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Hormonal regulations in insect metamorphosis: A review

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Abstract
Metamorphosis is an obligatory feature in insect physiology mediating to insect evolution, wherein the individual acquires characteristic adult features and stops molting during postembryonic development. Ecdysteroids and juvenile hormones (JHs) are key hormones that are responsible for insect molting and metamorphosis contributing in this process for successful adaptation. To understand the development process of insects, this paper reviews the mechanisms of the major hormones, especially how they control the insect metamorphosis process and their control over gene expression for stage speciation. It also focuses on the source of synthesis of those hormones, their active signalling pathway. This paper is supported by secondary information. According to the literature reviewed, ecdysteroids and Juvenile Hormones (JHs) are key hormones that are responsible for insect molting and metamorphosis, respectively. JH maintains the larval state and its decline along with the increase of ecdysteroid in the hemolymph are crucial to elicit transformation to the pupal stage; therefore, the precise control of JH and Ecdysteroid (ecdysone & 20E), is necessary for normal development and the initiation of metamorphosis. Juvenile hormone signalling pathway with referring to Kr-h1, BR-C and E93 genes have also been described.

Keywords: Metamorphosis, juvenile hormone, ecdysteroids, MET protein, hormone signalling

Introduction
Metamorphosis is a physiological process in insects leading to develop physically through conspicuous and relatively abrupt changes in body structure through cell growth and differentiation. Most insects undergo a radical transformation during their life cycle in order to reach adulthood -called metamorphosis. Mueller (2014) defined metamorphosis as fundamental remodeling of the whole body and fundamental change in the mode of life [1]. For example, the creeping caterpillar transforms into a lovely gossamer butterfly. In 2000, Buszczak & Segraves explained it as a mature organism is not only the product of a series of cellular divisions and differentiation events but also the product of a highly regulated series of cell deaths [2]. In metamorphosis, the individual acquires characteristic adult features and stops molting during postembryonic development [3]. And during insect metamorphosis, the steroid hormone ecdysone activates programmed cell death of larval tissues and the further development of adult tissues. Jiang *et al*. (2000) [4] and Lee *et al*. (2000) [5] expanded the understanding of the metamorphosis process by dissecting the molecular genetic mechanisms through which the hormonal triggers of metamorphosis direct the stage- and tissue-specific activation of cell death programs.
Insect metamorphosis often involves the destruction of larval tissues and their replacement by an entirely different population of cells. Insects grow by molting i.e. shedding old cuticle and growing new cuticle as their size increases. There are three major patterns of insect development. Primitive wingless insects such as springtails and mayflies have an ametabolous lifestyle, shows direct development. Immature and adults look identical except for size. But they continue to molt, typically alternating a molt with a bout of reproduction [6]. Winged insects have evolved diverse modes of metamorphosis [7]. Hemimetabolous derives from ametabolous ancestors [3]. In this case, embryogenesis gives rise to a first instar nymph, go through some successive molts, wings are gradually formed and finally, nymphs perform their last molt, after which the adult emerges with functional genitalia. Holometaboly emerged from hemimetaboly, which involved a fundamental alteration of embryonic development to produce a larval stage.

After the last larval instar, the insect changes into a pupa, covered by a protective cocoon. Eventually, the insect molts for the last time and emerges as an adult.

Hormones were first shown to be the signal for this transformation 70 years ago by Kopec (1922) [8]. In the intervening years, the primary hormones controlling insect growth and metamorphosis- prothoracicotropic hormone (PTTH), ecdysone and its bioactive derivative 20-hydroxyecdysone (20HE), and juvenile hormone (JH)-were isolated, chemically characterized, and much has been learned about their biological actions [9]. In most insects, ecdysone (E) is synthesized by the prothoracic glands (PGs) and converted systemically to the active ecdysteroid, 20-hydroxyecdysone (20E). JHs are synthesized by the corpora allata [3].

Insect metamorphosis is a fascinating and highly successful biological adaptation [10]. Typically, an insect's life cycle comprises embryonic, larval, pupal, and adult stages, where growth occurs exclusively during larval development. The succession of different stages and body plans begs the question as to how these transitions are regulated [11]. Small lipophilic hormones, such as ecdysteroids are responsible for this dramatic reprogramming of body plans of insets [12]. So, it is under hormonal control, which has been a subject of experimental study for almost a century. The importance of insects as competitors for food and fiber as well as their impact on human and animal health. Different life stages of the same insect species exploit different resources like foods and habitats could prevent intra-specific competition (i.e., competition for resources between organisms of the same species). Thus, the main functional sense of metamorphosis could be to minimize the intraspecific competition for resources. And the more specialized are the different stages of an insect, the greater would be the chance to exploit more and better the resources. These facts would mean a great advantage for these organisms, so that holometabolous development, which is characterized for being divided into very different stages, could have been more successful than the hemimetabolous or the ametabolous insects. These spurred extensive research into insect's development, and especially into how they control their metamorphosis, how different stages express by different genes, and their hormonal regulation.

Metamorphosis and it's requirements in the body

The rigid exoskeleton of insect constrains growth, an insect's life history is punctuated by molts, during which a new cuticle is formed and the old one shed, a process termed ecdysis. The span from one ecdysis to the next is termed an instar. In most insects, several number of instars, but the starting metamorphosis depends on reaching a species-specific size. Size is controlled by hormones [13]. To undergo larva-pupa transition, the larva must reach a threshold size (TS); but in some species, the larva can opt for an earlier instar to start metamorphosis because of poor food conditions [14, 15].

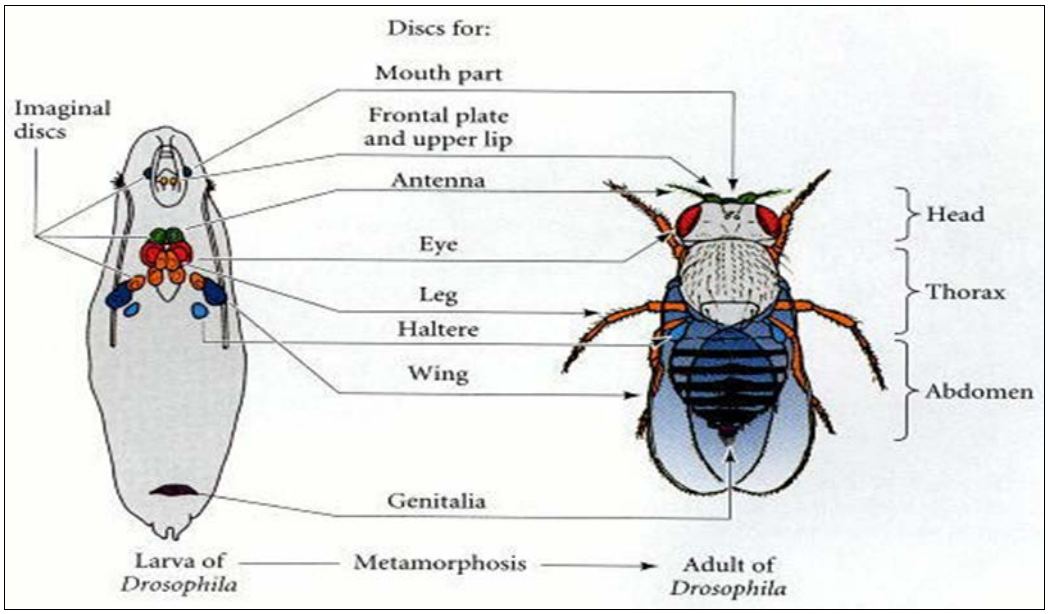

Fig 1: Fates of the imaginal discs in *Drosophila melanogaster*. [16]

In holometabolous insects, the transformation from juvenile into adult occurs within the pupal cuticle. Most of the old body of the larva is systematically destroyed by apoptosis, while new adult organs develop from undifferentiated nests of cells, the imaginal discs. Within larva, there are two distinct populations of cells: the larval cells and the thousands of imaginal cells (fig 1) will construct many of the adult organs, which lie within the larva in clusters, awaiting the signal to differentiate. When there a signal by hormone, imaginal disc differentiates and transform totally different stage [16].

Hormones responsible for metamorphosis

In both hemimetabolous and holometabolous insects, the developmental switch between immature and adult forms depends on mainly two hormones: the juvenile hormone (JH) and the ecdysone. Although the detailed mechanisms of insect metamorphosis differ among species, the general pattern of hormone action is very similar. Two hormones, which regulate the metamorphosis of insects, are controlled by neurohormones in the brain [17]. Neurosecretory cells in an insect's brain secrete a hormone, the prothoracicotropic hormone (PTTH) in response to neural, hormonal, or environmental signals. PTTH is a peptide hormone with a molecular weight of approximately 40,000 [16]. And it stimulates prothoracic glands in order to secrete ecdysone hormone. PTTH also stimulates the corpora allata to produce a sesquiterpenoid juvenile hormone. Ecdysone hormone is rapidly converted in peripheral tissues to its

biologically active form, 20-hydroxyecdysone (20E) called "active molting hormone" [3, 11, 16, 18]. Both ecdysone and 20E are steroids, collectively known as ecdysteroids, which have differing but overlapping actions [3].

Table 1: Major regulatory hormones & their origin and function

Hormones	Origin	Function
Prothoracicotropic hormone (PTTH)	Insect's brain	Stimulates prothoracic glands & corpora allatain to secrete ecdysone & juvenile hormone respectively
Juvenile hormone	Corpora allata	Prevent metamorphosis until larvae attain an appropriate size
Ecdysone	Prothorasic glands	Differenyiate imaginal disc
20-hydroxyecdysone (20E)	Biologically active form of ecdysone	Produce two impulses for molting & regulates the changes in gene expression

Source: [3, 11, 16, 18]

Mechanism of hormones

Juvenile hormone act as the developmental switch between immature and adult forms, a sesquiterpenoid produced by the corpora allata gland [19]. As long as the juvenile hormone is present in sufficient concentration, fosters the growth of larva, moults result in another larval instar but inhibits the differentiation of adult structures. In the last larval instar, an axonal nerve from the brain inhibits the corpus allatum from releasing juvenile hormone. The lowered JH level allows metamorphosis to proceed. Timely experimental removal of the corpora allata causes larvae to undergo premature metamorphosis from which small adults arise, while implantation of additional glands results in additional larval moults, delayed metamorphosis, and giant adults.

20E promotes the successive molts, including the metamorphic one, whereas JH is a terpenoids (lipid), which represses metamorphosis [3, 20]. 20-hydroxyecdysone initiates and coordinates each molt and regulates the changes in gene expression, induces ecdysis, which mean transfer one stage to another, metamorphosis. Each molt is initiated by one or more pulses of 20-hydroxyecdysone. For a larval molt, the first pulse produces a small rise in the hydroxy ecdysone concentration in the larval hemolymph (blood) and elicits a change in cellular commitment. A second, large pulse of Hydroxy ecdysone initiates the differentiation events associated with molting. The hydroxyecdysone produced by these pulses commits and stimulates the epidermal cells to synthesize enzymes that digest and recycle the components of the cuticle. Juvenile hormone prevents the ecdysone-induced changes in gene expression (from immature to mature) that means prevents the development of adult characteristics [16]. The presence of juvenile hormone during a molt ensures that the result of that molt produces another instar, not a pupa or an adult.

Regulatory pathway of insect metamorphosis

Ecdysteroid and juvenile hormone together cause molts and form another larval instar. When there is a lower concentration of juvenile hormone, the ecdysteroid-induced molt produces a pupa. When ecdysteroid (ecdysone & 20E) acts in the absence of the juvenile hormone, the imaginal discs differentiate, and the molt gives rise to the adult (Fig.2). Thus, in holometabolous insects, molts between larval instars have a high level of juvenile hormone, the moult to the pupal stage has a low level of juvenile hormone, and the final, or imaginal, molt has no juvenile hormone present at all [21].

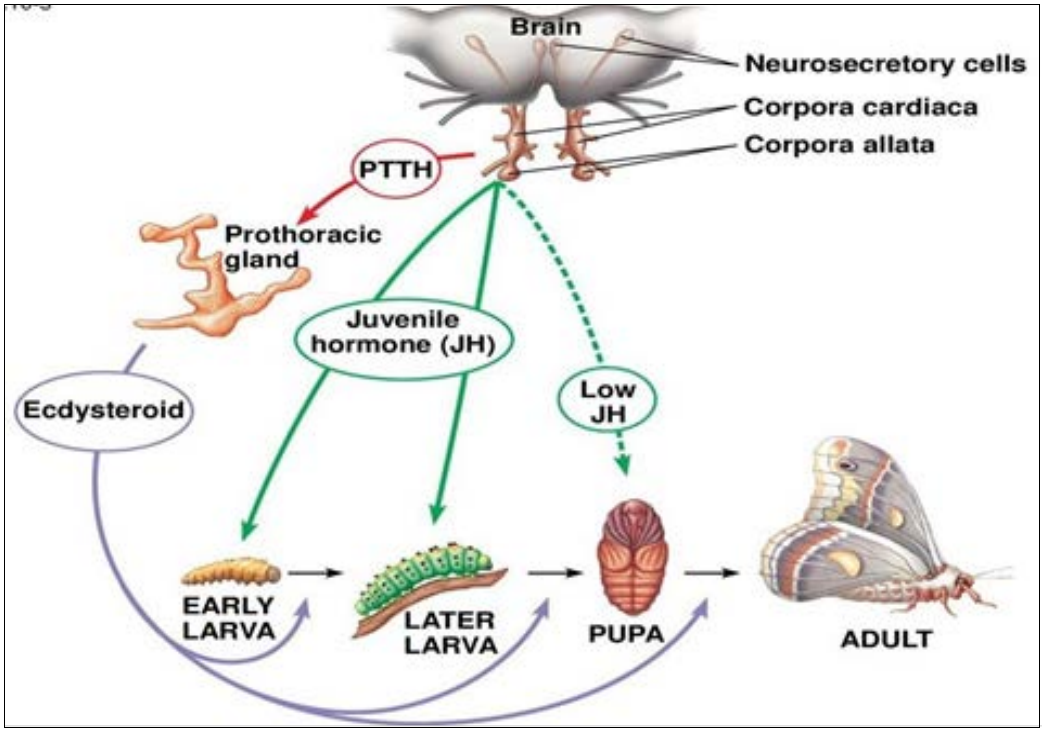

Fig 2: Hormonal regulation in insect metamorphosis.

Secretion of hormones as per required

Juvenile hormone is secreted by the corpora allata. The secretory cells of the corpora allata are active during larval molts but inactive during the metamorphic molt. As long as JH is present, the 20E stimulates to molts in a new larval instar. But in the last larval instar, the medial nerve from the brain to the corpora allata inhibits the production of JH.

Existing JH decreases with the simultaneous increase of body [22]. Both these mechanisms cause JH levels to drop below a critical threshold value. This triggers the release of PTTH from the brain [23]. PTTH, in turn, stimulates the prothoracic glands to secrete a small amount of ecdysone. In the absence of high levels of JH, 20E commits the cells to pupal development. Larva-specific mRNAs are not replaced yet, but new mRNAs are synthesized whose protein products inhibit the transcription of the larval messages. After the second ecdysone pulse, new pupa-specific gene products are synthesized [24], and the subsequent molt shifts the organism from larva to pupa. The first ecdysone pulse during the last larval instar triggers the processes that inactivate the larva-specific genes and prepare the pupa-specific genes to be transcribed. The second ecdysone pulse transcribes the pupa-specific genes and initiates the molt [25]. At the imaginal molt, when ecdysone acts in the absence of juvenile hormone, the imaginal discs differentiate, and the molt gives rise to the adult.

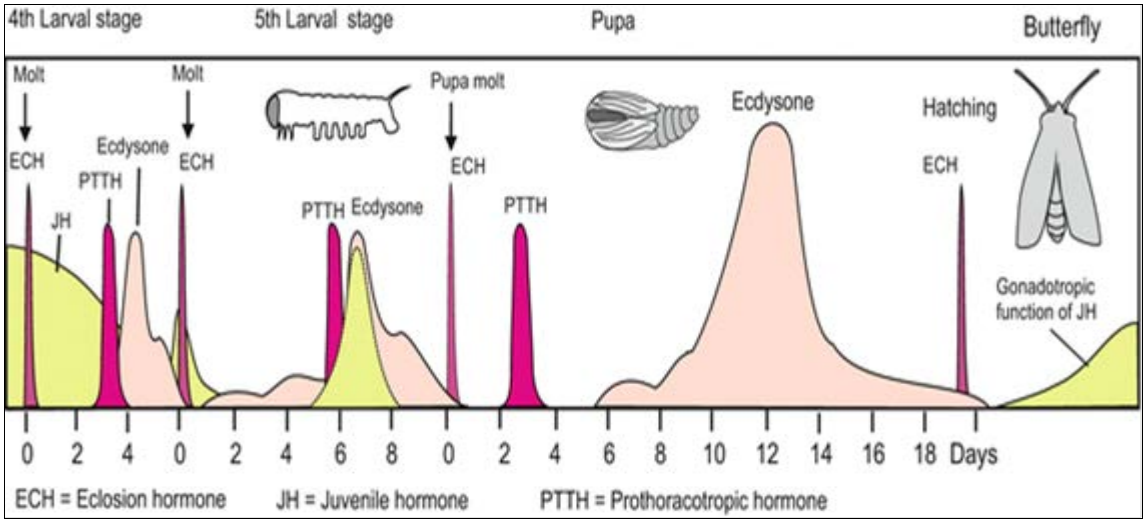

Fig 3: Hormonal secretion timing in case of butterfly *Manduca sexta* [1].

Figure 2 shows, 4th larval stage there is high level of JH, when molt into 5th larva PTTH release ecdusone for molting. In 5th or final instar JH decreases. And then molting of metamorphosis means transformation from larva to pupae occur. Pupal stage there is no JH, where huge transformation occurs, developmental structure as well as reproductive structure form for adult stage.

Molecular activity of JH

Methoprene-tolerant (Met) protein, belongs to an ancient family of basic helix–loop–helix Per/Arnt/Sim (bHLH-PAS) transcription factors. Which are considered as the intracellular JH receptor in JH signaling pathways, to which JH binds [3, 26, 27]. JH binds with Met) protein & Taiman, (it's also a bHLH-PAS protein) that act as co-receptor. The direct interaction between Met and Tai is stimulated by JH [3, 27, 28].

When activated by JH, the Met–Tai receptor complex directly induces Krüppel homolog 1 (Kr-h1) gene, which is the main transducer of the anti-metamorphic signal of JH [3, 20, 28, 29]. And Kr-h1 represses an 'adult specifier' gene E93, whereas the dramatic fall of Kr-h1 expression following the natural drop in JH titer during the final juvenile stages (particularly last-instar nymphs in hemimetabolans and pupae in holometabolan) permits adult development [20, 26]. The action of this JH-activated pathway in maintaining the juvenile status is dispensable during early postembryonic development when larvae/nymphs lack the competence to metamorphose.

Molecular activity of Ecdysteroid

Ecdysteroids (ecdysone (E) and its derivative 20-hydroxyecdysone (20E), are primary regulators of insect molting and metamorphosis [30]. The action of ecdysteroids in causing molting of the cuticle is an ancient function of these hormones that precedes their involvement in metamorphosis [15]. Each molt is initiated and coordinated by a large pulse of ecdysteroids that act through a conserved genetic circuit, the Ashburner cascade, that translates features of the steroid pulse into the synthesis of a new cuticle and the shedding of the old one [15].

The ecdysteroids are polyhydroxylated steroids; for synthesis, insects utilize cholesterol and/or plant sterols as precursors [18, 31, 32]. In the 1990s, the 20E signaling pathway was first described [11]. 20E is an active form, but ecdysone can function in its own right early in molting or metamorphic programs [33]. Ecdysone predominates at the start of the peak where 20E becoming predominant later [34]. Ecdysteroids act through nuclear hormone receptors and the ecdysone receptor (EcR) [35]. 20-hydroxyecdysone cannot bind to DNA by itself. At first, it binds with receptors. The receptors specifically binding 20-hydroxyecdysone are called the ecdysone receptors (EcR) [36]. An EcR protein pair with an Ultraspiracle (Usp) protein to form an active molecule [37]. The structure of EcR mediate DNA binding, spliced to make two major isoforms, EcR-A and EcR-B. These two isoforms are found throughout the insects [38], but it is not known if their functions differ with the different types of metamorphic development.

Although there is only one type of gene for Usp in *Drosophila*, and only one type of gene for EcR, the EcR gene transcript can be spliced in at least three different ways to form three distinct proteins. All three EcR proteins have the same domains for 20-hydroxyecdysone and DNA binding but

they differ in their N-terminal domains [36]. The type of EcR in a cell may inform the cell how to act when it receives a hormonal signal. All cells appear to have some of each type, but the strictly larval tissues and neurons that die when exposed to 20-hydroxyecdysone are characterized by their great abundance of the EcR-B1 form of the ecdysone receptor. Imaginal discs and differentiating neurons, on the other hand, show a preponderance of the EcR-A isoform. It is therefore possible that the different receptors activate different sets of genes when they bind 20-hydroxyecdysone.

Pulses of 20-hydroxyecdysone occur during insect development, whereupon this hormone binds to the ecdysone receptor, a ligand-activated transcription factor found in the nuclei of insect cells. This in turn leads to the activation of many other genes, as evidenced by the puffing of polytene chromosomes at over a hundred sites. Ultimately the activation cascade causes physiological changes that result in ecdysis (moulting). The temporal expression of the ecdysone receptor within neural stem cells mediates temporal patterning and neural diversity.

20-hydroxyecdysone with DNA

During molting and metamorphosis, certain regions of the polytene chromosomes of *Drosophila* puff out in the cells of certain organs at certain times [39]. These chromosome puffs represent areas where DNA is being actively transcribed. The puffing is mediated by the binding of hydroxyecdysone at specific places on the chromosomes. Hydroxyecdysone-regulated chromosome puffs occurring during the late stages of the third-instar larva (as it prepares to form the pupa) can be divided into three categories: "early" puffs that hydroxyecdysone causes to regress; "early" puffs that hydroxyecdysone induces rapidly; and "late" puffs that are first seen several hours after hydroxyecdysone stimulation. For example, in the larval salivary gland, about six puffs emerge within a few minutes of hydroxyecdysone treatment. No new protein has to be made in order for these puffs to be induced. A much larger set of genes are induced later in development, and these genes do need protein synthesis to become transcribed. These insights have been confirmed by molecular analyses. The three early puffs include the genes for EcR and two other transcription factors, BR-C and E74B [16].

Hormonal regulation in stage-specification gene expression

Three genes, *Kr-h1*, *broad-complex (BR-C)* and *Ecdysone-inducible protein 93F* (*E93*), are key genes that respond to the developmental hormones and control the characteristics of the different life stages. All were initially identified in *Drosophila* and associated with ecdysteroid action during metamorphosis [10, 15].

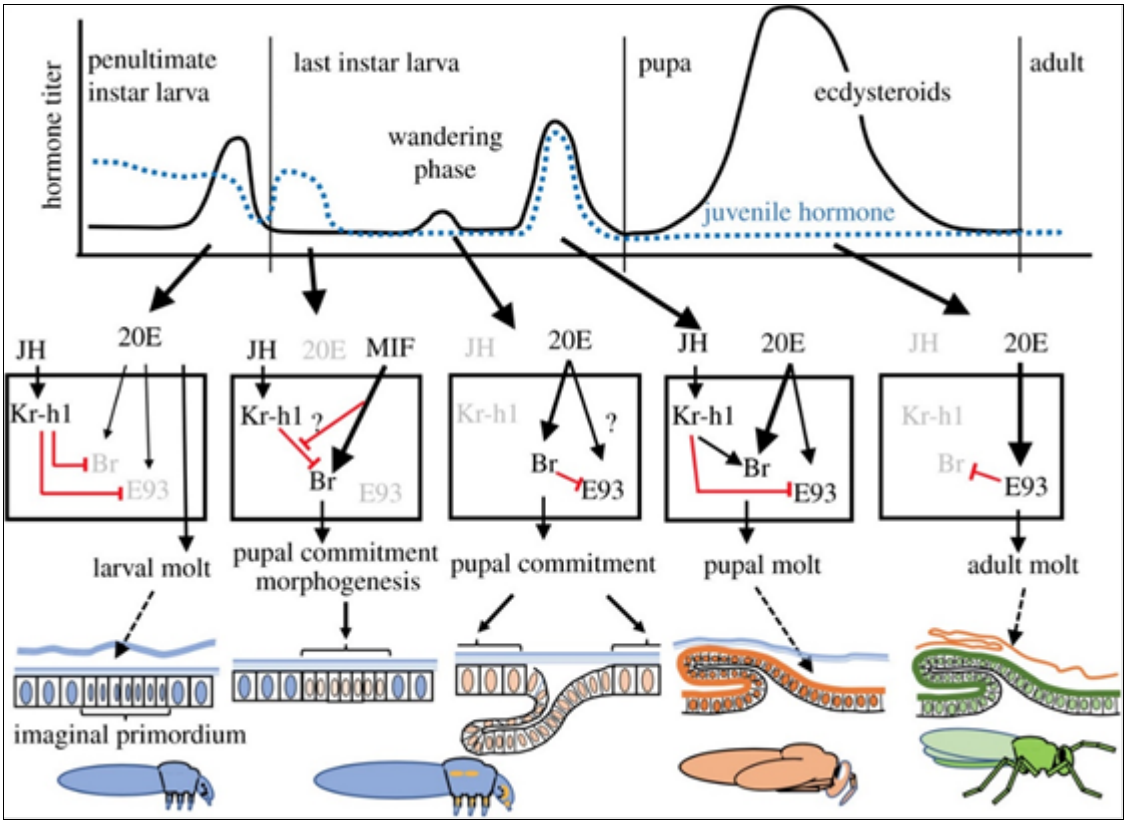

Fig 4: Stage specification genes in holometabolous insects [10].

A generalized scheme shows in holometabolous insects how stage specification genes relate to each other, to the hormonal environment that orchestrates metamorphosis and to the cellular responses of the imaginal primordium and the general epidermis. Symbols that are greyed out are either absent or suppressed. 20E, 20-hydroxyecdysone; Br, Broad; E93, Ecdysone-inducible protein 93F; JH, juvenile hormone; Kr-h1, Krüppel-homolog 1; MIF, metamorphosis initiation factor.

Kruppel homolog1 (Kr-h1)

Kruppel homolog1 (Kr-h1) is a juvenile hormone (JH) response transcriptional factor that transduces JH signaling to repress insect metamorphosis in both hemimetabolous and holometabolous insects [40]. *Kr-h1* was first described by Pecasse *et al*. based on the disruption of prepupal

development in *Kr-h1* mutants [41]. Although intimately associated with the maintenance of the larval or the nymphal condition, *Kr-h1* is a larval specifying gene. *Kr-h1* is the main target of JH acting through its receptor Met [42] and hence the main effector of JH action in *Drosophila melanogaster* the adult abdominal epidermis derives from larval histoblasts, which start proliferating after pupa formation. The experiments of Ashburner in 1970 showed that administration of JH prior to the prepupal stage prevents the normal differentiation of the abdominal epidermis and the bristles, that should be formed in the adult, are shorter or lacking [3]. In the flour beetle, *T. castaneum*, for example, the removal of either JH, Met or Kr-h1 produces the same developmental response: the larvae initiate premature metamorphosis [43]. Therefore, if JH is required for larval maintenance, then the same is true for Kr-h1 [10]. In 2008, the experiments of Minakuchi and co-workers indicated that Kr-h1 expressed ectopically in the abdominal epidermis during metamorphosis of *D. melanogaster* resulted in missing or short bristles, thereby suggesting that Kr-h1 mediates the antimetamorphic action of JH [3, 44]. In 2020, He *et al*. 2020 induced of miR-927 which decrease the expression of Kr-h1 that resulted in reduced oviposition [40], increased mortality, delayed pupation, and reduced pupal size. But the expression of miR-927 was found to be repressed by JH and its receptor Met/gce, forming a positive regulatory loop of JH signaling.

E93 as Transcription factor

Transcription factor E93 is a steroid hormone ecdysone early response gene and plays crucial roles in both the degradation of larval tissues and the formation of adult organs during insect metamorphosis with the prepupal-pupal-adult transition [45]. The function of broad to establish the pupal stage was initially demonstrated in Drosophila [46]. E93 determines the adult stage in both hemimetabolous and holometabolous insects. In hemimetabolous nymphs, JH, acting through Kr-h1, suppresses E93 and thereby maintains the nymphal state. In the absence of Kr-h1, E93 is expressed and causes adult differentiation. This has been referred to as the MEKRE93 pathway [47], with E93 providing the gateway into metamorphosis [15]. In beetle *Tribolium castaneum*, E93 depletion by RNAi prevented the pupal–adult transition, resulting in the formation of a supernumerary second pupa.

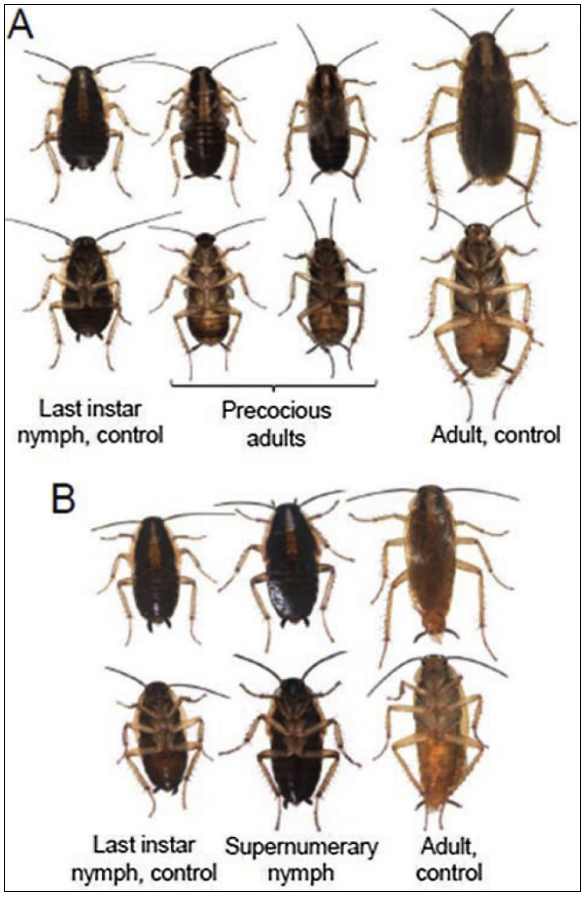

Fig 5: Regulation of metamorphosis by Krüppel homolog 1(Kr-h1) and E93 [3].

Figure 5 shows (Kr-h1) and E93 regulation in the cockroach, *Blattella germanica*. A. Depletion of Kr-h1 in penultimate nymphal instar triggers a precocious metamorphosis at the next molt, thus a miniature adult is produced instead of a last instar nymph. B. Depletion of E93 in last nymphal instar inhibits metamorphosis, thus a supernumerary nymphal instar is produced instead of an adult.

Broad-Complex (BR-C)

The Broad-Complex (BR-C) is an early ecdysone-inducible gene that encodes a family of DNA binding proteins defined by at least three lethal complementation groups: br, rbp, and l (1) 2Bc. The BR-C is critical for the appropriate regulation of all three classes of ecdysone-inducible genes [48]. The broad-complex (BR-C) gene is particularly interesting. BR-C gene can generate several different transcription factor proteins through differentially initiated and spliced messages. It appears that the variants of the ecdysone receptor may signal particular variants of the BR-C protein to be synthesized. Organs such as the larval salivary gland that are destined for

death during metamorphosis express the Z1 isoform; imaginal discs destined for cell differentiation express the Z2 isoform; and the central nervous system (which undergoes marked remodeling during metamorphosis) expresses all isoforms, with Z3 predominating [49, 16]. *Drosophila* mutants that lack *broad* function go through larval life but are blocked at the entry to metamorphosis [50]. In Diptera and Lepidoptera, *broad* mRNA and protein appear in tissues when they become committed to pupal differentiation.

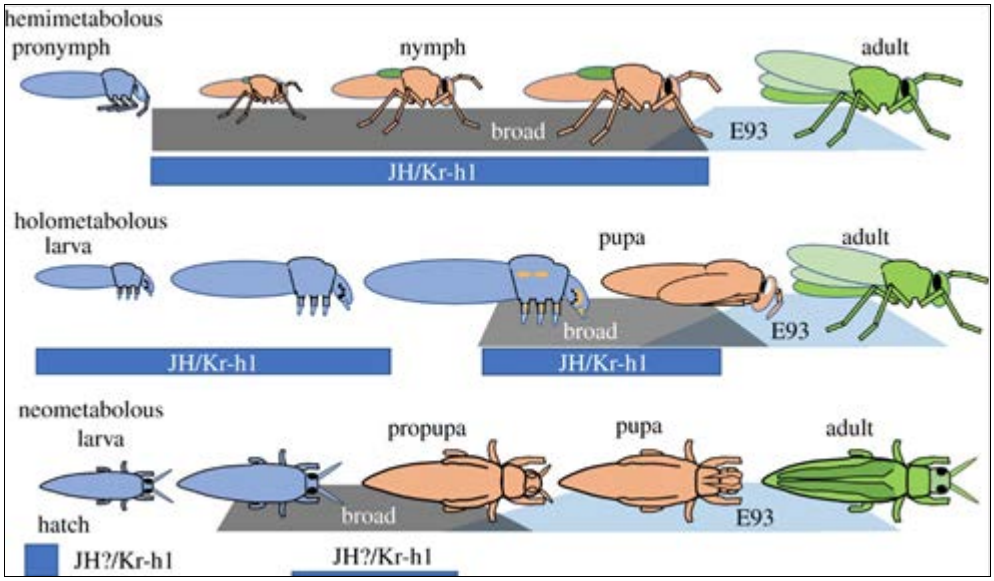

Fig 6: Relationship of Kr-h1, Broad and E93 expression [10].

Generalized diagram showing the relationship of Kr-h1, Broad and E93 expression to the various life stages of hemimetabolous, holometabolous and neometabolous insects.

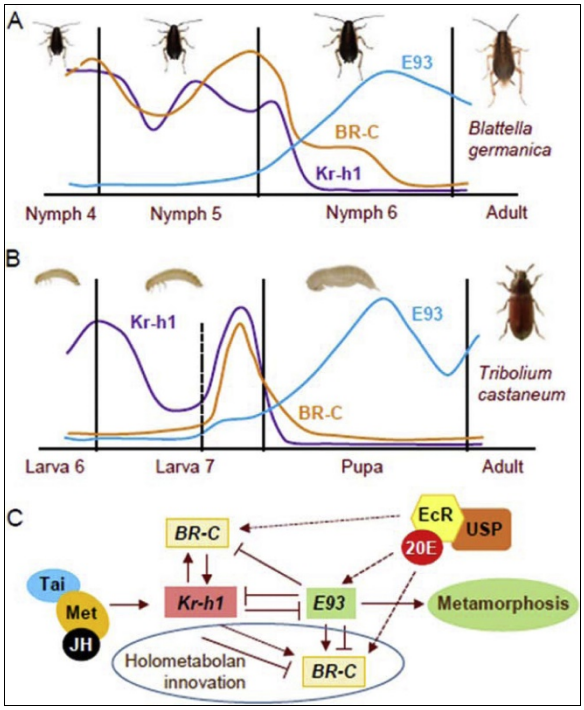

Fig 7: The MEKRE93 pathway. A. Expression patterns of Krüppel homolog 1 (Kr-h1), E93 and Broad complex (BR-C) in hemimetabolan insects (*Blattella germanica*). B. Expression patterns of Kr-h1, E93 and BR-C in holometabolan insects (*Tribolium castaneum*). C. The MEKRE93 pathway in hemimetabolan and holometabolan species [3]

The MEKRE93 pathway in hemimetabolan species

The observation that Kr-h1 represses E93 expression led to propose the MEKRE93 pathway as the essential axis regulating insect metamorphosis. Accordingly, in nymph–nymph transitions, JH acts
through its receptor Met-Taiman to induce the expression of Kr-h1, while Kr-h1 represses the expression of E93. In contrast, the decline of JH production in the final juvenile stage interrupts Kr-h1 expression, E93 becomes de-repressed, thus triggering adult morphogenesis [47]. RNAi experiments in *B. germanica* by the same authors also revealed that E93 depletion increases Kr-h1 expression, thus indicating that Kr-h1 and E93 are reciprocally repressed [3].

The MEKRE93 pathway in holometabolan species

The main difference between the hemimetabolan and holometabolan metamorphoses is the regulation and function of the Broad complex (BR-C) zinc-finger transcription factors. In hemimetabolan species, BR-C is mainly involved in promoting the growth of wing primordia. BR-C is repressed by E93 in the metamorphic transition. BRC triggers the formation of the pupal stage in holometabolan species, where JH inhibits the expression of BR-C during larval stages [12]. E93 is involved in triggering the pupal stage, as it promotes BR-C expression in *T. castaneum*. The whole data indicates that the MEKRE93 pathway is conserved in the holometabolan species, which added the E93/BR-C interaction loop to the ancestral (hemimetabolan) pathway during the evolutionary transition from hemimetaboly to Holometaboly [3].

Conclusion

Juvenile hormone and ecdysteroids (ecdysone & 20 E) are responsible for metamorphosis. A high level of the juvenile hormone causes the growth of larva from one instar to another. Without JH, the first instar of larva will remain in first instar. So, for proper growth and development juvenile hormone is a must. After reaching the last instar, JH concentration decreases, that switches transformation from larva to pupal and the adult stage has no JH present. Without ecdysone, there may be a giant larva, but no adult stage; and 20 hydroxyecdysone gives rise to two pulses, which ultimately causes pre-pupal to the adult stage. Thus, they ensure proper timing of metamorphosis by maintaining proper concentration throughout the insect's life-cycle. JH induce Kr-h1 which maintains the larval state through expression. Ecdysone inducible E93 & BR-C gene or transcription factors needed for adult and pupal differentiation, respectively. Thus, hormones exert major control over the stage specification genes such as the nymph and adult pattern of hemimetabolous insects and the larva/ pupa/adult pattern of the holometabolous insects.

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