromones, and cuticular lipids to their sites of utilization (Canavoso et al., 2001). The transporter contribution of lipophorin could be quite significant as they are highly abundant in the hemolymph, and they work as a reusable lipoprotein shuttle, yielding a continuous supply of binding sites for JH. The second class of high-affinity, high molecular weight JH transports molecules is hexamerins. These proteins are composed of six 70–80 kDa subunits and are not primarily hemolymph transporters, but storage proteins. Hexamerins are present at relatively low concentrations, not exceeding 2% of the total hemolymph protein. Nevertheless, their hexameric structure allows a single complex to bind up to six molecules of JH (Koopmanschap and De Kort, 1988).

1.2.2.2 LOW MOLECULAR WEIGHT JHBPS

Insect low molecular weight JHBPs have been related to two distinct protein families. First, the Tubular lipid-binding proteins (TULIP), including takeout

proteins found in many insects and the lepidopteran-specific hemolymph JHBPs (hJHBPs) (Alva et al., 2016; Wong and Levine, 2017). The second type recruits from the family of OBP with the recently identified mosquito JHBP (mJHBP) (Kim et al., 2017). The lepidopteran proteins belonging to the TULIP family contain a JHBP domain (pfam06585), whereas mJHBP lacks this domain and consists of α-helices (Figure 1.3). The molecular function of the insect takeout proteins is unclear except that their role as lipid carriers has been supported by crystal structures of the lepidopteran (*Epiphyas postvittana*) Takeout bound either by ubiquinone-8 or by myristic acid (Hamiaux et al., 2009, 2013). Binding of ligands with long aliphatic chains corresponds to the uninterrupted tubular cavity forming a single hydrophobic ligand-binding pocket of takeout. This structure is different from that of the lepidopteran hJHBPs whose tubular cavity is divided in the middle by hydrogen bonds to form a confined JH-binding pocket and a second cavity (see Section 1.2.2.3).

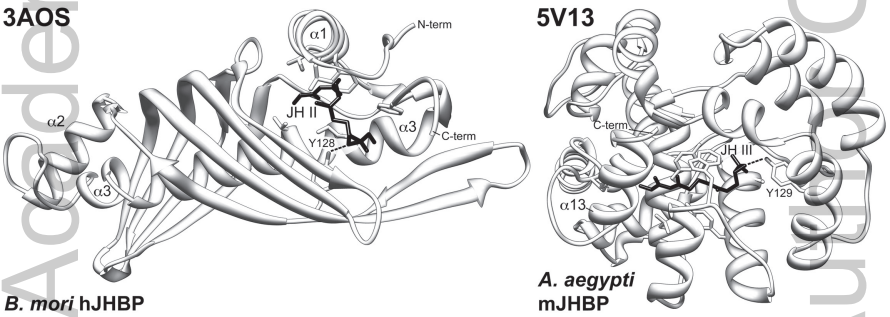

FIGURE 1.3 Structures of low molecular weight JH binding proteins.
Left, *B. mori* hJHBP bound by JH II (PDB 3AOS; Suzuki et al., 2011) belongs to the TULIP/Takeout protein family. Note the gate (helix α1 with the N-terminal arm) and latch (helix α3 with the C-terminal tail) mechanism enclosing the JH ligand. Right, *A. aegypti* mJHBP bound by JH III (PDB 5V13; Kim et al., 2017) related to the Odorant binding proteins. Note the C-terminal helix α13 covering the methyl ester side of the ligand. Only one of three mJHBP monomers of the 5V13 complex structure is shown. Structures of both proteins derive from X-ray diffraction crystallography. In both proteins, the hydroxyl groups of the indicated tyrosine phenolic rings form hydrogen bonds (dashed lines) with the epoxide oxygen of JH.

Lepidopteran hJHBPs, originally described in *M. sexta*, are secreted proteins consisting of a single polypeptide chain, typically 230–260 amino acids in length; a signal peptide is removed upon maturation (Goodman and Chang, 1985; Goodman and Cusson, 2012). Table 1.1 summarizes the hormone-binding affinities toward JHs, determined for hJHBPs from several

species, mostly emphasizing data reported after an earlier review by Trowell (1992). In general, hJHBPs show preference to natural epoxidated JHs over synthetic JH analogs such as methoprene (Goodman and Chang, 1985; Goodman and Cusson, 2012).

The OBP-type low molecular JHBPs are represented by the mJHBPs that have only recently been discovered in mosquitoes (Kim et al., 2017). Thus far mJHBP orthologues have been found in all available genomes of the *Culicidae* but not outside of this family. The mJHBPs are related to the mosquito salivary D7 proteins, which are secreted into the female saliva where they bind small molecular weight mediators of the vertebrate host hemostasis and inflammation response during blood feeding. The mJHBP occurs in the hemolymph of pupae and adults of the *A. aegypti* mosquitoes. The N-terminus of *A. aegypti* mJHBP binds with high specificity the epoxidated methyl esters JH III and JH II (Table 1.1), while MF or FA acid show no measurable interaction with the protein and FOL and methoprene interacted only weakly (Kim et al., 2017). Thus the ligand selectivity of mJHBP resembles that of lepidopteran hJHBPs. Both types of low molecular weight JHBPs also show moderate preference towards the natural enantiomers 10*R* of JH III (Schooley et al., 1978; Kim et al., 2017) and 10*R*, 11*S* of either JH II or JH III (Prestwich and Wawrzeńczyk, 1985; Prestwich et al., 1987; Ożyhar and Kochman, 1987).

1.2.2.3 JHBP STRUCTURE AND MECHANISM OF ACTION

The structures of low molecular weight JHBPs have been studied in order to elucidate the interactions between the amino acid residues of the binding pocket and the JH molecule. Four crystal structures of lepidopteran hJHBPs have been resolved to date (Table 1.1). A hJHBP structure from the wax moth *Galleria mellonella* was obtained in its apo-form, without a ligand (PDB 2RCK; Kolodziejczyk et al., 2008). The *B. mori* hJHBP was crystallized in the apo-form (3AOT) and in complex with either JH II (3AOS; Suzuki et al., 2011) (Figure 1.3) or with an artificial ligand 2-methyl-2,4-pentanediol (MPD) (3A1Z; Fujimoto et al., 2013). Suzuki et al. (2011) also reported a solution structure of *B. mori* hJHBP with bound JH III using NMR spectroscopy (2RQF). A crystal structure of the unrelated mosquito *A. aegypti* mJHBP in complex with JH III was also resolved (5V13; Kim et al., 2017) (Figure 1.3). The available structural information has greatly improved our understanding of JH interactions with the specific carrier proteins and suggested the mode of hormone transport and delivery.

The lepidopteran hJHBPs form an unusual fold which resembles that of lipid-binding mammalian proteins (Kolodziejczyk et al., 2008; Suzuki et al., 2011) and insect takeout proteins (Hamiaux et al., 2009, 2013). This fold mainly consists of a long, C-terminal helix α3 (annotated as α4 in *G. mellonella*; Kolodziejczyk et al., 2008) opposed by a curved wrap of antiparallel β-sheets (Figure 1.3). Of the additional shorter helices, the N-terminal helix α1 located close to α3 acts as a gate for bound JH (Kolodziejczyk et al., 2008; Suzuki et al., 2011). A disulfide bridge that connects helix α1 with the disordered N terminus had been previously suggested to play a role in JH binding (Wojtasek and Prestwich, 1995). The structure of *G. mellonella* hJHBP revealed a second disulfide bridge between the central part of helix α4 and the middle of the β-wrap, as well as N-acetyl-glucosamine glycosylation at Asn-94 (Kolodziecjzyk et al., 2008). The proteins contain two hydrophobic cavities, originally dubbed West and East in *G. mellonella* hJHBP (Kolodziecjzyk et al., 2008), arranged in the opposite poles of the protein. Of these, only the "West" cavity near both protein termini binds the hormone, whereas the other remains empty in hJHBP-JH complexes (Suzuki et al., 2011). The JH-binding pocket is confined by a network of hydrogen bonds forming among the side chains of conserved residues in the middle of the protein structure, between helix α3 and the β-wrap. These hydrogen-bonded residues are not conserved in the takeout proteins, resulting in a continuous tubular cavity.

Studies of the crystal and solution structures of hJHBP from *B. mori* have shed light on the mechanisms of JH recognition, binding, and release (Suzuki et al., 2011). When free in the hemolymph, the unliganded hJHBP assumes either a closed or an open conformation. This is achieved through a gate-latch interaction between the N terminus/helix α1 and the C-terminal tail following helix α3. In the closed conformation, a contact between helix α1 (the gate) and the C-tail (the latch) blocks access to the JH-binding cavity. Binding of JH, initiated by the epoxide end of the hormone, induces a conformational change that completely buries JH within the protein. Multiple hydrogen bonds then form between the N terminus and the C-tail, effectively sealing the JH-binding cavity and protecting JH from enzymatic degradation. The closed conformation is further stabilized by multiple non-polar interactions between residues of the hydrophobic pocket and the non-polar skeleton of JH. A hydrogen bond forming between Tyr-128 and the epoxide oxygen of JH III (Figure 1.3) is essential for the hormone binding (Suzuki et al., 2011).

Interestingly, the artificial ligand MPD, which is less than half-size of JH, competed for *B. mori* hJHBP against JH III, and bound to the JH-binding cavity, with the hydroxyl group of MPD mimicking the hydrogen bond interaction of JH III with Tyr-128 (Fujimoto et al., 2013). A crystal structure of the hJHBP complex with two molecules of MPD revealed that the JH-binding pocket was flexible as it adjusted the position of the gate α1 helix to the smaller size of the ligand; the second MPD molecule occupied the second (East) cavity that does not bind JH.

Although the precise mechanisms of JH loading to hJHBP in the CA and hormone delivery to target tissues are incompletely understood, the NMR-based solution structure of the *B. mori* hJHBP-JH III complex has provided a preliminary model (Suzuki et al., 2011). Because the sealed gate-latch pocket opened to release JH III in the presence of 30% ethanol, the authors proposed that reduction of dielectric constant near cell membrane may trigger another conformational change leading to JH release and delivery to the target cell. Whether the delivery involves a membrane-bound transporter protein is unknown.

The crystal structure of the mosquito mJHBP-JH III complex (5V13; Kim et al., 2017) has revealed a binding pocket that finely adapts in size, shape, and hydrophobicity to accommodate the native JH of mosquitoes. JH binds to the N-terminal domain, which is comprised of seven α-helices crosslinked with two disulfide bonds. The C-terminal domain consists of another six α-helices, also with two disulfide links. The C-terminal helix α13 distinguishes mJHBP from the relative D7 salivary proteins as it extends over the mJHBP surface and covers the JH-binding pocket (Kim et al., 2017). The epoxide moiety of JH III is buried deep in the cavity and, similar to *B. mori* hJHBP; it forms a hydrogen bond with the hydroxyl group of Tyr-129 (Figure 1.3). The rest of the JH-binding pocket is lined with hydrophobic residues engaging in non-polar interactions with JH. The methyl ester end of JH III locates near the protein surface and is covered by closely packed helix α13 (Figure 1.3), which excludes both hydrophilic ligands of those of larger chains including methoprene (Kim et al., 2017). Like lepidopteran hJHBP, also mJHBP preferentially bound the correct optical isomer. In the presence of racemic 10*R, S*-JH III, the complex crystallized as a structure composed of three identical mJHBP monomers, each occupied by a single 10*R* enantiomer of JH III in its ligand-binding pocket (5V13; Kim et al., 2017).

1.2.2.4 JHBP EXPRESSION

There is limited information on the control of expression of the hemolymph low molecular weight JHBP genes (*jhbp*). hJHBPs are mainly expressed in the fat body of larval and adult insects (Orth et al., 2003). They are also expressed in other tissues, including epidermis, testes, and ovaries (Vermunt et al., 2001; Wei et al., 2015), and in the antennae and taste organs (Saito et al., 2006), suggesting an involvement in chemoreception (Fujikawa et al., 2006). The expression of *jhbp* in the fat body is tightly regulated during larval development in several insect species (Hidayat et al., 1994; Orth et al., 1999; Kim et al., 2017). Exogenous JH treatment specifically and rapidly increased fat body *jhbp* expression levels up to five-fold in an age-dependent fashion in *B. mori* larvae (Orth et al., 1999; Vermunt et al., 2001). In *G. mellonella*, the *jhbp* mRNA is high during the first two days of development in the last instar, and significantly decreases just before pupation (Rodriguez et al., 2002). Although JH modulates *jhbp* expression, there is no correlation between JH titers and *jhbp* expression, suggesting that additional factors are important in the regulation of these genes. Computational analysis of the *M. sextajhbp* upstream regulatory region revealed eleven recognition sites for transcription factors, including GATA 1 and 3, as well as C/EPB α and β sites (Orth et al., 2003). In *G. mellonella* the *jhbp* promoters are TATA- and Inr-driven, while a high-affinity element shown to bind components of the 20E receptor (Usp/EcR-DBD) but distinct from the canonical 20E response elements, inhibits *jhbp* expression (Sok et al., 2008).

Changes in protein levels of hJHBP mirror *jhbp* mRNA expression in *B. mori* fat body cells (Vermunt et al., 2001). Fluctuation of hJHBP titer during insect development is crucial for effective JH signaling (Suzuki et al., 2011). The levels of JHBP in the hemolymph were found relatively constant (Goodman and Gilbert, 1978; Koopmanschap and deKort, 1988), but always exceeding the concentration of the hormone (Goodman and Gilbert, 1978; Kim et al., 2017). Therefore, almost every molecule of JH in the hemolymph is potentially bound to a hJHBP for its transport and protection (Braun et al., 1995; Kim et al., 2017).

1.2.3 JH DEGRADATION

The mechanisms of JH catabolism have remained a critical area of study due to the negative effects that inappropriate or mistimed JH titers (or application of JH mimics) have during development, molting, and reproduction of

insects (Staal, 1975). The manipulation of JH degradation processes (and therefore, titers) remains an important goal for those interested in using JH signaling to control insect development and reproduction. In pursuit of this goal, JH Epoxide hydrolase (JHEH), JH esterase (JHE), and JH diol (JHD) kinase (JHDK) have been identified as key enzymes in the catabolism and inactivation of JH (Goodman and Cusson, 2012). Together these three enzymes remove active JH species from circulation and directly participate in the regulation of JH titers and the overall developmental or reproductive programs of insects (Goodman and Cusson, 2012).

In general, a physiologically active extracellular JH molecule will ultimately become an inactive intracellular JH diol phosphate (JHDP) or JH acid diol (JHAD) through two alternative pathways (Figure 1.4). The first catabolic pathway is the hydrolysis of the methyl ester group on the one end of the molecule, yielding an extracellular JHA (White, 1972; Kort and Granger, 1981; Iga and Kataoka, 2012; Goodman and Cusson, 2012). JHA may then enter the cell to be processed further by JHEH (Figure 1.4). The second catabolic pathway for JH (or JHA) occurs intracellularly. Microsomal JHEH modifies the epoxide ring of JH (as well as the epoxide ring of intracellular JHA) to yield JHD or in the case of JHA, JHAD (White, 1972). JHE may also catalyze the reaction of JHD into JHAD within the cell, although some questions remain about whether this alternate degradation pathway exists (Maxwell et al., 2002) (Figure 1.4).

The final step in the permanent inactivation of JH occurs when JHD is phosphorylated by juvenile hormone diol kinase (JHDK) to yield JH diol phosphate, a polar molecule with no known hormonal activity (Maxwell et al., 2002). Interestingly, JHAD is likely not metabolized by JHDK, indicating that the final fate of a JH may be either JHAD or JHDP depending on whether the molecule was first processed outside of the cell by JHE or inside the cell by JHEH. In either case, it is likely that JHAD and JHDP are ultimately excreted with other polar metabolites (Hua-Jun et al., 2011; Yang et al., 2016; Fu et al., 2015; Shapiro et al., 1986).

Although JHE, JHEH, and JHDK have all been implicated in the regulation of JH titer, they should probably not be considered as interchangeable. The cellular localization of each enzyme seems to be an important factor in how each enzyme participates in the regulation of JH titer and contributes to the overall developmental program. In hemolymph incubation experiments utilizing radiolabeled JH, JHA was typically the only metabolite produced indicating the presence and activity of JHE but not JHEH or JHDK (Shapiro et al., 1986). The hydrolysis of the methyl ester from JH is catalyzed by

JHE, and occurs in the hemolymph where JHE is thought to be primarily localized (Kort and Granger, 1981; Kamita and Hammock, 2010) (Figure 1.4). JHE is also clearly capable of processing JH bound to JHBPs (Touhara et al., 1995). The JHA product of JHE may then go on to exert hormonal effects within the cell that are distinct from JH itself, which suggests that JHE may play roles in both the degradation of one hormonal product and the formation of another (Ismail et al., 1998). Later work with JHEH has

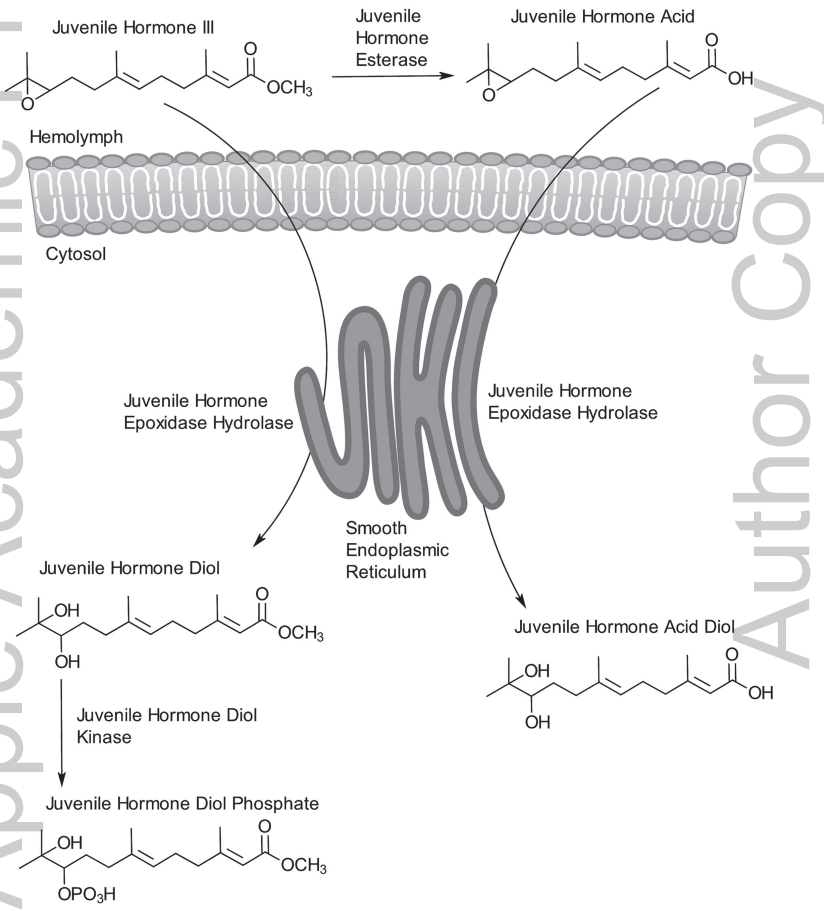

FIGURE 1.4 JH degradation pathways.
An early bifurcation leads to alternate pathways: In the first one, JH enters the cell and is converted to JH diol (JHD) through the action of the membrane-bound JH epoxide hydrolase (JHEH). JHD is phosphorylated by JH diol kinase (JHDK) to yield a polar metabolite likely excreted through the Malpighian tubules and the hindgut. In the second pathway, JH is converted to JH acid (JHA) extracellularly. JHA crosses the cell membrane and is processed by JHEH into the final polar metabolite, JH acid diol (JHDP).

shown that this enzyme is localized primarily within microsomal fractions, contains a membrane anchor and is not located in the hemolymph (Touhara and Prestwich, 1993). Not surprisingly, JHEH is incapable of processing JH bound to JHBPs as it would be found in the hemolymph (Touhara and Prestwich, 1993). Together these observations demonstrate that the metabolism of JH contains alternative pathways, both inside and outside of cells, with potentially different metabolic outcomes for a JH molecule. In many species of lepidopterans, dipterans, and coleopterans, JHEH, and JHDK are primarily expressed in the hindgut and Malpighian tubules, likely reflecting the role these tissues in excretion of the final JH metabolites (Shapiro et al., 1986; Fu et al., 2015; Yang et al., 2016). However, Fu et al., (2015) detected trace amounts of JHDK mRNA in a variety of additional tissues, including the thoracic muscles, brain-CC-CA complex, foregut, midgut, and ventral ganglia.

The relationship and relative participation of JHE, JHEH, and JHDK in the regulation of JH titer is complex and depends on a variety of other identified factors. JHE, JHEH, and JHDK expression are responsive to 20E application (Yang et al., 2016; Zeng et al., 2017). JHEH, JHDK, JHE can also respond to feeding or starvation (Duan et al., 2016; Yang et al., 2016; Zeng et al., 2017). Several transcription factors play a role in the regulation of expression of JH catabolic enzymes. FOXO mutants exhibit an upregulation of JHE, JHEH, and JHDK. FOXO regulates JH degradation, thereby altering JH titers and developmental programming (Zeng et al., 2017). Mutation and RNAi experiments have demonstrated that the various extracellular and intracellular pathways for JH catabolism can be compensatory. Reductions in one enzyme may cause increases in others. Reduction of JHE in *B. mori* causes an upregulation of JHEH and JHDK, indicating that, at least in this species, the various JH catabolic pathways are interrelated and complementary (Zhang et al., 2017).

1.2.3.1 JUVENILE HORMONE ESTERASE (JHE)

Juvenile hormone esterase (JHE) displays all the critical attributes of a JH-specific esterase with a vital role in the regulation of JH titers. That includes (1) inverse relationship with JH-titers;(2) high specificity for a JH substrate, and (3) the ability to efficiently process low levels of JH (i.e., to "scavenge" JH) (Ward et al., 1992; Kamita and Hammock, 2010). Furthermore, JHE is capable of processing JH in the presence of JHBP, a key requirement for a hemolymph catabolic enzyme (Kamita and Hammock, 2010).

Many studies have demonstrated a clear inverse relationship between JHE and JH titers. Reducing JHE activity either through enzyme inhibitors or reverse genetic techniques, yields a predictable rise in JH titer coupled with a delay or blockage in development typical of increased JH (Edgar et al., 2000). The inverse relationship between JHE and JH was also revealed using CRISPR/Cas9-mediated knockout of JHE in *B. mori* (Zhang et al., 2017). Depletion of JHE caused predictable alterations of developmental timing and body size, indicative of high JH titers. Loss-of-function experiments with CRISPR/Cas9 FOXO mutants in *B. mori* indicated that JH degradation genes such as JHE are under the control of FOXO. *B. mori* FOXO mutants display up-regulated JHE, JHEH, and JHDK as well as growth delays and precocious metamorphosis expected for JH deficiency phenotypes (Zeng et al., 2017). Conversely, overexpression of JHE causes phenotypes associated with low JH titer, namely precocious metamorphosis (Tan et al., 2005). In *A. aegypti*, inhibition of JHE with the irreversible inhibitor, BEPAT (s-benzyl-O-ethyl phosphoramidothiolate), resulted in the absence of the product JHA and a reduction of JHAD (Shapiro et al., 1986). It is important to note that JHA also retains a distinct hormonal activity and can enter cells to exert effects (Ismail et al., 1998; Kamita and Hammock, 2010). Because of the evidence of hormonal activity for JHA, JHE might also be considered a biosynthetic enzyme capable of producing a JH species with hormonal activity (Ismail et al., 1998).

JHE also seems to participate in the reproductive physiology of insects. JHE in *Bactrocera dorsalis* was most highly expressed in adults rather than larvae and exhibited sex-specific expression patterns indicating a potential undetermined role in sexual development (Yang et al., 2016). Since JH is fairly well understood as a gonadotropin in many insects, it would make sense for catabolic enzymes of JH, such as JHE, to similarly play a role in sexual development. Surprisingly few papers have explored the role of JHE in sexual development and reproduction, and it seems to be an area of research worthy of more study.

In vitro work with JHE indicates that it has a moderate *Kcat* (rate of catalysis) and a high affinity for JH, which indicates that this enzyme likely participates as a "scavenger" of hemolymph JH during critical developmental transformations (Ward et al., 1992). Site-directed mutagenesis experiments have demonstrated that the catalytic core of JHE contains a His-Ser-Glu catalytic triad involved in the hydrolysis of JH (Ward et al., 1992; Goodman and Cusson, 2012). Other important residues include a Phe-259 and a Thr-314, which appear to be vital for maintaining the high specificity of JHE, as well

as some polar residues at the entrance to the catalytic portion of the enzyme responsible for creating a polar area (Goodman and Cusson, 2012).

1.2.3.2 JUVENILE HORMONE EPOXIDE HYDROLASE (JHEH)

Juvenile hormone epoxide hydrolase (JHEH) is a microsomal hydrolase similar to mammalian microsomal EHs and is found exclusively within the cell. The JHEH has a strongly hydrophobic transmembrane anchor (Craft et al., 1990; Friedberg et al., 1994). JHEH is also critical to JH degradation (Campbell et al., 1992; Halarnkar et al., 1993; Lassiter et al., 1995; Debernard et al., 1998). However, much still remains to be determined about the specific developmental timing as well as the interaction between JHE and JHEH to determine final JH titers and developmental outcomes. In the brown planthopper, *Nilaparvata lugens*, RNAi silencing of JHEH caused polyphenic alterations (short wing length), consistent with increased JH titers (Zhao et al., 2017). The timing and expression profile of JHE and JHEH differed between the two wing morphs, which suggest that although both enzymes are capable of catabolizing JH, they do not act interchangeably and that JHEH plays a specific developmental role (Zhao et al., 2017). In many studies, it is clear that JHEH is highly expressed during larval-pupal metamorphosis, further indicating a clear developmental role in clearing JH from within specific cells during critical developmental stages (Lü et al., 2015; Fu et al., 2015; Yang et al., 2016). RNAi knockdown of JHEH caused a predictable rise in JH titers in the Colorado potato beetle (*Leptinotarsa decemlineata*) (Lü et al., 2015). Interestingly and perhaps related to the caste differentiation properties of JH in eusocial insects, JHEH in *Apis mellifera* does not seem to play a lead role in JH degradation but instead may play a role in dietary lipid metabolism (Mackert et al., 2010). Tissue-specific expression studies have indicated that JHEH is present in a variety of tissues, including the brain-CC-CA, hindgut, Malpighian tubules and ovaries, suggesting that multiple tissues may play a role in JH degradation, with the catabolic product transported to the hindgut for final degradation by JHDK (Lü et al., 2015; Fu et al., 2015; Yang et al., 2016). Despite the progress made in the understanding of the role of JHEH in JH catabolism, some important questions remain. Namely, how is the expression of JHE and JHEH coordinated to control JH titers? Central to this question is understanding the importance of having catabolic pathways both inside the cell (JHEH) as well as outside of the cell (JHE) and the comparative contributions of each degradation pathway to the overall developmental or reproductive outcome.

1.2.3.3 JUVENILE HORMONE DIOL KINASE (JHDK)

JHDK, in combination with JHEH, is very important for the inactivation and metabolism of JH inside the cell. JHDK displays high specificity for a diol with an ester moiety, but not for acid diols (such as JHAD) (Maxwell et al., 2002). These results indicate that two alternative pathways exist for the metabolism of JH. In one pathway, JH enters the cell and is processed by JHEH and then JHDK. In the other pathway, JH is converted to JHA outside of the cell, and JHA can then enter the cell to be catabolized by JHEH. This bifurcation in the JH degradation pathway with certain intermediates possessing hormonal activity (JHA and possibly others) is interesting and points to a much more complicated system of hormonal signaling and degradation than was previously supposed.

JHDK is important for secondary metabolism and inactivation of JH by phosphorylation of the JHD within the cell (Goodman and Granger, 2005). The final product of JH catabolism (JHDP) is highly water-soluble and likely hormonally inactive (Maxwell et al., 2002). Not surprisingly, JHDK, and JHEH are highly expressed in the hindgut and Malpighian tubules, which is likely related to the hydrolysis of JHD and JHA and final excretion of the inactive polar metabolite, JHDP (Hua-Jun et al., 2011; Yang et al., 2016; Fu et al., 2015). Experimental evidence further supports the idea that JHAD is a polar metabolite excreted in the urine of insects. In *A. aegypti*, it has been estimated that up to 50% of radiolabeled JH was excreted in urine as JHAD (Shapiro et al., 1986). The overall structure of a JHDK from *Spodoptera litura* was recently modeled. The predicted 183-amino acid protein contains three calcium-binding motifs (EF-hand) as well as a GTP-binding motif and shares a high degree of conservation with other lepidopteran JHDKs, possibly indicative of a pivotal role in the regulation of JH titer (Fu et al., 2015; Zeng et al., 2016). Characterization of a JHDK from *L. decemlineata* revealed similar results; a 184-amino acid protein with a high degree of similarity to other characterized JHDKs (Fu et al., 2015).

Since JHEH and JHDK seem to act in concert within the cell, it is not surprising that these two enzymes appear to be expressed in parallel, indicative of their strong association and related roles (Goodman and Granger, 2005). Other work has shown that JHDK expression in the midgut and fat body is higher during earlier stages of larval development (Zeng et al., 2016). Similar results were obtained in *L. decemlineata*. In this species, JHDK was most highly expressed in earlier instars as well. However, the highest mRNA levels were detected in the hindgut and Malpighian

tubules (Fu et al., 2015). Silencing of JHDK with dsRNA led to a clear increase in JH levels and an alteration of the developmental program (Fu et al., 2015). An analysis of the upstream regulatory response elements of the JHDK gene indicated that this enzyme might be under the control of a diverse array of transcription factors including the Broad-Complex, myeloblastosis family, and FOX family of transcription factors (Zeng et al., 2016). Other work on factors that control the expression of JHDK has indicated that this enzyme is sensitive to 20E and highly expressed around metamorphosis and is likely vital during this time to clear JH (Cheng et al., 2014; Fu et al., 2015; Yang et al., 2016).

1.2.3.4 OVERALL IMPORTANCE OF THE JH CATABOLIC PATHWAYS

In nearly every study that has attempted to modify the expression or activity of one of the JH catabolic enzymes, an alteration in JH titers or some related developmental factor was observed. Together with this body of literature repeatedly demonstrates that the catalysis of JH is as important as the synthesis of JH in determining JH-linked developmental outcomes. The JH degradative enzymes are very clearly localized in species, tissue, and temporally specific ways and very few broad generalizations can be made other than each enzyme (JHE, JHEH, and JHDK) can participate in JH degradation depending on species, timing, and tissue. However, it is very likely that an underlying pattern or principle does exist. Perhaps the complicated degradation of JH, with various JH metabolites possessing various degrees and targets of hormonal activity, can help to account for the high levels of "hormonal pleiotropy" seen with JH (Flatt et al., 2005).

1.3 MECHANISM OF ACTION OF JH

Section 1.2 explains how JH is produced in the CA, transported around the insect body, and finally degraded. We will now discuss signaling events following JH delivery to target tissues and cells. While the biological effects of JH on development, polyphenism, diapause, reproduction, and other aspects of insect life have been recognized for many decades, the molecular mode of action of this multitasking hormone remained enigmatic until recently. We will, therefore, start this section with a short historical excursus.

1.3.1 FINDING A JH RECEPTOR

The early parabiosis experiments (joining of two individual insects) by Wigglesworth in the 1930s indicated that like other hormones, JH was a systemically acting blood-borne signal (Wigglesworth, 1934, 1936). It also became clear that JH acted upon a target tissue, such as the epidermis, in a cell-autonomous manner, i.e., that its effect remained restricted to the cells exposed to JH and did not propagate throughout the animal. Therefore, Wigglesworth could etch his initials into the cuticle of a *Rhodnius prolixus* bug: the epidermal cells treated with JH synthesized the larval type of cuticle, making the letters "VBW" stand out when the larva molted into an adult. This was a striking visual demonstration that JH preserved, at the cellular level, the juvenile state of the epidermis; in other words that it prevented its transition to the adult state.

In this regard, JH counters the action of ecdysone, which promotes the onset of metamorphosis and adult differentiation. Luckily for ecdysone, its effect on gene expression could be readily observed through the induction of "puffs" (sites of active transcription) on the giant salivary gland chromosomes, originally in the *Chironomus tentans* midge larvae (Clever and Karlson, 1960). Later work on puffs in *D. melanogaster* led to the visionary Ashburner's model, where 20E bound its hypothetical receptor to directly activate a specific set of early-responding genes (Ashburner et al., 1974). This model was fully confirmed when the 20E receptor complex, formed by the Ecdysone receptor (EcR) and Ultraspiracle (Usp) proteins of the nuclear receptor (NR) family, was shown to regulate 20E-response genes (Koelle et al., 1991; Yao et al., 1992; Hill et al., 2013).

It was naturally thought that JH must direct an analogous genetic program opposing or modulating that of 20E (Riddiford, 1994). However, tracking down the molecular players in JH signaling proved difficult due to the lack of a robust effect of JH on *D. melanogaster* (Gilbert et al., 2000). The lipophilic nature of JH encouraged the idea that, like the steroid 20E, JH may regulate transcription through a ligand-activated intracellular receptor of the NR type. The apparent similarity between JH and retinoids favored Usp, the partner of EcR and an ortholog of the vertebrate *9-cis*-retinoic acid receptor, RXR. However, initial findings that Usp bound JH III or its precursor MF (Jones and Sharp, 1997; Jones et al., 2006) have been supported neither by structural and *in vivo* functional data (Iwema et al., 2007), nor by *usp* loss-of-function phenotypes in *D. melanogaster* or other insects. Rather than inducing precocious metamorphosis, a hallmark of perturbed JH signaling,

removal of Usp prevented molting as would be expected given its role in 20E signaling.

A second JH receptor candidate arose from early genetic screens in *D. melanogaster* performed by Wilson and colleagues. The screens yielded several mutants exhibiting increased tolerance to lethal doses of the JH analog and insecticide methoprene, all allelic to a single gene named *Methoprene-tolerant* (*Met*) (Wilson and Fabian, 1986). *Met* encodes a transcription factor of the basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) family (Ashok et al., 1998). Rather than a hormone receptor function, the bHLH-PAS signature of Met might have suggested a xenobiotic-induced function, such as of the closely related Aryl hydrocarbon receptor (AhR) that mediates dioxin response in vertebrates. The idea that Met is a JH receptor was further discouraged by the absence of a robust phenotype in the Met-deficient flies. As was discovered much later, the $Met^{-/-}$ mutants were viable and fertile owing to a partly redundant *Met* paralog, *germ cell-expressed* (*gce*) (Abdou et al., 2011a), which indeed encodes a functional JH receptor protein (Jindra et al., 2015b; Bittova et al., 2019).

The situation changed with the demonstration in 2007 that RNAi knockdown of the single *Met* ortholog in the beetle *Tribolium castaneum* triggered precocious metamorphosis (Konopova and Jindra, 2007), which finally produced a developmental phenotype that was clearly consistent with JH deficiency. Stimulated by that result and by the initial evidence of JH binding to a recombinant Met protein (Miura et al., 2005), the conclusive definition of a *bona fide* JH receptor followed a few years later (Charles et al., 2011; Jindra et al., 2015b). The gap separating the identification of the ecdysone and the JH receptors thus amounted to two frustrating decades. Relative to the great knowledge of molecular mechanisms of NR signaling, our grasp of JH receptor action is still rudimentary. However, owing to recent progress in several laboratories, some elementary features of JH signaling begin to emerge (Jindra et al., 2015a; Zhu and Noriega, 2016; Roy et al., 2018).

1.3.2 JH RECEPTOR ACTION

JH has set an unprecedented case of a hormone acting through a receptor of the bHLH-PAS protein family; all other known lipophilic animal hormones have intracellular receptors of the NR type. Besides Met, there are other bHLH-PAS proteins activated by low-molecular ligands, of which AhR is the best-studied example (Denison et al., 2011). Although not a true ortholog, AhR is a close relative to Met. While the ligand-sensing bHLH-PAS and NR

proteins are structurally different, both types have an analogous modular architecture with DNA-binding, ligand-binding, dimerization, and transcriptional activation domains (Mangelsdorf et al., 1995; Kewley et al., 2004). In the case of bHLH-PAS proteins, a bipartite DNA-binding domain assembles from the N-terminal basic regions of two interacting partners, such as AhR and ARNT (AhR nuclear translocator). Their dimerization is mediated by the HLH and each of the tandemly arranged A and B PAS domains of both AhR and ARNT; the ligand-binding function resides exclusively in the PAS-B domain of AhR (Figure 1.5). The action of NR and bHLH-PAS receptors follow a common logic: a small signaling molecule enters the cell, where it's binding to a specific pocket within the receptor stimulates the formation of a DNA-bound protein complex, capable of transcriptional activation of specific target genes. The above scheme essentially applies to the JH receptor (Jindra et al., 2015a; Zhu and Noriega, 2016; Roy et al., 2018).

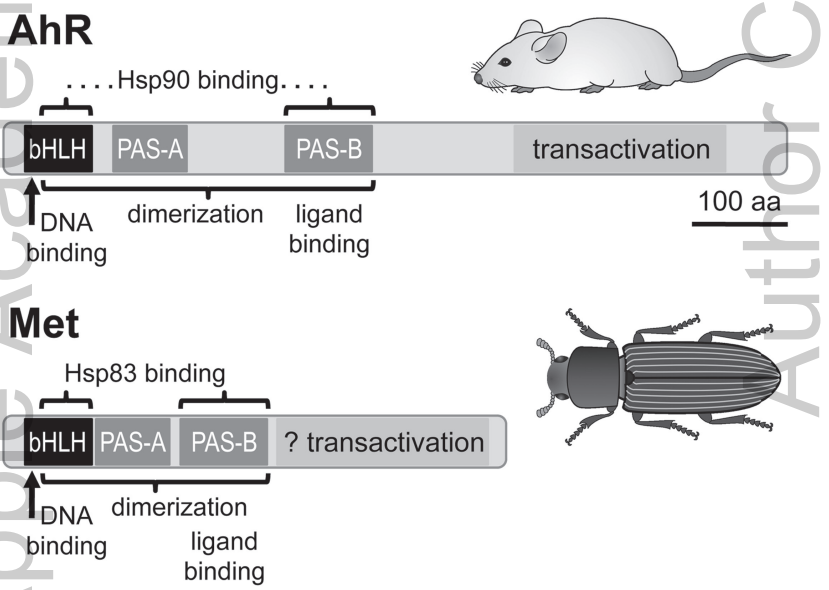

FIGURE 1.5 Functional domain organization of bHLH-PAS proteins Aryl hydrocarbon receptor (AhR) from mouse, and the JH receptor Met from *T. castaneum*.
The basic helix-loop-helix (bHLH) regions form a bipartite DNA-binding domain upon dimerization of AhR and Met with their bHLH-PAS partner proteins ARNT and Taiman, respectively. All the bHLH, PAS-A, and PAS-B domains engage in the dimerization; PAS-B does so in a ligand-dependent manner. Interaction with the chaperone Hsp90 (Hsp83 is a *D. melanogaster* ortholog) facilitates nuclear import. A Q-rich C-terminal domain which mediates transcriptional activation in AhR remains to be defined in insects. (Image by www.biographix.cz).

1.3.2.1 LIGAND BINDING

Using the dextran-coated charcoal assay, previously employed for the hemolymph JHBPs (Touhara et al., 1993), Miura and colleagues initially reported a K_d value of 5.3 nM for binding of [^{3}H]-JH III to the *D. melanogaster* Met (DmMet) protein (Table 1.2), translated in the rabbit reticulocyte lysate (Miura et al., 2005). However, their experiment was difficult to repeat, either when DmMet was translated in the rabbit reticulocyte lysate (Charles et al., 2011) or expressed using baculovirus in insect Sf9 cells (M. Jindra, W. J. McKinstry, T. Nebl, R. J. Hill, unpublished data). A recent study based on ligand-activated receptor dimerization (Miyakawa et al., 2017) indeed suggests that of the two *D. melanogaster* JH receptors, DmMet is the weaker one. In contrast, the single *T. castaneum* Met and the ancestral *D. melanogaster* paralog Gce both have proven robust JH III binders for which K_d of 2.9 nM and 19.3 nM, respectively, were determined (Charles et al., 2011; Jindra et al., 2015b; Bittova et al., 2019). High-affinity (4.4 nM K_d) binding to JH III was also reported for *A. aegypti* Met (Li et al., 2014). Affinities thus far determined for binding of recombinant JH receptor proteins to native insect JHs are summarized in Table 1.2. The specific JH III binding has been confirmed for *T. castaneum* and *A. aegypti* Met proteins expressed from baculoviral constructs and purified from the Sf9 cells (M. Jindra, W. J. McKinstry, T. Nebl, R. J. Hill, unpublished data).

In vitro assays with truncated proteins enabled localization of the JH-binding activity to the PAS-B domain of *T. castaneum* Met (Charles et al., 2011), which corresponded to the site of ligand interaction in AhR (Figure 1.5). Because the PAS-B domain is to some extent conserved among bHLH-PAS proteins, a structural model of *T. castaneum* Met PAS-B could be developed (Charles et al., 2011) based on homology to the Hypoxia-inducible factor 2α (HIF2α) whose crystal structure had been resolved (Scheuermann et al., 2009). The modeling and ligand docking highlighted amino acid residues lining a putative hydrophobic JH-binding pocket. When individually replaced with bulkier side chains of similar chemical nature, these mutations prevented *T. castaneum* Met from binding JH III, thereby establishing the specificity of the receptor-hormone interaction (Charles et al., 2011). This study was followed by modeling and mutagenesis of Met from *A. aegypti* (Li et al., 2014), essentially arriving to a common set of about ten highly conserved residues within the ligand-binding pocket, which are required for JH binding. Importantly, mutations of some of these critical amino acids in the *D. melanogaster* receptors Gce and Met rendered either

TABLE 1.2 Characteristics of Selected[a] Insect bHLH-PAS Intracellular JH Receptors

Protein	Species	Accession No.	Size (kDa)	JH Bound	Affinity (nM)	References
Met	*D. melanogaster*	NP_511126.2	79	10*R, S*-JH III	5.3[c]	Miura et al., 2005
			79	10*R, S*-JH III	ND	Charles et al., 2011
Gce	*D. melanogaster*	NP_511160.1	77[b]	10*R, S*-JH III	ND	Charles et al., 2011
Gce	*D. melanogaster*	NP_511160.1	77[b]	10*R, S*-JH III	19.3[c]	Jindra et al., 2015b
				10*R, S*-JH III	11.0[d]	Bittova et al., 2019
				10*R*, 11*S*-JH I	13.8[d]	
				JHB3	83.3[d]	
				MF	89.8[d]	
Met	*T. castaneum*	NP_001092812.1	58	10*R, S*-JH III	2.9[c]	Charles et al., 2011
Met	*A. aegypti*	AAX55681.1	105	10*R, S*-JH III	4.4[c]	Li et al., 2014

[a] Recombinant proteins with determined JH-binding affinities are listed.
[b] Size of initially annotated protein (NP_511160.1); size of full-length Gce (NP_511160.2) is 106 kDa.
[c] Determined as K_d from direct binding of racemic [^{3}H]-10*R, S*-JH III.
[d] Determined as K_i from competition against [^{3}H]-10*R, S*-JH III.
ND, not determined.

protein incapable of responding to JH *in vivo* and insufficient to support the normal development of the fly (Jindra et al., 2015b). This genetic evidence has unequivocally proven that the JH-binding capacity is essential for both Met and Gce to function as JH receptors. Recent data by Bittova et al., (2019) have shown that at least Gce binds all three of the circulating native hormones in *D. melanogaster* (i.e., JH III, JHB3, and MF) (Table 1.2), and that it prefers the native (10*R*) enantiomer of JH III. The ligand binding by Gce is also highly sensitive to the correct geometrical isomerism of the JH skeleton (Bittova et al., 2019) (Figure 1.6). Thus, the JH receptor shows an exquisite stereoselectivity towards its native hormonal agonists.

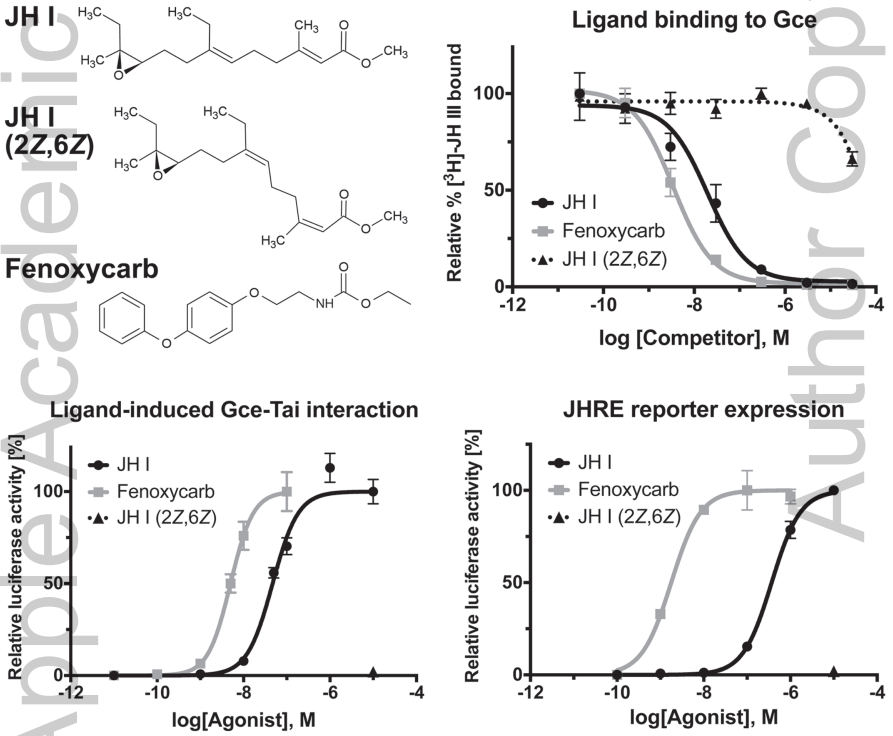

FIGURE 1.6 Comparison of agonist activities between JH I [the natural 10*R*, 11*S*-(2*E*, 6*E*) configuration], its inactive 10*R*, 11*S*-(2*Z*, 6*Z*) geometric isomer, and a super-active JH mimic fenoxycarb.

The three compounds were tested for their ability to compete with radiolabeled JH III for binding to the Gce protein *in vitro* (top right), to stimulate Gce-Tai interaction in a two-hybrid assay in the HEK293 mammalian cells (bottom left), and to transcriptionally activate a JHRE1-driven reporter in the *D. melanogaster* S2 cells (Bittova et al., 2019).

There are numerous synthetic compounds that exert JH-like effects on insects (Slama et al., 1974; Parthasarathy et al., 2012). Some of these JH mimics, including methoprene, pyriproxyfen, or fenoxycarb, are widely used as insecticides. It is therefore important to know whether these compounds, in spite of their diverse chemistries, activate the Met/Gce receptors as true agonists. Indeed, the few synthetic JH mimics tested thus far competed against the native JH III hormone for binding to the *D. melanogaster* and *T. castaneum* receptor proteins (Charles et al., 2011; Jindra et al., 2015b; Bittova et al., 2019; Jindra and Bittova, 2019). For the potent pyridine derivative pyriproxyfen or the carbamate fenoxycarb (Figure 1.6), the binding affinity exceeded that of JH III itself (Bittova et al., 2019). The entire repertoire of Met/Gce agonists is yet to be explored, and attempts have begun to identify JH antagonists that might inhibit the JH receptor function. A few such compounds have been found in plant extracts based on their ability to interfere with the assembly of the JH receptor complex (Lee et al., 2015; Shin et al., 2018) but whether these plant compounds bind Met/Gce and whether they compete with JH for the receptor has not been reported.

1.3.2.2 INTERACTING PROTEINS

To form active transcription factor complexes, bHLH-PAS proteins of class I dimerize with bHLH-PAS proteins of class II (Kewley et al., 2004). Examples of such heterodimers include AhR and its class II partner ARNT, or the circadian proteins Clock and its class II partner BMAL (called Cycle in insects). The interaction between AhR and ARNT is induced by the binding of ligands to AhR (Denison et al., 2011). The JH receptor Met/Gce fits the role of a class I, ligand-activated monomer, which is required to combine with another bHLH-PAS protein (Jindra et al., 2015a). So far, two bHLH-PAS members, Taiman, and Cycle have been shown to interact with Met/Gce proteins in a JH-dependent manner.

Taiman (also called SRC) is an ortholog of the steroid receptor coactivator SRC-1 (aka NCoA-1 or p160). In insects it was originally described as a transcriptional coactivator of EcR in *D. melanogaster* (Bai et al., 2000) and later, under the name FISC, in *A. aegypti* as a coactivator to Ftz-F1, another NR in ecdysone signaling (Zhu et al., 2006). In keeping with current nomenclature (Roy et al., 2018; Santos et al., 2019), we encourage the use of the original name Taiman (Tai). The JH-induced association between Met and Tai was reported in various two-hybrid systems, initially for the *A. aegypti* proteins (Li et al., 2011) and then for their *T. castaneum* (Zhang et

al., 2011) and *B. mori* (Kayukawa et al., 2012) orthologs. Recent data have confirmed JH-dependent binding also between the *D. melanogaster* Tai and either Met or Gce proteins (Miyakawa et al., 2017), which could be achieved with JH I, JH III, JHB3, or MF (Bittova et al., 2019). Chemically diverse JH mimics such as fenoxycarb could substitute for the native JHs in this assay and even exceed their activity (Figure 1.6).

Co-immunoprecipitation of *T. castaneum* Tai with normal and mutated Met variants has demonstrated that the JH-binding capacity of Met is indeed necessary for JH to stimulate the interaction (Charles et al., 2011), and a two-hybrid assay has confirmed this to be the case for the *A. aegypti* Met and Tai proteins as well (Li et al., 2014). Tai itself neither binds JH nor is required for the hormone-binding activity of Met (Charles et al., 2011). The above studies simply presume that, by analogy with other bHLH-PAS proteins, the Met-Tai complexes are dimers. This 1:1 stoichiometry has been conclusively determined by size-exclusion chromatography combined with multi-angle laser-light scattering once the *T. castaneum* and *A. aegypti* JH receptor proteins were expressed and their complexes purified from insect cells (M. Jindra, W. J. McKinstry, T. Nebl, R. J. Hill, unpublished data).

The circadian clock protein Cycle was shown to bind Met in a yeast two-hybrid screen among *A. aegypti* bHLH-PAS proteins (Shin et al., 2012). The interaction required JH and implicated the Met-Cycle complex in the daily rhythmic regulation of certain JH-response genes, which are important to prepare the mosquito female for oogenesis. The study also identified *A. aegypti* Tango, the insect ortholog of ARNT, as a protein interacting with Met regardless whether or not JH was present (Shin et al., 2012). Co-immunoprecipitation revealed similar, JH-independent binding between Met and Tango from *T. castaneum* (M. Jindra and J. Rynes, unpublished data).

The last presently known bHLH-PAS partner of Met is Met itself. Contrary to the binding of Tai, Cycle, or Tango, this homophilic interaction was observed in the absence of JH, originally for DmMet and its paralog Gce (Godlewski et al., 2006). As detected by co-immunoprecipitation in insect cells, the formation of either DmMet-Gce or DmMet-DmMet complexes was reduced by the addition of JH III or methoprene. The same effect was found for the *T. castaneum* Met protein (Charles et al., 2011). The latter study also showed that the dissociation of the Met-Met complex indeed required JH binding, as a complex formed by the mutated Met variants, incapable of binding JH, persisted in the presence of JH III or methoprene. Moreover, the ligand-binding PAS-B domain of *T. castaneum* Met was dispensable for the JH-inhibited Met-Met interaction, whereas it was required for the

JH-dependent Met binding to Tai (Charles et al., 2011). These results suggest that Met might occur in an "inactive homodimer" form until binding of JH makes it accessible to partners such as Tai or Cycle (Jindra et al., 2013).

Effects of JH and ecdysone are intimately linked and can be antagonistic depending on the context (e.g., Wu et al., 2006; Riddiford, 2012; Liu et al., 2018b). However, it is unclear whether the two hormonal signals are mutually modulated at the receptor level. It is therefore of interest that DmMet has been shown to interact with some NRs constituting the ecdysone signaling pathway, namely both of the subunits of the functional 20E receptor, the EcR and Usp proteins (Bitra and Palli, 2009). Another study has indicated that both DmMet and Gce bind Ftz-F1 (Bernardo and Dubrovsky, 2012). All of these protein-protein interactions appear to be independent of JH, and their biological significance is as yet unknown. Being both a functional component of the JH receptor and one of EcR coactivators, Tai is another attractive point where the JH and 20E pathways likely intercept.

1.3.2.3 SUBCELLULAR LOCALIZATION

While DmMet (Pursley et al., 2000) and Gce (Jindra et al., 2015b) were found in the nuclei of *D. melanogaster* tissues or cultured S2 cells regardless of JH presence or absence, others have found that DmMet resided in the cytoplasm in the fat body of larvae experiencing low JH titer, and in the nuclei when JH titer was high (He et al., 2014, 2017). Nuclear localization of DmMet could also be induced by the addition of methoprene. The authors further showed that the translocation and subsequent activation of a JH-response gene required direct interaction between DmMet and the chaperon Heat shock protein 83 (Hsp83, a *D. melanogaster* ortholog of the vertebrate Hsp90) (He et al., 2014). DmMet contacts Hsp83 via the HLH and PAS-B domains, corresponding to the regions in AhR that are responsible for binding Hsp90 (Figure 1.5). The nuclear import of DmMet depends on the conserved importin β pathway and involves binding of Hsp83 to a component of the nuclear pore complex, a nucleoporin Nup358 (He et al., 2017). This mechanism resembles the nuclear import of the AhR-Hsp90 complex, which also relies on importin β and is induced by activating ligands (Denison et al., 2011). One difference in the model proposed for DmMet (He et al., 2014, 2017) is that Hsp83 is considered to take part in the DmMet transcription-activating complex, whereas AhR dissociates from Hsp90 and instead combines with ARNT upon transport to the nucleus.

In an attempt to identify functional nuclear export and nuclear localization signals (NES and NLS, respectively) within the *D. melanogaster* JH receptors, YFP-tagged DmMet and Gce proteins were expressed in mammalian cell lines (Greb-Markiewicz et al., 2011, 2015). By monitoring the localization of a set of systematic deletion and point mutation variants of the proteins, the authors delimited two specific and one putative NES, respectively, to the PAS-A, PAS-B, and the C-terminal region of each DmMet and Gce. Both receptors contain two NLS motifs, such that one NLS resides in each of the two PAS domains of DmMet, whereas Gce harbors one NLS in its ligand-binding PAS-B, and the other in the C-terminal region. The presence of an NLS in the C terminus of Gce is intriguing, as this non-conserved region is intrinsically disordered and thus may be prone to post-translational modifications (Kolonko et al., 2016).

1.3.2.4 JHRE DNA BINDING

An active JH-receptor complex is expected to bind specific *cis*-acting JH response elements (JHREs) and through them, regulate target genes. A JHRE for the Met-Tai dimer was found in the enhancer region of the *A. aegypti early trypsin* (*ET*) gene (Li et al., 2011). The element JHRE1 (CCA**CAC-GCG**AAG) contained an imperfect palindrome (in bold) resembling binding sites (E-boxes) of some bHLH-PAS proteins. Chromatin immunoprecipitation together with electrophoretic mobility shift assays with nuclear extracts from female mosquitoes experiencing high JH titer revealed occupancy of the *ET* enhancer by the Met and Tai proteins, which activated a luciferase reporter with multiple copies of JHRE1 in response to JH III (Li et al., 2011). A JHRE1-driven reporter was also strongly activated by native JHs and synthetic JH agonists through the endogenous Gce and Tai proteins in the *D. melanogaster* S2 cells (Jindra et al., 2015b) (Figure 1.6). Systematic analyses of another direct JH response gene, *Krüppel-homolog 1* (*Kr-h1*), from *B. mori* (Kayukawa et al., 2012) and *T. castaneum* (Kayukawa et al., 2013) led to the identification of a functional *k*JHRE core (CCTC**CACGTG**) with an E-box-like palindrome (in bold). Independently, functional JHREs with both the perfect and imperfect palindromes were found upstream of the *A. aegypti Kr-h1* gene (Shin et al., 2012; Cui et al., 2014); the latter was bound by the Met-Cycle complex rather than by Met-Tai (Shin et al., 2012). Genomic analyses later identified consensus JHRE sequences in a number of JH/Met-regulated mosquito genes (Zou et al., 2013; Saha et al., 2016).

Li et al. (2014) performed an unbiased selection of oligonucleotides bound by a complex of bacterially expressed *A. aegypti* Met and Tai proteins, essentially arriving to the already known consensus with the CACGTG palindrome. A double-stranded DNA probe carrying this sequence bound the Met-Tai complex with the highest achieved affinity (5.8 nM K_d) relative to a 103.0 nM K_d measured for JHRE1 (Li et al., 2014). This important study has firmly established that the basic regions of both Met and Tai are required to contribute to the DNA-binding function, which itself is, however, independent of the JH ligand. Experiments with *T. castaneum* and *A. aegypti* Met and Tai proteins purified from the Sf9 cells confirmed that while JH stimulates the formation of the receptor dimers, it is dispensable for their further interaction with the JHREs (M. Jindra, W. J. McKinstry, T. Nebl, R. J. Hill, unpublished data).

1.3.3 DOWNSTREAM JH SIGNALING

Attempts to identify JH-response genes generally aimed at processes best known to depend on JH, i.e., metamorphosis, and vitellogenesis, and these attempts predate finding of the intracellular JH receptor. We will describe JH target genes before discussing their roles in development and reproduction. In this section, we will also consider the modulation of JH signaling by second-messenger pathways.

1.3.3.1 Direct and Indirect Target Genes

Three transcription factor genes, *Broad-Complex* (*Br-C*) (Bayer et al., 1996), *Kr-h1* (Pecasse et al., 2000), and *E93* (Baehrecke and Thummel, 1995), were all originally characterized as 20E-response genes involved in the regulation of *D. melanogaster* metamorphosis. Seminal work from the Riddiford laboratory uncovered that the critical requirement of *Br-C* (Zhou and Riddiford, 2002) and *Kr-h1* (Minakuchi et al., 2008b) in metamorphosis is linked to JH signaling. Using complementary insect models, the beetle *T. castaneum* and the cockroach *B. germanica*, others later discovered that *E93* is an essential, JH-regulated driver of adult development, which interacts with *Br-C* and *Kr-h1* (Ureña et al., 2014, 2016; Bellés and Santos, 2014; Kayukawa et al., 2017). All of these three genes are now known to orchestrate the pupal and adult developmental programs (see Section 1.4.1). While *Kr-h1* is a direct target of the JH receptor Met (Kayukawa et al., 2012; 2013; Cui et al., 2014),

Br-C, and *E93* are regulated by JH indirectly, via Kr-h1-mediated transcriptional repression (Kayukawa et al., 2016; Ureña et al., 2016; Kayukawa et al., 2017) (Figure 1.7).

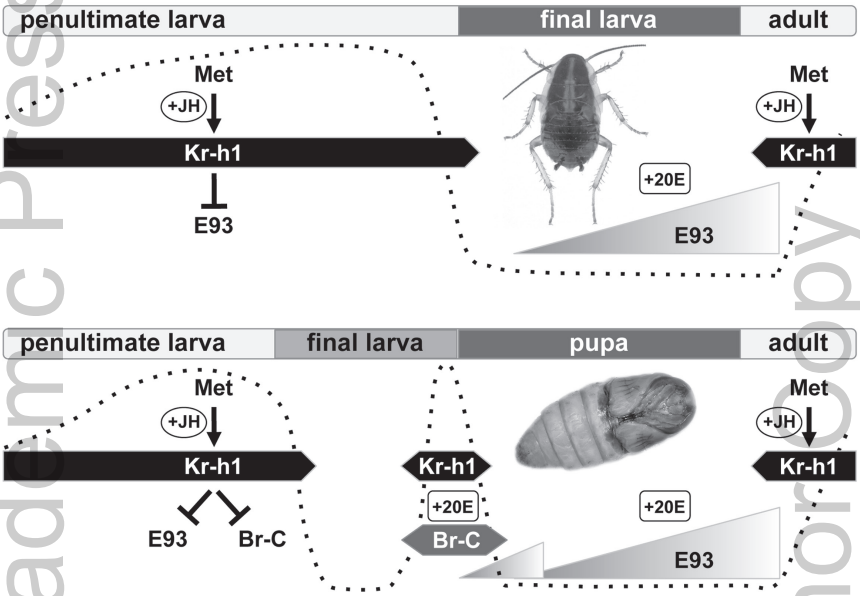

FIGURE 1.7 A schematic temporal diagram of gene expression activities that regulate hemimetabolan (top) and holometabolan (bottom) metamorphosis in response to JH and 20E. Kr-h1 **is** a JH/Met-induced keeper of the larval state, and E93 specifies adult development in the absence of JH and Kr-h1 expression during the final juvenile stage in both types of metamorphosis. Br-C has a unique role in pupa formation in holometaboly. The intermittent prepupal pulse of Kr-h1 and Br-C prevents precocious adult development in holometabolans. The dotted line shows an approximate trend of JH titer.

Induction of the "canonical" 20E early-response gene *E75A* by 20E was found to be directly enhanced by JH (Jindra et al., 1996; Zhou et al., 1998; Dubrovsky et al., 2004). However, the significance of this regulation has not been clarified. Similarly, many genes identified as putative JH targets in the transcriptome (Beckstead et al., 2007; Zhu et al., 2010; Zhang et al., 2011) or genetic (Abdou et al., 2011b) screens remain to be connected with JH signaling.

In insects whose vitellogenesis directly depends on JH, primarily in orthopterans, cockroaches, and some hemipterans, vitellogenin (*Vg*) genes encoding yolk protein precursors had been traditionally studied as JH-activated targets (reviewed in Wyatt and Davey, 1996; Raikhel et al., 2005; Roy

et al., 2018; Santos et al., 2019). More recent RNAi knockdown studies in the representatives of these three insect orders have shown that *Vg* expression in the fat body indeed requires Met (Smykal et al., 2014a; Marchal et al., 2014; Guo et al., 2014) and its partner Tai (Smykal et al., 2014a; Guo et al., 2014; Wang et al., 2017c). Some authors have also implicated *Kr-h1* in *Vg* induction (Song et al., 2014; Yue et al., 2018). However, *Vg* transcription is not directly activated by the JH-receptor complex. A model developed in the migratory locust (*Locusta migratoria*) instead suggests that the JH receptor directly induces transcription of genes *Mcm4*, *Mcm7*, and *Cdc6* that all promote polyploidization in the fat body, which presumably aids the massive *Vg* transcription (Guo et al., 2014; Wu et al., 2016). Enhancers of *Mcm4*, *Mcm7*, and *Cdc6* genes contain JHREs bound by the Met-Tai complex. Another direct target of Met in *L. migratoria* encodes a chaperone Grp78-2, which facilitates proper folding of the Vg protein (Luo et al., 2017).

In the anautogenous *A. aegypti* mosquito females, where JH does not directly induce vitellogenesis but rather primes the female for egg production after the blood meal, *ET* was among the first JH-response genes identified (Noriega et al., 1997; Zhu et al., 2010). As already mentioned above, the transcription of *ET* is directly activated by binding of the JH/Met-Tai complex to its JHRE (Li et al., 2011; Li et al., 2014). Additional genes, activated by Met in response to JH in *A. aegypti* females, encode ribosomal proteins and the regulator of ribosome synthesis 1 (RRS1) (Wang et al., 2017b). At least *RRS1* is a direct target containing a JHRE motif. Regulation of ribosome biogenesis genes reflects the role JH plays in preparing the mosquito female for massive Vg protein synthesis. More recently, JH has been shown to regulate Tai expression at the level of alternative splicing (Liu et al., 2018a). Of four Tai isoforms occurring in *A. aegypti*, JH stimulates production of those two that preferentially interact with EcR to facilitate the vitellogenic response to 20E in the mosquito females.

The JH response was further investigated at the transcriptome level in *A. aegypti* females undergoing post-eclosion, previtellogenic development. Microarray and RNA sequencing data from the Raikhel laboratory uncovered substantial portions of the mosquito genome to be differentially regulated in response to the changing JH titer during this 3-day period (Zou et al., 2013; Saha et al., 2016). It is worth noting that many genes activated in a JH/Met-dependent manner contain consensus JHREs in their upstream regions, whereas most genes repressed through Met do not, suggesting that the JH receptor activates transcriptional repressors (Zou et al., 2013). One of these is Kr-h1 (Kayukawa et al., 2016; Ojani et al., 2018). Another is Hairy,

which forms a complex with a corepressor Groucho (Saha et al., 2016). The *A. aegypti hairy* gene itself is a direct target of JH/Met activation.

1.3.3.2 AN ALTERNATIVE PATHWAY AND POST-TRANSLATIONAL REGULATION

It has always been thought that similar to some steroid hormones, JH also may have a non-genomic site of action at the cell membrane (Wyatt and Davey, 1996). Early genetic evidence showed that JH III, acting at subnanomolar concentrations, stimulated protein synthesis in the *D. melanogaster* male AG in a Ca^{2+} and protein kinase C (PKC) dependent manner (Yamamoto et al., 1988). When the intracellular JH receptor Met became known decades later, work from the Zhu laboratory has revealed that JH III indeed triggers a Ca^{2+} and phospholipase C (PLC) mediated response in *A. aegypti* cells and fat body (Liu et al., 2015). Based partly on inhibitor treatments and partly on RNAi, the response was ascribed to both Ca^{2+}/calmodulin dependent kinase II (CaMKII) and PKC (Liu et al., 2015; Ojani et al., 2016). A membrane receptor for JH III has been postulated based on these experiments, but such a receptor still remains to be found. Surprisingly, this kinase-dependent branch of JH signaling culminates by phosphorylation of both Met and Tai subunits of the intracellular JH receptor, and stimulates their interaction with DNA. It thereby enhances the transcriptional activation of the primary JH-response genes such as *ET* or *Kr-h1* (Liu et al., 2015; Ojani et al., 2016). Thus, quite paradoxically, while adding a potentially important level of regulation, this membrane-initiated pathway has not yet uncovered novel targets of JH signaling. Mass spectrometry on *T. castaneum* and *A. aegypti* Met proteins purified from the Sf9 cells has putatively identified multiple phosphorylation sites in non-conserved, intrinsically disordered regions of JH receptors from both species (M. Jindra, W. J. McKinstry, T. Nebl, R. J. Hill, unpublished data).

1.4 ROLES OF JH IN DEVELOPMENT AND REPRODUCTION

It is not possible to discuss here all the effects JH has been found to exert on various processes and consider how these effects can differ depending on the species at hand. It would probably be easier to list what JH does not influence. Excellent reviews and books have covered the mass of knowledge accrued on the multitude of roles JH plays in insect tissues during juvenile

stages, in the adult life, particularly reproduction, and overall in growth, development, polyphenism, and behavior of diverse insect species (Nijhout, 1994; Riddiford, 1994; Wyatt and Davey, 1996; Hartfelder and Emlen, 2012). Given the vastly pleiotropic and species-specific JH effects, it is often difficult to discern between genuine response to the hormone and secondary consequences that follow. We will therefore mainly discuss those functions of JH where there is molecular support for the involvement of identified JH signaling pathway components.

1.4.1 GROWTH AND DEVELOPMENT

Progressing through the molts and successive instars allow insects to grow and finally develop to reproductive adults. Allocating resources to growth and reproduction involves critical nutrition-dependent decisions. JH is an important component of a signal transduction mechanism that connects changes in the nutritional status with activation of specific physiological events during development and reproduction. JH is part of a nutrient-sensing system that includes both insulin/TOR and ecdysone signaling. It is the fine-tuning of mutual interactions among these pathways what permits growth while nutrients are available, and at the same time triggers developmental transitions, particularly metamorphosis to reproductively competent adults, once the nutritional and size thresholds have been reached. The endocrine control of body size has been comprehensively reviewed (Nijhout et al., 2014). We will, therefore, focus on the role of JH and its receptor.

In general, JH promotes growth in larvae. Genetic ablation of the CA in *D. melanogaster* caused smaller body size at pupariation but also a slight delay of pupariation; both effects could be averted by supplementing a JH mimic (Riddiford et al., 2010). A follow-up in-depth study (Mirth et al., 2014) has shown that JH deficiency does not affect the critical weight required for pupariation but rather increases the time needed before the critical weight is attained. In other words, JH deficiency slows the growth rate, and it appears to do so throughout the three *D. melanogaster* larval instars. Feeding pyriproxyfen could partly restore the growth rate of the CA-deprived larvae, although it could not enhance the growth of normal larvae. The study further revealed that depletion of JH attenuates insulin/insulin-like signaling (IIS) and that FOXO is responsible for the reduced body size. 20E, which limits larval growth by antagonizing IIS (Colombani et al., 2005), was elevated in larvae lacking the CA (Mirth et al., 2014). The authors have therefore proposed that JH normally maintains the growth-promoting activity of IIS

indirectly, via reducing ecdysone production in the PG. Their RNAi data have implicated DmMet in this PG-specific role of JH (Mirth et al., 2014) although Gce is expressed in the steroidogenic gland as well (Baumann et al., 2017).

A recent study (Liu et al., 2018b) has demonstrated that JH signaling through Gce, DmMet, and Kr-h1 indeed inhibits ecdysone synthesis in the PG without affecting ILP synthesis in the brain. Removal of either the JH receptors or Kr-h1 from the PG led to abnormally high steroid titer and to an earlier onset of pupariation. Conversely, overexpression of Kr-h1 in the PG blocked the ecdysone pulse and pupariation altogether, and this phenotype could be remedied with dietary 20E (Liu et al., 2018b; Zhang et al., 2018). The negative effect of JH and Kr-h1 on ecdysone synthesis was recently corroborated in the PGs explanted from *D. melanogaster* and *B. mori* (Zhang et al., 2018). These authors implicated Kr-h1 as transcriptional repressor acting directly on the promoters of the key steroidogenic genes. Finally, Liu et al., (2018b) showed that 20E, acting on its receptor EcR in the CA, negatively regulated the synthesis of JH. In summary, these data have suggested how the two systemic hormonal signals act in mutual antagonism to set the proper time limit to feeding and growth, and to initiate metamorphosis.

An important role of JH in adapting to starvation has been demonstrated through experiments on *M. sexta* (Truman et al., 2006; Suzuki et al., 2013). When last-instar larvae experience an episode of starvation prior to attaining the critical weight, then once given food again they prolong the feeding duration, proportionally to the time spent fasting, to surpass the critical weight before initiating metamorphosis. However, larvae surgically deprived of the CA lack this compensatory feeding and instead start the wandering behavior after a constant amount of time, irrespective of size attained after re-feeding (Suzuki et al., 2013). Naturally, in severely undersize animals, this leads to a failure to develop. It is intriguing that JH-deficient larvae lose the sense of their own size and instead metamorphose based on a default timer. Another type of JH-dependent response to starvation could be observed in the imaginal primordia and wing discs of *M. sexta* larvae (Truman et al., 2006). Under starvation, the proliferative growth of these primordia in the final instar ceases. However, the growth continues regardless of starvation in larvae deprived of JH, suggesting that JH is necessary to limit the disproportional growth of imaginal tissues under a nutrient shortage. The molecular basis of JH action in these nutritional decisions remains to be defined.

1.4.1.1 EMBRYOGENESIS

Whether endogenous JH plays a role during embryogenesis remains a matter of debate. Part of the problem is the difficulty to obtain JH-free embryos where the impact of JH deficiency could be assessed. Functional JH signaling is essential not only to complete adult development but typically also to produce progeny. Therefore, it is difficult to obtain embryos lacking not only zygotically expressed but also maternally contributed JH receptors. Such an experiment is feasible in *D. melanogaster*, where germline clones simultaneously mutant for *Met* and *gce*, both X-linked genes, need to be generated.

So far the most conclusive genetic evidence for a dispensable role of embryonic JH has been achieved in *B. mori*. The silkworm is exceptional as it does not require JH to reproduce. Therefore, a *dimolting* (*mod*) mutant strain that lacks the CYP15C1 epoxidase, and thus, any epoxidated JH, can be maintained as a homozygous, fertile stock (Daimon et al., 2012). The *mod* adults are miniature due to precocious pupation of the mutant larvae after only three or four, rather than the normal five instars. Yet the absence of JH has no appreciable effect on embryogenesis and early larval development (Daimon et al., 2012). Although FA epoxidation by CYP15C1 precedes methylation by JHAMT in the Lepidoptera (see Section 1.2.1.3), it is theoretically possible that MF might substitute for the lack of epoxidated JH in the *mod* strain. However, Daimon and colleagues have employed the gene-editing TALEN technique to generate null mutants also for *JHAMT* and for both of the *B. mori* JH receptor genes, *Met1* and *Met2* (Daimon et al., 2015). Prominent expression of *JHAMT* mRNA during the latter half of *B. mori* embryogenesis tightly coincides with *Kr-h1* expression, indicating a JH peak produced by the embryonic CA between days 5 and 10 (hatching). As expected, embryos homozygous for a *JHAMT*-null mutation lost *Kr-h1* expression; however, they still formed normal larvae. The only anomaly resulting from JH depletion was difficulty to hatch, which could be mitigated either by JH application or simply by breaking the eggshells (Daimon et al., 2015). Why the depletion of JH impairs hatching is not clear. Double mutants deficient in both JHAMT and the CYP15C1 epoxidase displayed the same phenotype, and also embryos lacking both Met1 and Met2 formed morphologically normal larvae.

These results suggest that neither JH nor its receptors play any role in embryonic development of holometabolous insects, at least in *B. mori*. The lack of essential JH role in embryos is also indicated in *D. melanogaster* by the fact that neither genetic ablation of the CA nor the complete zygotic loss

of *Met* and *gce* in double-mutant, hemizygous male progeny, kill the animals no earlier than before the outset of metamorphosis (Riddiford et al., 2010; Abdou et al., 2011a).

The situation might be different in hemimetabolous embryos where, unlike in holometabolans, application of JH mimics has been known to accelerate the appearance of postembryonic characters (reviewed in Truman and Riddiford, 2007). Hemimetabolous species such as *B. germanica* also display a major JH surge during the second half of embryogenesis, which is accompanied by a strong *Kr-h1* upregulation (Fernandez-Nicolas and Bellés, 2017); a similar *Kr-h1* mRNA peak occurs in embryos of the linden bug, *Pyrrhocoris apterus* (Konopova et al., 2011). The facility of systemic maternal RNAi in these species provides a tool for probing the potential role of JH signaling in embryogenesis. However, in agreement with the essential role of JH in vitellogenesis, *P. apterus* females produced no eggs upon *Met* knockdown (Smykal et al., 2014a). Surprisingly, maternal *Kr-h1* RNAi had no effect on embryogenesis (Smykal et al., 2014b). The issue was recently revisited in *B. germanica* with maternal knockdown of *JHAMT*, *Met*, and *Kr-h1* (Fernandez-Nicolas and Bellés, 2017). The results have shown a collection of mostly low-penetrance phenotypes at various phases of embryogenesis, which are difficult to reconcile with perturbed JH signaling. Perhaps the most compelling defect, observed for all three tested genes, was premature tanning of the cuticle, which correlated with laccase 2 upregulation (Fernandez-Nicolas and Bellés, 2017). There also was a reduced hatching rate, reminiscent of the situation seen in *JHAMT*$^{-/-}$ *B. mori* embryos. The study, therefore, supports a view that JH may have an auxiliary effect on the embryo fitness rather than an essential developmental function, even in hemimetabolous insects.

Clearly, more research is needed to resolve the role of JH in embryogenesis. Future studies should be cautious in interpreting the effects of knocking down players such as Kr-h1 as being equal to blocking JH signaling. Ancient transcription factors such as Kr-h1 are likely to have JH-independent functions. An example has been provided in the crustacean *Daphnia pulex*, where *Kr-h1* does not seem to be regulated by JH, and a developmental arrest upon *Kr-h1* knockdown occurs much earlier during embryogenesis than arrest induced by *Met* RNAi (Miyakawa et al., 2018).

1.4.1.2 MAINTAINING THE LARVAL STATUS

Maintenance of the juvenile status until larvae have attained a size appropriate for metamorphosis is the nominal function of JH. However, when

precisely this role of JH begins, is not clear. The traditional view is that larvae of both hemimetabolous and holometabolous species need JH protection from premature activation of adult morphogenesis from the moment they hatch (Wigglesworth, 1954). This view was supported by parabiosis or transplantation experiments where epidermis as young as from first-instar larvae (L1) could metamorphose when exposed to a complex milieu of a final-instar host, not just to JH depletion. For example, L1 epidermis from the *G. mellonella* wax moth, implanted into a final-instar larva, produced pupal rather than larval cuticle when it experienced pupation of the host (Piepho, 1938).

However, the paradigm was challenged by unsuccessful attempts to induce early metamorphosis in whole animals by surgical allatectomy of early-instar *B. mori* larvae (Bounhiol, 1938; Fukuda, 1944), by chemical allatectomy in locust embryos (Aboulafia-Baginsky et al., 1984), or by overexpression of JHE (Tan et al., 2005). In all cases the treated animals did not manifest premature metamorphic development before they have completed at least two larval instars (summarized in Feyereisen and Jindra, 2012). A strong case against an early role of JH came from the CYP15C1-deficient *B. mori mod* mutants, which pupate after two or three molts, i.e., at the end of the L3 or L4 instars, even though they lack epoxidated JH during the entire life cycle (Daimon et al., 2012). Remarkably, a single dose of methoprene restored the normal number of five larval instars in the *mod* homozygotes.

The controversy between the old tenet and the new genetic evidence inspired experiments comparing *B. mori* with the hemimetabolous model *P. apterus*, which develops through five larval instars before molting to adult (Smykal et al., 2014b). After subjecting each of the four pre-final instars of this linden bug to *Met* or *Kr-h1* RNAi, the authors concluded that the propensity to develop precocious adult character, evident as advanced wings, genitals, and adult pigmentation, was greatest at L4, modest at L3, and nil at L2 and L1. They proposed that insects require a minimum of two larval instars, or molts, before gaining competence to metamorphose – only then the role of JH in safeguarding the juvenile status becomes critical (Smykal et al., 2014b).

This idea was fully verified when Daimon et al. (2015) presented TALEN-generated *B. mori* mutants lacking JHAMT or the JH receptors Met1 and Met2, of which Met2 seems to play no role in larvae. Both JHAMT and Met1 KO larvae developed pupal characters, but again no earlier than at L3, confirming that the two earliest instars are larval by

default, independently of JH signaling. Consistently, neither the early *P. apterus* nor *B. mori* larvae deficient in JH signaling initiated metamorphosis, even though the JH-inducible *Kr-h1* gene was severely underexpressed in them (Smykal et al., 2014b; Daimon et al., 2015). *Kr-h1* encodes a critical repressor of metamorphosis and its removal at later instars very efficiently triggers precocious adult development (Minakuchi et al., 2009; Konopova et al., 2011; Lozano and Bellés, 2011; Smykal et al., 2014b). Interestingly, JHAMT/JH deficiency resulted in premature pupation, whereas loss of *Met1* often retarded larval growth or caused arrest at the L2-L3 molt. Surviving *Met1*$^{-/-}$ L3 larvae displayed patches of heterochronic pupal cuticle, and genetic mosaic analyses showed that as much as half of a larva bearing *Met1*$^{-/-}$ cells can become pupally committed (Daimon et al., 2015). The power of these genetic data is that they leave no room for speculations about residual hormone after allatectomy or residual protein after RNAi.

Not satisfied with the above answers, Inui and Daimon (2017) revisited the 80-year old Piepho's implantation experiments using *B. mori* and its present-day genetics. They confirmed that while as whole animals L1/L2 *B. mori* larvae are indeed incompetent to pupate, their epidermis has this capacity when transferred to a final-instar (L5) larva and allowed to pupate with it. Even epidermis from fresh hatchlings could produce first pupal, and then adult cuticles as the host progressed from L5 to pupa to adulthood (Inui and Daimon, 2017). However, when implanted into an L4 host and observed after the larval molt, the L1 graft could only produce new larval cuticle. To test whether this latter effect was caused by JH, as was expected, the authors used L1 implants from the *Met1*$^{-/-}$ KO larvae (Daimon et al., 2015). Strikingly, once molted with the L4 host to L5, the mutant graft, incapable of receiving the JH signal, developed patches of pupal rather than purely larval cuticle (Inui and Daimon, 2017).

Thus, the competence of the epidermis to metamorphose has two essential components, none of which can suffice by itself. One is the absence of JH, the other must be informed by a systemic, perhaps nutritional factor, present in the circulation of an appropriately mature host. It will be of great interest to know the nature of this competence factor. In any event, the important work of Inui and Daimon (2017) extends the fundamental concept of JH regulation of pupal commitment, originally developed in *M. sexta* (Riddiford, 1976), and settles the debate on JH role in preventing precocious metamorphosis.

1.4.1.3 METAMORPHOSIS

The metamorphic transition of insect larvae to adults entails the acquisition of primarily functional wings and reproductive organs, but also of reproductive behaviors. In hemimetabolans, the relatively minor morphogenetic change occurs in a single molt, whereas holometaboly necessitates the formation of a pupa before the adult body plan may be laid down. Internalization of development of adult structures, such as the wings and other appendages, has been a great innovation, reaching a pinnacle in highly advanced dipterans such as *D. melanogaster*. Metamorphosis, and particularly holometaboly, which permits the highest differentiation and specialization between larvae and adults, is a key driver of insect success and species diversity (Rainford et al., 2014).

Discussion of the evolutionary origin of holometaboly is out of the scope of this chapter, and has been covered elsewhere (Sehnal et al., 1996; Truman and Riddiford, 2002, 2019; Bellés, 2011; Bellés and Santos, 2014; Redei and Stys, 2016; Jindra, 2019). Nonetheless, based primarily on the nature of JH signaling that precedes metamorphosis (Konopova et al., 2011; Bellés and Santos, 2014; Ureña et al., 2016, Ishimaru et al., 2019), we hold the view that the pupal stage has evolved as an adaptation to the increasing diversification between the larval and adult forms, and that it represents a modified equivalent of the hemimetabolous final juvenile instar (Jindra, 2019).

As explained above, absence or at least a temporal decline in JH production is a signal for otherwise competent larvae to commence metamorphosis (Figure 1.7). Up until the penultimate instar, the larval status requires JH to drive Met-dependent expression of *Kr-h1*, which is the key repressor of metamorphosis (Minakuchi et al., 2009; Konopova et al., 2011; Lozano and Bellés, 2011; Ureña et al., 2016; Kayukawa et al., 2017). Final-instar hemimetabolous larvae experience a JH-free period during which *Kr-h1* mRNA naturally drops to undetectable levels, providing a window to commit to the adult program (Figure 1.7). Because the JH receptor Met is still present, ectopic JH administration at this time re-induces *Kr-h1* and drives the animal into a supernumerary larval instar (reviewed in Jindra et al., 2013). As has been demonstrated in *B. germanica*, the disappearance of Kr-h1 enables upregulation of the *E93* gene which is driven by 20E and which informs the adult developmental program (Ureña et al., 2014, 2016; Bellés and Santos, 2014) (Figure 1.7). Removal of *E93* causes reiteration of larval molts (Ureña et al., 2014).

The JH/Met/Kr-h1/E93 signaling axis plays a conserved role in holometaboly, where the situation is slightly complicated with the two-step transition via the intermediate pupal stage. Again, *Kr-h1* decline is necessary to initiate metamorphosis; however, the downregulation is temporary, just sufficient to lift the JH-imposed repression off the *Br-C* gene and thereby initiate pupal development (Ureña et al., 2014, 2016; Kayukawa et al., 2017) (Figure 1.7). As shown in *T. castaneum*, a new peak of *Kr-h1* expression, concomitant with the rise of *Br-C* mRNA during the prepupal period, is necessary for the pupal program to be properly accomplished (Minakuchi et al., 2009; Ureña at al., 2016). Indeed, removal of *Kr-h1* led to partial skipping of the pupal stage and to heterochronic appearance of adult characters in the beetle pupae (Minakuchi et al., 2009; Ureña at al., 2016), similar to the effect of knocking down *Br-C* itself (Konopova and Jindra, 2008; Suzuki et al., 2008; Parthasarathy et al., 2008; Ureña at al., 2016). The recent work of Ureña et al. (2016) has indicated that Kr-h1 harmonizes the timely progression through the pupal stage, again by suppressing a premature rise of *E93* expression. This function of Kr-h1 likely reflects the presence of JH during the prepupal phase, invariably observed in holometabolous insects (Riddiford, 1994).

While the intermittent remission of Kr-h1 sets the stage for metamorphosis to take off, it is *Br-C* that specifies and executes the pupal program. The requirement of *Br-C* for pupal development has been confirmed for every holometabolan species thus far examined (Zhou and Riddiford, 2002; Uhlirova et al., 2003; Konopova et al., 2008; Suzuki et al., 2008), most recently using a TALEN-based genetic mosaic approach in *B. mori* (Daimon et al., 2015). The data clearly show that *Br-C* is cell-autonomously required for every epidermal cell to abandon the larval program and instead synthesize pupal cuticle. Gain-of-function experiments in *D. melanogaster* (Zhou and Riddiford, 2002) indicated that *Br-C* is not only required but also sufficient to drive the pupal program, as misexpression of particular Br-C isoforms led to ectopic re-expression of pupal cuticle genes, whereas adult cuticle gene expression was suppressed. A similar effect was achieved by treating the fly pupae with JH, which re-induced Br-C expression and with it, the pupal program in the abdominal epidermis (Zhou and Riddiford, 2002). It is quite interesting to note that while JH suppresses *Br-C* transcription through most of the holometabolous larval development, it can strongly induce it when given to pupae, where JH is normally absent. This induction requires both *Met* and *Kr-h1* (Konopova et al., 2008; Minakuchi et al., 2009). While Br-C is functionally linked to

the holometabolous pupa, its role in hemimetaboly appears to be limited to advancing growth and differentiation of the wing pads of true bugs and *B. germanica* (Erezyilmaz et al., 2006; Konopova et al., 2011; Huang et al., 2013; Ureña et al., 2016). A recent report has implicated Br-C in metamorphosis of the cricket, *Gryllus bimaculatus* (Ishimaru et al., 2019).

Like the final-instar larvae of bugs or cockroaches, the pupae of beetles, moths, and flies experience a long JH-free period; the prepupal peaks of JH, Kr-h1, and Br-C are gone and will not recur until the adult is (nearly) formed (Konopova et al., 2011; Lozano et al., 2011; Santos and Bellés, 2014) (Figure 1.7). This is the time in both types of metamorphosis when *E93* can finally be fully expressed to bestow the adult fate (Ureña et al., 2016; Kayukawa et al., 2017).

Next to finding of the JH receptor Met, deciphering of the above intricate signaling presents a great advance in our understanding of insect developmental endocrinology. The effort has continued, primarily through work of the Shinoda laboratory, by uncovering the molecular interactions among the main players. After demonstrating the JHRE-dependent transcriptional activation of *Kr-h1* by the JH-receptor complex (Kayukawa et al., 2012), the group has defined the Kr-h1 protein as a transcriptional repressor acting directly through specific binding sites in the regulatory *cis*-elements of both the *Br-C* and *E93* genes of *B. mori* (Kayukawa et al., 2016, 2017). The same studies have also identified 20E-response elements in both *Br-C* and *E93* genes. They have shown how these genes are induced, after the JH-mediated repression is lifted, by the steroid hormone during the prepupal and the pupal stages, respectively, to specify the pupal (*Br-C*), and the adult (*E93*) developmental programs (Figure 1.7).

1.4.2 REPRODUCTION

The JHs regulate many aspects of reproductive maturation after adult emergence with the specific effects depending on the insect group. In non-social insects, JH has an evolutionarily conserved pro-reproductive and pro-aging effect. Downregulation of JH signaling in response to low nutrient availability switches the physiological state of the organism to a pro-maintenance, pro-survival mode at the expense of reproduction (Rodrigues and Flatt, 2016).

1.4.2.1 JUVENILE HORMONE (JH) AS A GONADOTROPIN

The JHs act as gonadotropins, regulating vitellogenesis and oogenesis in most insects. There are evolutionary differences among highly eusocial and non-social insects in the 'coupling strength' between JH as a gonadotropic hormone and the synthesis of vitellogenin (Vg). Non-social insects, particularly species with long lasting adult stage, exhibit diet-dependent reproductive cycles whose initiation requires a gonadotropic signal that induces Vg production in the fat body (Rodrigues and Flatt, 2016). This signal is typically JH that pleiotropically controls most aspects of female reproduction, including Vg synthesis and uptake, lipophorin synthesis, endoplasmic reticulum proliferation, and courtship behavior. Recent reviews have extensively covered the pleiotropic roles of JH in controlling female insect reproduction (Roy et al., 2018; Santos et al., 2019). We will, therefore, highlight in this section some of the studies on model insects that have advanced the knowledge of molecular mechanisms of JH action in reproduction.

The role of JH signaling in reproduction has been studied in several hemimetabolans. In *L. migratoria*, knockdown of Met or Tai markedly suppressed vitellogenesis and egg production, and this suppression was linked with insufficient ploidy of the fat body cells (Guo et al., 2014; Wu et al., 2016). As already described in section 1.3.3.1 above, the JH-induced increase in ploidy requires direct activation by the Met-Tai complex of the *Mcm4*, *Mcm7*, and *Cdc6* genes involved in DNA replication. The pro-vitellogenic action of JH in *L. migratoria* has also been shown to involve Kr-h1 whose knockdown resulted in a substantial reduction of Vg expression in the fat body and lipid accumulation in the primary oocytes, along with blocked follicular epithelium development, oocyte maturation and ovarian growth (Song et al., 2014). Similarly, in the linden bug *P. apterus*, Met and Tai were required for Vg synthesis and oogenesis to take place, whereas *Kr-h1* RNAi had no appreciable effect on egg production, suggesting either that the effect of JH on oogenesis in the bug might be mediated by players other than Kr-h1, or that the RNAi knockdown was inadequate to reveal Kr-h1 function (Smykal et al., 2014a). In the cockroach *D. punctata*, knockdown of Met resulted in an arrest of oocyte development, suppression of Vg production in the fat body and of Vg uptake by the ovary. In addition, follicle cells did not develop whorls of rough endoplasmic reticulum and failed to form the chorion (Marchal et al., 2014). In the cockroach *B. germanica*, JH also induces Vg production (Cruz et al., 2003), with the insulin pathway mediating the activation of JH biosynthesis, and Vg expression elicited by nutrient signaling (Abrisqueta et al., 2014).

In holometabolans, the hormonal control of oogenesis is more diverse. In the beetle *T. castaneum*, JH has been shown to stimulate Vg production indirectly, via the insulin-signaling pathway (Sheng et al., 2011). Both JH and feeding induced the expression of specific ILPs in the brain and fat body, and these ILPs, in turn, triggered Vg synthesis. *T. castaneum* thus seems to differ from other insects where JH biosynthesis is downstream of the nutrient-sensing insulin/TOR pathways (Tu et al., 2005; Maestro et al., 2009; Pérez-Hedo et al., 2013).

Rather than JH, ecdysteroids exert more direct control over Vg synthesis in some representatives of the Diptera, Hymenoptera, and Lepidoptera (Raikhel et al., 2005; Hansen et al., 2014). Nevertheless, in anautogenous mosquitoes, JH is still responsible for preparing the female organs for oogenesis (Hansen et al., 2014; Roy et al., 2018). This JH-controlled preparatory development for oogenesis involves the effects of JH on multiple tissues and a range of processes. JH prepares the midgut for a blood meal digestion (Noriega and Wells, 1999), activates ribosomal biogenesis in the fat body (Wang et al., 2017b), and increases the lipid content. In the ovary, JH stimulates expression of the lipophorin and Vg receptors, as well as expression of heavy chain clathrin and of ribosomal proteins (Clifton and Noriega, 2012). In addition, JH directly controls nutrient allocation into the ovaries before a blood meal, as well as indirectly during the vitellogenic phase (Clifton and Noriega, 2011). Transcriptome studies have revealed that by acting through Met, JH regulates both carbohydrate (Hou et al., 2015) and lipid (Zou et al., 2013) metabolism genes in adult female mosquitoes. Specifically, RNAi knockdown leads to a reduction of lipid reserves in the fat body and to increased lipid utilization during the post-eclosion, pre-blood meal phase (Wang et al., 2017a). This study has also shown how JH and 20E exert opposite effects on lipid catabolism by suppressing (JH/Met) versus activating (20E/EcR) expression of an evolutionarily conserved regulator of lipid homeostasis, the NR member Hepatocyte nuclear factor 4 (HNF4). Thus, while JH supports lipid storage before the blood meal is taken, 20E and HNF4 together with the nutrient-sensing TOR pathway are important for efficient mobilization of the fat reserves for egg production post-blood meal (Wang et al., 2017a).

Oogenesis in *D. melanogaster* depends upon balanced levels of JH and 20E (Soller et al., 1999). Under normal conditions, JH stimulates yolk protein synthesis in the fat body. In the ovary, JH, in combination with other factors, promotes endocytosis of yolk proteins into developing oocytes (Yamamoto et al., 2013). CA-deficient *D. melanogaster* adults had greatly reduced fecundity, inhibited oogenesis, impaired adult fat body development, and

extended lifespan. Treating these adults with the JH analog methoprene restored all traits toward wild type (Yamamoto et al., 2013). The role of JH in *D. melanogaster* oogenesis is most likely mediated by the JH receptors, as egg-laying and overall egg production are temporally retarded in females lacking either DmMet or, less so, Gce (Wilson and Fabian, 1986; Abdou et al., 2011a).

A recent paper describes how 20E, ETH, and JH are repurposed to function as an endocrine network essential for reproductive success in *D. melanogaster* (Meiselman et al., 2017). JH also acts as an anticipatory endocrine signal released after mating in flies. In mated females, JH signals directly through its receptor Gce to intestinal stem cells to proliferate, triggering an increase in gut size. In addition, JH adjusts gene expression in the enterocytes to support increased lipid metabolism in preparation for oogenesis (Reiff et al., 2015).

Results on the role of JH in the regulation of *D. melanogaster* male reproduction are ambiguous (Yamamoto et al., 2013). Although a variety of insects show reduced AG production and a decrease in reproductive fitness when JH titers are reduced, often these changes have not been associated with changes in the reproductive potential of adult males. *D. melanogaster* males lacking DmMet are less active in courtship and mating, but in terms of sperm transfer, their fertility was unaffected (Wilson et al., 2003). Recent results have revealed that egg production in *D. melanogaster* females mated with JH-deficient males was reduced, but rescued by topical treatment of JH-deficient males with methoprene (Meiselman et al., 2017).

Similarly, in *A. aegypti,* male mosquitoes, nutrition, JH titers, AG contents, and insemination rates are connected. Application of a JH analog increases AG contents and insemination rates (Ramalingam and Craig, 1977). Starvation decreased both JH titers and insemination rates in male mosquitoes, whereas increased nutrition had the opposite effect (Nouzova et al., 2018) and led to increased accumulation of JH in the AG as well as the amount of JH III transferred to the female at mating (Clifton et al., 2014). The JH contents in the AG decreased after mating (Clifton et al., 2014).

1.4.2.2 JUVENILE HORMONE (JH) AND SEXUAL BEHAVIOR

JH plays several roles in female reproductive behavior in *D. melanogaster*, including stimulation of pheromone production and regulation of virgin female receptivity to courting males (Argue et al., 2013; Belgacem and Martin, 2002; Bilen et al., 2013; Lin et al., 2016). In *D. melanogaster*, JH is essential for courtship memory in adult males (Lee et al., 2017). Reduction

of JH levels impaired short-term courtship memory, a phenotype that was rescued by the JH analog methoprene. JH deficit-induced memory impairment involved rapid decay rather than the failure of memory acquisition. A critical period directed memory performance during the first three days of adulthood. JH acted through dopaminergic neurons, and this signaling cascade was required during a critical period for promotion of social context-dependent memory (Lee et al., 2017). These brain modulatory effects of JH have been also described in the honeybee, where JH determines social status and regulates olfactory memory of adult animals, most likely through modulation of aminergic circuits in the brain (McQuillan et al., 2014).

Other reproductive behaviors have been linked to JH. Sperm and sex peptide (SP) stimulate aggression in female *D. melanogaster* (Bath et al., 2017). SP stimulates JH production in the CA; it is, therefore, possible that SP acts to increase female aggression by stimulating JH production. Indeed, the amount of JH present in the hemolymph has been linked to aggression in both sexes in other insect species, such as burying beetles, paper wasps and cockroaches (Scott, 2006; Kou et al., 2009; Tibbetts et al., 2013).

JH plays a critical role in the normal timing of the onset of female mating and sex pheromone production in *D. melanogaster* (Bilen et al., 2013). The *D. melanogaster* sex pheromones are a subgroup of the cuticular hydrocarbons (CHC) that mediate chemical communication for both sex and species recognition. Removal of JH through genetic ablation of the CA delayed both sex pheromone production and mating; a decrease in male courtship suggested reduced female attractiveness. There were drastic changes in the CHC profiles in the allatectomized females, and treating these females with methoprene advanced the onset of mating and increased the attractiveness of the females (Bilen et al., 2013). Because some of these JH-deficiency phenotypes correlated with the loss of DmMet in the fly females, the authors have suggested that DmMet, rather than its paralog Gce, mediates JH effect on female mating.

1.5 CONCLUDING REMARKS

The different sections included in this chapter illustrate the progress that has been made in our understanding of JH synthesis, transport, degradation, signaling, and actions. We comprehend the steps involved in building the JH molecule starting with acetyl-CoA, as well as many of the factors involved in the regulation of synthesis inside the CA. On the other hand, less is known about the export of JH from the CA into the hemolymph, and the

loading of JH into the binding pocket of the hJHBP. Does this process take place inside an exocytotic vesicle? Is JH transfer to the hJHBP a membrane-bound process? Is the loading into an hJHBP assisted by "loading" factors? Because of the lipophilic nature of JH, the loading of JH to the hJHBP and the unloading at the target cell are particularly puzzling. In particular, how the hJHBP-JH complex recognizes a target cell and delivers the hormonal signal is also unclear. Does the hJHBP recognize a receptor or a docking site in target cells? How does JH cross the membrane to reach the intracellular receptor Met/Gce, and what is the precise mode of interaction between the receptor and its hormonal ligand? What is the role of the plasma membrane-initiated JH signaling in this process? Once the hormone binds the receptor complex, which translocates to the nucleus and activates the transcription of target genes, the hormone needs to be degraded, transported to the excretory tissues and eliminated; once more, these processes are understudied. It seems that these transitions among the sites of synthesis, target cells, and excretory tissues are areas with fascinating questions, which need to be addressed before we have a clear understanding of JH regulation and actions.

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KEYWORDS

- **juvenile hormone binding proteins**
- **juvenile hormone receptors**
- **methoprene-tolerant**
- **basic helix-loop-helix PAS domain**
- **transcriptional regulation**
- **development**
- **metamorphosis**
- **vitellogenesis**
- **oogenesis**
- **mating behavior**

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