From One Site of Insect Juvenile Hormone Synthesis, No Identified Receptors, and a Denomination as “Status Quo Hormone” in the 1960s to Multiple, Sometimes Conflicting, Possibilities to Date\

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Abstract: Juvenile hormones (JHs) are a group of acyclic sesquiterpenoids. They are biosynthesized in the mevalonate biosynthetic pathway as esters of farnesol (F). F is best known as an intermediate in this pathway that is omnipresent in all eukaryotes. Since 2019 it became apparent that F also has an important role on its own, namely in Ca\textsuperscript{2+}-homeostasis, as a receptor of some types of Ca\textsuperscript{2+}-channels in mammals. JHs occur in 6 forms, they are poorly soluble in water, and they regulate many aspects of insect physiology. In 1967, when the chemical structure of the first insect JH (JH I) was published, the corpora allata (CA), which are located in the head, were assumed to be the only site, or source, of JH-synthesis. However, the fact that JH I had not been extracted from active CA but from abdomens of young (maximum 96 hours old) adult male, not female, Cecropia moths, Hyalophora cecropia, with intact CA presented a problem. Given the large amounts of JH present in the extract, which was called “golden oil”, this JH had apparently started to accumulate during metamorphosis. How did JH I end up in the abdomen, why was it quantitatively sex- and tissue-specific, and what was its function(s) and mode of action? First, in 1976 it turned out, unexpectedly, that not the fat body as initially presumed, but the accessory sex glands (MAGs) acted as the exclusive repository for the huge amounts of JHs, namely 2.1 µg JH I and 0.3 µg JH II. It was then thought that the JHs had been synthesized by the CA, although presumed to be inactive during metamorphosis. After a long period with moderate progress, in 2014, it became established MAG-JHs are not synthesized in the CA, but by the MAGs themselves, that they not released into the hemolymph, but secreted, along with other MAG secretions, into the genital duct system of the female during copulation. MAG-JH was named “exocrine JH”, to functionally distinguish it from “endocrine/hormonal JH” that is chemically identical but synthesized by the CA in young larvae and adults. Furthermore, the focus on how JH exert its functions also shifted considerably in the past decade, namely towards molecular genetic approaches, in particular to its nuclear- and membrane receptors. Concurrently, the importance of Ca\textsuperscript{2+}-homeostasis and of the hydrophobic properties of JH also got more attention. In the pioneering days of insect endocrinology, JH had become known as the “master-“ and as the “status quo hormone”, denominations that continue to be used in some textbooks to date. What did status quo originally mean, and how did its meaning gradually evolve towards one of today’s interpretations that says that “keeping the cytoplasmic free Ca\textsuperscript{2+}...
concentration below the toxicity limit of Ca$^{2+}$ (when rising above 100 nanomolar) for a longer period of time” is a, if not the key issue. Indeed, without such very low free Ca$^{2+}$ in the cytoplasm, the well documented secondary signaling systems such as “Ca$^{2+}$ as a secondary messenger”, “Ca$^{2+}$-induced Ca$^{2+}$-release from intracellular storage sites (e.g., the endoplasmic reticulum)”, and “Control of the activity of some enzyme systems which are anchored in endomembranes, and which can be inhibited by a high intraluminal Ca$^{2+}$ concentration” could not become functional. Maintaining such low free cytoplasmic concentration of Ca$^{2+}$ in resting cells is indeed a big challenge because the average Ca$^{2+}$ concentration, in aqueous extracellular environments, is thousands of times higher (about 2 millimolar or higher). After optimal conditions for maintaining low Ca$^{2+}$-conditions can be realized, a second level of activity for JHs can start. It is mediated through intranuclear targets/receptors. In our model, extranuclear (membrane-) and nuclear targets/receptors (e.g., transcription factors) are necessarily complementary.

Key words: Calcium homeostasis, farnesol, Golgi, insect hormones, insect hormone receptors, Methoprene-tolerant

1. Introduction

Juvenile Hormone (JH) has been long known as “the status quo hormone” because it prevents metamorphosis initiated by the molting hormone 20-hydroxyecdysone (Wigglesworth 1934,1936; Williams 1959, 1961, 1963; Willis 1974, 2018; Tobe and Stay 1980; Riddiford 1996; Hartfelder 2000; Zhou and Riddiford 2002; Huang and Riddiford 2007, 2019; Li et al. 2019; Truman 2019; Riddiford 2020; etc.). This idea emerged primarily from four sets of observations: 1. Allatectomy, the surgical removal of the corpora allata (CA), in combination with reimplantation of extra active CA to overrule the effect of allatectomy. These procedures have to be seen in a historical context. Indeed, for a long time the CA were thought, erroneously, to be the only site of synthesis of JH (see subsequent sections); 2. The occurrence of supernumerary larvae in extreme natural conditions; 3. The application of natural JHs or of synthetic JH-analogs in bioassays resulting in the induction of supernumerary larval instars; 4. JH-receptor studies, both membrane- and nuclear, obtained by molecular and electrophysiological methods are more recent approaches.

The number of immature instars may not have been rigorously fixed in the evolutionarily ancient ancestors of insects. This can be inferred from the current developmental profile in some ametabolous insect species. Incomplete metamorphosis as in the Hemimetabola, and complete metamorphosis as in the Holometabola, were much later developments in evolution. Holometaboly came into being when in the last larval instar, the JH titer drops to zero by not yet fully understood mechanisms (regulatory peptides, esterases etc.) (Peferoen and De Loof 1980, Nijhout et al 2014, De Loof and Schoofs 2019a). The absence of JH stops the status quo molting scenario (from larval type into another larval type), and induces pupation, and later the adult stage. Key questions are: Which

3 A list of abbreviations used in this paper is given in Box 1.
signaling pathway(s) is (are) at the onset of metamorphosis so drastically halted or/and redirected that the status quo modus operandi of JH no longer applies? Which is its modus operandi at the molecular level?

Box 1. Abbreviations used in this paper. [Ca\(^{2+}\)]: intracellular free Calcium concentration; 20E: 20-hydroxy Ecdysone or Ecdysterone; ATP: Adenosine triphosphate; bHLH-PAS: basic Helix-Loop-Helix-Palindromic Sequence; CA: corpora allata; E: Ecdysone; EcR: Ecdysone Receptor; ER: Endoplasmic Reticulum; Erβ: Estrogen receptor beta; F: Farnesol; FLS: Farnesol-like endogenous sesquiterpenoids; FXR: farnesoid X Receptor; Gee: Germ cell expressed; JH(s): Juvenile Hormone(s); JHA: Juvenile Hormone Analog; Kr-h1: Krüppel homolog1; L3 L4: third, fourth larval stage; LXR: Liver X Receptor; MAG(s): Male Accessory Sex Gland(s); Met: Methoprene-tolerant; MF: Methyl Farnesoate; miRNA(s): microRNA(s); PCD: Programmed Cell Death; PTTH: Prothoracicotropic hormone; RER: Rough Endoplasmic Reticulum; RNAi: RNA interference; RXR: Retinoid X Receptor; SER: Smooth Endoplasmic Reticulum; SERCA: Sarco/endoplasmic Reticulum Calcium ATPase; sNPF: short Neuropeptide F; Tai: Taiman; TGF-β: Transforming Growth Factor beta; USP: Ultraspiracle.

2. Insights into the basic principles of animal development

To understand the above-mentioned cell physiological synthesis of JH as a status quo hormone, some basic principles of animal development should be kept in mind.

2.1. The broad picture: All animals are built according to the folded epithelium principle.

All animals, insects inclusive, develop from a blastula, a crucial stage in development in which all animals organize themselves into a tightly closed epithelium (De Loof 1992, De Loof et al. 1992). Maintenance of epithelial integrity (Sumigray and Lechler 2015) is of particular importance during metamorphosis of holometabolous insects. If the integrity would be lost (e.g., by local programmed cell death activity), the animals would bleed to death. This does not happen during metamorphosis, indicating that the sharp changes in either JH- or ecdysteroid titer do not affect epidermal epithelial integrity.

2.2. The picture at the level of the whole (differentiating) cell: Basic principles of differentiation during development

In its very essence the most basic rule operating during differentiation says: “Keep the genome constant during the numerous successive mitotic cell divisions, but change again and again the plasma membrane/cytoskeletal properties of the resulting cells” (De Loof et al. 1992, Sumigray and Lechner 2015). These determine to a large extent which (sets of) genes will be activated or inactivated at all stages of development. Of course, there are some exceptions to this general
rule (e.g., random second X chromosome inactivation in human females, eventual epigenetic changes, etc. (References can be found in textbooks of developmental biology, such as Wolpert et al. 2019, Barresi and Gilbert 2019).

Another basic concept is the generation of functional asymmetry as the result of “the double asymmetry principle” (De Loof et al. 1992). It says that if perfect spherical symmetry of cells exists at all (probably not), such symmetry gets lost not later than the third cleavage of the zygote into blastomeres. From then on, all differentiating cells are no longer spherical. Keeping in mind that the majority of membrane proteins does not float freely in the plasma membrane, but instead are anchored to extremities of the cytoskeleton, this means that the distribution of membrane proteins can get organized in a multitude of different constellations. The ultimate consequence of this principle is that in the whole body no two cells have a fully identical plasma membrane-cytoskeletal complex.

A third principle is the potency of cells to drive a self-generated electric current, carried by inorganic ions, through themselves, thereby becoming miniature electrophoresis chambers (De Loof 1986). This is the result of the uneven but ordered distribution of ion pump and ion channel combinations over the plasma membrane. All cells differ in their “electrome properties” (De Loof 1986, 2016). When a fertilized egg starts dividing, it uses two complementary mechanisms for transfer of “developmental information” from the parental generation to the F1 generation. One is through the classical mechanisms of genetics (DNA-RNA-proteins), the other is through sharing a part of its plasma membrane with its embedded/anchored electrical system with the daughter cells (De Loof 2016). This is the cell-physiological basis of the mechanism of the “electric rewiring” of some cell types during development and in particular during metamorphosis (De Loof et al. 2014). This aspect should be included in any explanation of the “status quo” principle.

A fourth principle says that cells are continuously confronted with the presence of huge extracellular concentrations of Ca\textsuperscript{2+} in their aqueous extracellular environment. Above the very low limit of about 100 nanomolar, Ca\textsuperscript{2+} becomes toxic. This means that cells have to engage in lifelong efforts to keep their intracellular/cytoplasmic free ([Ca\textsuperscript{2+}]i) concentration very low, this being the essence of Ca\textsuperscript{2+} homeostasis. JH is a key player in this respect (De Loof and Schoofs 2019b, 2020). This aspect of cellular physiology continues to be highly undervalued in insect endocrinology up to the present day.

In addition to these principles, one should also keep in mind that JH is not a single molecule that universally occurs in all insect species. According to Tsang et al. (2020) different insects have evolved with different sesquiterpenoid biosynthetic pathways which could yield different products.

Thus, development is much more than just the story of genes getting sequentially (in)activated by transcription factors. Non-coding RNAs such as microRNAs have recently been explored in the regulation of sesquiterpenoid biosynthesis (Tsang et al. 2020, Ling et al. 2017). Therefore, all the above-
mentioned aspects need to be considered when investigating the role of hormones, in particular that of juvenile hormone, a master hormone of insect development.

3. **Hormonal control of the status quo**

3.1. **Precocious metamorphosis and formation of supernumerary larvae in natural (extreme) conditions**

Supernumerary larvae and pupae can occasionally form in extreme living conditions, thus without surgical manipulation of the CA. In the tobacco hornworm *Manduca sexta* (Linnaeus, 1763) (Lepidoptera: Sphingidae), Safranek and Williams (1984) reported that fifth instar larvae underwent a supernumerary larval molt under severe feeding regimes. Supernumerary molting occurred only when initiated at a live weight of less than about 3 g. At higher weights pupation took place. With what is known on hormonal control of feeding to date, an important role for the neuropeptide sNPF can be suggested (Kaneko and Hiruma 2014, Fadda et al. 2019).

According to Malczewska and Cymborovsky (1988) a cold shock (cooling) at the onset of the last larval instar of the greater wax moth *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) induces supernumerary molts. In the beetle *Tribolium freeman* Hinton 1948 (Coleoptera: Tenebrionidae), a crowded environment in the last larval instar delays the development into a pupa, and the beetle continues to engage in larval-larval molting (Ruangsrit and Park 2018). This raises the question whether the different regimes that yield a supernumerary larval instar involve a common hormonal inducer and similar transcription factors (see section 6.2).

3.2. **A role for the corpora allata and their juvenile hormone?**

In the pioneering days of insect endocrinology, the comparison of effects of microsurgical removal of the CA with a sham operation was the most straightforward way to learn about the physiological and morphological effects that are under control of hormone(s) secreted by the corpora allata (Wigglesworth 1936, Williams 1959, Tobe and Stay 1980). Extirpation of active CA in an early larval instar resulted in an abnormal molt, namely in a precocious molt into the pupal form instead of the next larval instar. Implantation of supernumerary active CA in the penultimate larval instar resulted, in some but not all species, in the formation of a supernumerary larval instar. Application of some synthetic JH analogs also induce a supernumerary instar. For example, in the beetle *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae), application of juvenile hormone analogs (JHA) during the penultimate and final instars blocked larval-pupal premetamorphosis and induced supernumerary larval molts (Parthasarathy and Palli 2009).
3.3. Effects of allatectomy: most relevant for understanding the complexity of “status quo”

The effects of allatectomy have been studied in a variety of insect species. The Colorado potato beetle, Leptinotarsa decemlineata Say, 1824 (Coleoptera: Chrysomelidae) served as a particularly good model to examine the diverse cell-physiological processes that are under control of JH. This insect is sensitive to photoperiod (de Wilde 1965). When living under long day conditions (≥ 12 hours light per day), the adults engage in reproduction. When experiencing short day (≤ 12 hours light per day) conditions, they refrain from reproduction, but instead start preparing for entering diapause/hibernation. Diapause can also be induced in long day animals by allatectomy, indicating a key role for juvenile hormone.

The changes induced by the sudden drop to zero of JH were already described fifty years ago by De Loof and Lagasse (1970). They mapped the changes observed in electron microscopic pictures of the fat body of adult Colorado beetle females which were either allatectomized or raised in short day conditions (zero JH condition) versus in females that were ovariectomized (normal high JH titer). They showed that absence of JH affected nearly all cell physiological processes: reduced multiplication of mitochondria, dysfunction of the protein secretory activity by the Golgi system, reduced lipid and glycogen synthesis when compared to the ovariectomized female situation, altered architecture of the nucleus and nucleolus, altered protein synthesis, altered membrane characteristics as indirectly revealed by dysfunctional membrane fusion, and changes in cytoskeleton/nucleoskeleton. Similar changes were found in the larval fat body after the JH titer drops to zero in the last larval instar (De Loof 1972). Briers et al. (1982) added the finding that it is the absence of JH that makes the ecdysteroid titer in adult beetles rise as part of the induction of diapause. All these changes could be overruled/rescued by application of synthetic JH or implantation of active CA. Thus, JH indeed is the “master hormone” in cell physiology. No indication from these experiments that in addition to JH, another factor secreted by the CA is necessary for the rescue. This, however, does not exclude that a factor other than JH is secreted by RER-Golgi systems present in the CA (e.g., a protein/peptide with growth promoting properties, De Loof and Schoofs 2020).

By complementing this electron microscopic study with a comparison of the protein composition of the hemolymph (De Loof and de Wilde 1970a, b) it could also be demonstrated that JH has drastic effects on the transcription of specific genes. Indeed, following allatectomy or forced entry into diapause by short-day conditions, the fat body starts synthesizing three major “diapause or storage proteins” which accumulate to high concentrations in the hemolymph. Concurrently, the synthesis of “reproduction-related proteins”, in particular of vitellogenins (De Loof and de Wilde 1970b) in females is terminated. Thus, in the absence of JH, transcription switches from transcribing one set of proteins to another one. How such a switch is realized remains unexplained until this very
day. It challenges the current ideas on the mode of action of JH (nuclear and membrane) receptors (see later). The major result of these classical experiments is that JH has many targets, and that, given this multitude, it is highly improbable that all these effects can be explained by the interaction of JH with one single nuclear receptor molecule (see later). One typical example is that farnesol, the precursor of JHs and of methyl farnesoate (Teal et al. 2014) that itself has JH-bioactivity (Schmialek 1961), may also act as a hormone in various insect orders. It plays an important role in the functioning of quite some G-protein coupled receptors through “prenylation”. This is a non-genomic, partially Ca\(^{2+}\)-homeostasis linked, effect of farnesol-like endogenous sesquiterpenoids (FLS) (De Loof and Schoofs 2019c). Another probable non-genomic effect that recently emerged (De Loof and Schoofs 2020) is that farnesol/JHs, which seem to be synthesized by the Golgi apparatus itself, also sort effects within the Golgi, namely as membrane fluidizers and, according to Murgolo et al. (1989) probably also in glycosylation of proteins.

4. Classical bioassays for detecting JH activity: changes in the cuticle

In the pioneering days of insect endocrinology, first allatectomize an experimental animal and next evaluate whether or not the application of a tissue extract could overrule the “negative effects” of allatectomy, was used as a JH-bioassay. Similarly, application of such extracts to induce a supernumerary larval instar has been tried as well. However, such bioassays are not very sensitive and not suited for testing large numbers of samples. More sensitive bioassays have been developed. Two of them have been of outermost importance. One employed a hemimetabolous species, namely the bug *Rhodnius prolixus* Stål, 1859 (Hemiptera: Reduviidae) and was developed by Wigglesworth (1973, see also Riddiford 2020). The second bioassay, developed by Gilbert and Schneiderman (1960), employed the holometabolous lepidopteran *Galleria mellonella*, commonly known as the greater wax moth (Figure 1). Both enabled observing JH-dependent changes in the cuticle following application of compounds with JH activity. In both, it was soon observed that inflicting an artificial wound in the epidermis before applying an extract that contains a JH-active substance greatly enhanced the sensitivity of both assays. Both assays have also been widely used for detecting JH activity in extracts from various animal- or plant sources (Wigglesworth 1969, Sláma 2013). To date, these classical bioassays for detecting and quantifying JH are no longer used: they have been replaced by modern chemical assays, in particular mass spectrometry and molecular methods. In this paper, we only elaborate on experiments with the *Galleria* bioassay.
Figure 1. The life cycle of the greater wax moth *Galleria mellonella*. Pupae less than 24 hours old after the onset of pupation are used in the JH-bioassay. Older pupae are no longer sensitive. From Abdalla and Battaglia (2018), Open Access with Copyright © 2018 by authors and Scientific Research Publishing Inc., licensed under CC BY 4.0. This species has been reported to have up to ten larval stages (Wojda et al. 2020).
4.1. The essence of the *Galleria* bioassay

The essence of this bioassay is: Application of JH (analsogs) induces the formation of a patch of “supernumerary” pupal cuticle in young pupae, treated less than 24 hrs after the larval-pupal molt. Inflicting a wound in the epidermis/cuticle greatly enhances the sensitivity of the bioassay. It induces regeneration of the damaged epidermis. Later than 24 hrs after the larval-pupal molt, the pupal epidermis becomes insensitive to JH. This bioassay can also be used for detecting compounds with anti-juvenile properties (Van Mellaert et al.1983).

4.2. How was the assay performed? Technical aspects

For (digital era-native) researchers who are not familiar with the basics of this classical assay for detecting JH activity, a brief summary of its (re)description by De Loof and Van Loon 1980) is given. Its principle is as follows. *Regenerating* epidermal cells induced by inflicting a wound in the integument are exposed to JH for a long time. The wound is made by cutting away a piece of integument with a piece of a sharp razor blade. Next, the wound is sealed with a drop of warm, molten paraffin mixed with an oil containing the JH extract. This paraffin-JH mixture is used as a slow-release formula, an essential element in this and other JH-bioassays. Prolonged exposure to JH of about one week is necessary in order to observe the JH-induced changes in the newly forming cuticle.

4.3. Effects of JH on the regenerating epidermis and cuticle formation

The regenerating cells will form a closed epithelium (restoring epithelial integrity) and form a new cuticle. The structure of the cuticle of the regenerating JH-exposed cells differs from that of the surrounding adult type cells. The normal, non-JH-treated adult cuticle is grey and has scales, whereas the cuticle under the paraffin-JH seal is brown and wrinkled as is typical for a normal pupa (Fig. 2). This is the qualitative effect of the assay: all compounds with JH activity [i.e., thousands of synthetic JH-active compounds exist (Sláma 2013)] yield a similar effect. This means that they likely act on the very same cell-physiological target. However, the potency of different compounds can be very different. Some compounds (e.g., farnesol), are only weakly active, while others are very active in low concentrations. Comparative quantitative assaying can be done by making use of a dilution series and testing all dilutions until the dilution is found in which only half of the treated animals produce the brown wrinkled cuticle as shown in Figures 2 and 3. The assay is extremely sensitive given that one *Galleria* Unit corresponds to 2 pg for JH I, to 2 pg for JH II and to 70 pg for JH III (De Loof and Van Loon 1980). In fact, if one considers that only a small percentage of the JH-active compound in the paraffin-oil mixture reaches the regenerating wound cells of the young pupa, the effective amount of JH I that corresponds to 1 GU must be orders of magnitude lower. That illustrates that a small gland like the CA can produce enough hormone to accommodate the whole body in its need for JH.
Figure 2. The *Galleria* bioassay for detecting Juvenile Hormone activity. (A) The assay is performed on pupae less than 24 h after pupation. (B) A hole is cut through the cuticle and epidermis of the pronotum. Next the wound is sealed with molten paraffin mixed with a mineral- or vegetal oil (the whitish spot in the middle of the square). (C) One week later (at 30°C) the epidermis underneath the paraffin drop has regenerated and has formed a new cuticle. If the oil did not contain a compound with JH activity, the regenerated cuticle is of the adult type, namely white gray (arrow). (D) If the oil contained JH activity, in this example it was JH III, part of the newly formed cuticle is pupal like, namely brown and wrinkled (arrow). (E) A positive reaction, weaker than with JH III (arrow) was obtained when farnesol was mixed with the oil. This figure (= our work) and the legend is partially modified from Figure 4 in De Loof et al. (2014) in *General and Comparative Endocrinology*, with permission from the publisher (Elsevier).
5. Ecdysteroids during metamorphosis: key actors in the termination of the *status quo*

5.1. Ecdysteroids as sex hormones: the first major E peak marks the end of the juvenile state and the beginning of the reproductive phase (“puberty”)

Part of the reasons why it took so long to grasp the very essence of “status quo” resides in the original naming of “ecdysone”, which referred to its role in
molting. Ecdysis in Greek means molt. The bioassay used to monitor the purification of the molting hormone from an extract of silkworm pupae focused on its effects on pupariation (Karlson and Sekeris 1966). It took another decade before Huybrechts and De Loof (1977) reported that injection of ecdysterone (20E) into adult males of the flesh fly Sarcophaga bullata (Parker, 1916) (Diptera: Sarcophagidae) induced the synthesis of vitellogenin, a typical reproduction-related yolk protein that normally only occurs in females. That resembled the situation that treating roosters with 17 beta-estradiol induces the appearance of vitellogenin-mRNA in their liver (Burns et al. 1974) and vitellogenin in their blood. This triggered the idea that ecdysteroids might also act as sex steroids in insects (De Loof 2006). It should be noted that at that time the general consensus view was that insects “do not have sex hormones, that reproduction was totally genetically controlled”, which is impossible.

In retrospect: if it had been known in the early sixties that the molting hormone also had sex steroid activity, the naming of “ecdysone (E)/ecdysterone (20E)” might have been different, such as sex-ecdysteroids. This changes the interpretation of data: the high concentration of 20E that manifests itself in the pupa in fact indicates that for the first time in its development the insect body is flushed with high titers of sex steroids for a long time. In juvenile stages there are also ecdysteroid peaks at molts, but they are of shorter duration. It is sometimes overlooked that sex steroids have to be present lifelong for sustaining sex-dependent physiological processes. One should consider the possibility that not so much the height of a steroid hormone peak, but the length of its duration is important. This may be particularly true for hydrophobic hormones. This is one of the lessons from the Galleria bioassay: for obtaining a lasting effect, JH has to be present in a slow-release mode (diffusion out of a paraffin droplet) for almost a week. In such circumstances, the amount of JH that comes into contact with the regenerating epidermal cells is very low but sufficient for eliciting an effect (De Loof and Van Loon 1980). Hence, rising concentrations that last long can be considered as the functional counterpart of the increase of androgens and estrogens at puberty of mammals, humans inclusive. Thus, the appearance of the first major post-larval ecdysteroid peak marks the end of the juvenile stage. In anthropomorphic wording, the newly formed “puber insects” (named “pupae”) start becoming sexually mature.

5.2. When does the ecdysteroid titer peak?

During early larval instars, the ecdysteroid titer is low, with some small rise at each molt. A high JH titer inhibits the biosynthesis of large amounts of ecdysteroids (De Loof et al. 2015; De Loof and Schoofs 2019a). The normal situation is that during metamorphosis the ecdysteroid titer rises to high values after the JH titer has fallen to zero.

Which mechanism(s) is/are causal to the pupal E peak(s)? The major one is the drop to zero (in holometabolous insects) of the JH titer. With the Colorado
potato beetle as an example, Meng et al. (2018) postulated that this may function as follows. A high JH titer inhibits the production and release of prothoracicotropic hormone (PTTH) before the young larvae attain critical weight. Once the critical weight is reached, JH production and release are stopped, and the JH present in the hemolymph is cleared from the body. This allows the brain to release PTTH that, in its turn, stimulates ecdysteroid biosynthesis and release to start larval-pupal transition.

At the subcellular level of the multiple (De Loof et al. 2015) tissues that can engage in ecdysteroidogenesis, the absence of JH may be explained as follows. Some of the ecdysteroidogenic enzymes reside in membranes, in particular of the smooth endoplasmic reticulum (SER). As long as the JH titer is high, the SERCA pumps actively pump Ca\(^{2+}\) into the SER’s lumen. The role of farnesol/JH is likely that of a natural endogenous agonist of the SERCA pump (the “thapsigargin argument” in De Loof et al. 2014, 2015). At high intraluminal concentration, Ca\(^{2+}\) apparently inhibits the ecdysteroidogenic enzymes, thereby keeping the ecdysteroid titer low during larval life. When the farnesol/JH titer drops to low values (i.e., under the influence of changing activities of allatoregulatory neuropeptides, allatostatins, allatotropins, short NPF-like peptides, etc.), growth factors (e.g., TGF-\(\beta\), Ishimaru et al. 2019) or neurotransmitters, the pumping activity of the SERCAs decreases, and the inhibition of the ecdysteroidogenic enzymes decreases. One or more (Briers et al. 1983) ecdysteroid peaks will result. The hormonal regulation of ecdysteroidogenesis is much more complex than being just JH-titer- and transcription factor-dependent (e.g., by PTTH, etc.; De Loof et al. 2015, Marchal et al. 2010, Roy et al. 2018). This topic will not be discussed in depth in this paper.

If ecdysteroids play a role in inducing scales in normal cuticle forming adult cells, the regenerating wound cells that experience the same ecdysteroid titer, “catch” the same hormonal message, but they respond differently. This suggests that during metamorphosis, the regenerating wound cells become differentially “(electrically) wired” (De Loof et al. 2014) with respect to their ecdysteroid receptors and inorganic ion-driven signaling pathways. It has been shown a long time ago that 20-hydroxyecdysone (20E) activates the transcription of specific genes. A clear illustration is that injection of 20E, but not of JH, induces the synthesis of vitellogenin in adult males of the flesh fly *Sarcophaga bullata* (Huybrechts and De Loof 1977). Stoppie et al. (1981) fed 20E to adult males of *Sarcophaga bullata* and observed that their fat body cells acquired an extensive RER, while JH was completely ineffective. This showed that although JH is needed for reproduction in adults, it is the ecdysteroids that induce the appearance of the machinery for synthesis and secretion of secretory proteins. However, this does not hold true for all insects: different modes of hormonal regulation of reproduction have been reported (e.g., in mosquitoes, locusts etc.; Wyatt and Davey 1996, Roy et al. 2018).
5.3. Synthesis of cuticular proteins: developmental variability and specificity

Insects can synthesize various types of cuticular proteins during development. At the time, the *Galleria* bioassay was in use, molecular methods were not yet widely used. The question on how the JH present in the paraffin drop overlying the regenerating epidermal tissue switches on the genes for pupal cuticle synthesis instead of the typical early larval instar genes, or how JH inhibits adult cuticle-type genes has never been thoroughly investigated. With the methods which are available to date, these interesting questions could be addressed. Rondot et al. (1996) reported that in *Tenebrio molitor* Linnaeus, 1758 (Coleoptera: Tenebrionidae) a larval-pupal-specific cuticular protein (TMLPCP-22) is regulated in a stage-specific and tissue-specific manner. The transcript is present during the secretion of pre-ecdysial larval and pupal cuticles and is restricted to epidermal cells. It is not expressed during adult cuticle deposition. In supernumerary pupae induced by a juvenile hormone analogue that is known to inhibit the adult program, TMLPCP-22 is expressed again, confirming its larval-pupal specificity.

RNAi has been used by Parthasarathy and Palli (2009) to analyze the effects of the pupal specifier gene *broad (br)* in a basal holometabolous insect, *Tribolium castaneum*. Application of hydroprene, a juvenile hormone analog, during the prepupal period caused a repeat of the penultimate *br* expression patterns, and the formation of supernumerary larvae. The authors suggest that the *br* isoform expression may have played an important role in the evolution of the pupa in holometabolous insects. The literature on *broad* is extensive and will not be reviewed here.

Over the years, the knowledge on the composition and control of the synthesis of cuticular proteins and the molting rhythm has substantially increased. This topic will not be dealt with here in extenso: the reader is referred to (Ishimaru et al. 2019, Bouhin et al. 1992, Bellés and Santos 2014, Daimon et al. 2015, Zhou et al. 2019).

6. Hormones act through receptors: which ones for JH?

A key question in this review relates to the type of JH-receptor(s) that is/are involved in JH signaling: only a membrane receptor(s), or only a nuclear receptor(s), or a combination of both acting with a secondary messenger, in particular Ca^{2+} (De Loof and Schoofs 2019a)? To date (early February 2021), this remains a controversial issue.

6.1. Introductory remark: the tricky “hydrophobicity issue” for intracellular and intranuclear JH-signaling

The hydrophobic nature of JH implies that it dissolves well in oils, that it enters the lipic membrane system of cells, in fact of all cells of the body, and that it can engage in hydrophobic interactions with the hydrophobic parts of some macromolecules, in particular proteins. Proteins that are anchored in membranes
have hydrophobic amino acid strands, otherwise they could not accommodate in membranes. The poor solubility of JH in water implies that for transport through the watery cytoplasm, if it happens at all, a lipophilic carrier would be needed (De Loof and Schoofs 2020). Such JH-specific carrier has not yet been identified. In the past decades numerous attempts have been undertaken to chromatographically purify the tentative JH receptor(s). They all failed. A major reason was that JH was found “to stick to everything”, hence many chromatographic fractions tested positive in JH-binding studies.

Only a few cases (Osir and Riddiford 1988, Palli et al. 1990) have as yet been published on whether JH, being hydrophobic, can enter the nucleus. Osir and Riddiford (1988) showed using both tritiated JH and an iodinated analog of Methoprene that JH was taken up by Manduca larval epidermis and that about 1/3 was found in the nuclear fraction. Moreover, within the nuclear fraction, there was a high affinity binder and a second binder of about 13-fold lower affinity. The nuclear fraction comprises both the nucleoplasm and the nuclear envelope. In section 8 the potential importance of the latter will be mentioned. One should keep in mind that control of transcription of specific genes by JH does not necessarily require that JH enters the nucleus. The activation of target genes can be achieved with the help of inorganic secondary messengers, such as Ca^{2+}. This possibility was already experimentally documented half a century ago by Lezzi, Kroeger and others (references in Lezzi 1970, Ashburner and Cherbas 1976, De Loof et al. 2014). Section 8 deals with novel insights on this topic.

6.2. A controversial dichotomy in thinking and approaches on the nature of JH receptor(s)

In particular during the 2010-2020 decade, as compared to the pioneering days of the discovery of JH dating from about half a century ago, a drastic shift in thinking took place on how to analyze the effects and mode of action of JH. In the “old days” the mode of action of JH was framed into the principles of general cell physiology. The formulation of the “central dogma of molecular biology” by F. Crick dates from 1970. Subsequently, a huge array of molecular biological techniques was invented and applied in nearly all disciplines of biology, endocrinology inclusive. The resulting literature is huge. To keep it digestible, ever more subdisciplines and ever smaller “bubbles” were created. The Met/Gce topic (see later) became such a recent microbubble that started to overshadow the much bigger classical cell-physiological bubble of the mode of action of JH.

The advent of molecular genetic techniques, in particular comparative whole genome sequencing projects in model organisms yielded, among numerous other novel insights, the discovery of numerous transcription factors. Approximately 10% of genes in the genome code for transcription factors. In humans this means that this family is the single largest family of human proteins. It is also well documented that genes are often flanked by several binding sites for distinct transcription factors, and that efficient expression of each of these genes requires
the cooperative action of several different transcription factors (Multiple Authors 2020, Ortega-Dominguez et al. 2015, Lambert et al. 2018). This also holds true for insects in general. According to Truman and Riddiford (2019) the key transcription factors that are associated with the holometabolous life stages are: Krüppel-homolog 1 (Kr-h1) in the larva, Broad in the pupa and E93 in the adult. Kr-h1 mediates JH action (Truman 2019, Dubrovsky and Bernardo 2014, Saha et al. 2019). The transcription factor Methoprene-tolerant (Met) and its paralog Germ cell expressed (Gce) are currently thought to be of crucial importance for explaining the molecular mode of action of JH (Wilson and Fabian 1986, Baumann et al. 2010, Jindra et al. 2015, Wang et al. 2007, Abdou et al. 2011, Marchal et al. 2014, Konopova and Jindra 2007, Konopova et al. 2011, Zhu et al. 2019, Jindra and Bittova 2020).

Methoprene is one of the numerous chemical analogs of JH that was developed as a candidate insecticide. The rationale was that, if applied in a sensitive stage of development, a potent analog might cause death or at least infertility. Most wild type Drosophila (Diptera: Drosophilidae) are sensitive. In 1986, Wilson and Fabian reported that in an EMS mutagenesis screen with Drosophila melanogaster Meigen, 1830 as a model, they selected two mutants, one of which was nearly 100 times more resistant than the wild type to either JH III or Methoprene. The mutation was named Methoprene-tolerant (Met). It altered juvenile hormone reception in target tissues. A second mutant was isolated much later (Bauman et al. 2010) and named Germ Cell Expressed (gce). It was shown to be a paralogous gene to Met. They are members of the of bHLH-PAS domain domain (basic Helix-Loop-Helix-Palindromic Sequence) transcriptional factor family (Jindra et al. 2015). The bHLH structural motif characterizes one of the largest families of dimerizing transcription factors are often important in development or cell activity. However, in general, they have much narrower cell physiological effects than nuclear receptors that can modify/change entire developmental programs.

Not Met but gce is the parent gene. The gce gene has many introns as compared to a very few in Met. Since then, Met has been isolated from many different insects (Jindra et al. 2015; Wang et al. 2007) and found to be more similar to the Drosophila gce than to Drosophila Met. In Drosophila Met and gce are partially redundant in transducing JH action (Abdou et al. 2011). Both Met and gce products promote JH action in preadult but not adult stages (Jindra et al. 2015). Silencing of gce in transgenic flies results in preadult lethality in the absence of Met. Both Met and gce null single mutants are fully viable, but the Met gce double mutant dies during larval-pupal transformation. Exogenous application of JH rescues JH-deficient animals but not the Met27 gce2.5k mutants. Thus, unlike Met, gce is a vital gene. Met knockdown in adult female cockroach, Diploptera punctata (Eschscholtz, 1822) (Blattodea: Blaberidae), completely inhibits ovary development (Marchal et al. 2014). The loss of Met in Tribolium by RNAi prevents normal metamorphic responses to JH (Konopova and Jindra
Recently, Zhu et al. (2019) reported that in *Aedes aegypti* (Linnaeus in Hasselquist, 1762) (Diptera: Culicidae), during the L3 and L4 stages, Met mediates JH suppression of pupal/adult genes involved in the synthesis and melanization of the cuticle and blood meal digestion.

The view of Jindra et al. (2015) as well the hypothesis that the JH receptor might prove to have potential as a juvenoid “insect growth regulator” (Jindra and Bittova 2020) gained wide acceptance. Some publications even refer to Met as being the JH receptor (that explains it all) (Jindra and Bittova 2020), Met/Gce fulfills all the classical criteria for a JH receptor: specific high affinity binding, necessity in many different developmental and physiological situations where JH is known to play a major role, and regulation of Kr-h1 in different insect nymphs and larvae where Kr-h1 is known to be necessary for preventing metamorphosis.

In *Drosophila* Met always enters the nucleus, irrespective of whether JH is present or not, whereas Gce distributes itself throughout the cell in the absence of JH and only stays in the nucleus in the presence of JH (Greb-Markiewicz et al. 2011, 2015). However, we disagree, not with the importance of Met/Gce, but with referring to Met/Gce as the receptor, because that obscures the role of JH as “master hormone” that plays a role in various signaling pathways (De Loof and Lagasse 1970, De Loof and Schoofs 2019a) There are also non-genomic JH-dependent processes. Furthermore, even to date (2021) it has not yet been shown that in Met/Gce experiments JH, Met’s/Gce’s supposed natural ligand, ever enters the nucleus (see also section 6.1). To date, this is an absolute requirement for its classification as a nuclear JH receptor, but novel insights that focus on the role of the nuclear envelope are emerging (see section 8).

6.3. In retrospect

A brief historical oversight summarizes some major reasons why we adhere to the view that as long as this situation persists, Met/Gce should be denominated as a JH target and not as the JH receptor.

- In 1967 Röller et al. (see also Röller and Dahm 1968) described for the first time the chemistry of a juvenile hormone (JH I).

- In 1969 Wigglesworth hypothesized that JH first acts at the level of the plasma membrane of its target cells, and that JH-dependent changes in nuclear transcription activity are a secondary effect.

- In 1970 De Loof and Lagasse (1970) and De Loof and de Wilde (1970a, b) showed that in the Colorado potato beetle, JH is indeed a master hormone that plays a role in major signaling pathways in cell physiology, either by being present or by being absent: differential protein-, lipid- and glycogen synthesis, differential functioning of the mitochondria, of the nervous system (with effects
on behavior, and in particular of the secretory activity of RER-Golgi system, of the structure of the nucleus and the cytoskeleton, were major effects.

- In 1976 Shirk et al. reported that the accessory sex glands (MAGs) are the repository for juvenile hormone in male Cecropia moths, *Hyalophora cecropia* (Linnaeus, 1758) (Lepidoptera: Saturniidae). Weirich and Culver (1979) showed that the enzyme to convert JH acid to JH was only present in the male Cecropia accessory gland, a suggestion that the MAGs might, perhaps, be a site of synthesis of JH. It took until 1996 and 2014 before this was further explored (see next).

- In 1982 Briers et al. (1982) described that disappearance of JH from the hemolymph of the adult Colorado potato beetle results in a drastic rise of the ecdysteroid titer.

- In 1988 Yamamoto et al. (1988) showed that in accessory glands of virgin *Drosophila* the signaling of JH III on stimulation of protein synthesis requires both the presence of Ca\(^{2+}\) in the *in vitro* incubation medium as well as protein kinase C activity. JH III mimicked the effect of copulation-induced response of increased protein synthesis. Thus, signaling at the level of the plasma membrane precedes intranuclear signaling, as had already been hypothesized earlier by Wigglesworth (1979).

- The long-held view that the corpora allata are the unique site of synthesis of JH had to be abandoned in 1994 when Borovsky et al. (1994a, b) showed that the gonads of a mosquito biosynthesize JH. Later Paroulek and Sláma (2014) also demonstrated that the male accessory glands (MAGs) of *H. cecropia* are not just a passive repository of JH I, but its site of synthesis as well, and that this JH is not released into the hemolymph. The MAGS also produce Vitamin E. This resulted in the term “exocrine JH”.

- In 1997-1999 Roullet et al. (1997, 1999) and Luft et al. (1999) using electrophysiological methods reported that a Ca\(^{2+}\)-channel-type acts as a genuine membrane receptor for farnesol (in mammals). This demonstrated that endogenous sesquiterpenoids apparently have a function in Ca\(^{2+}\)-homeostasis, apparently in keeping the cytoplasmic free Ca\(^{2+}\)-concentration low by keeping Ca\(^{2+}\)-channels closed as much as possible. A very low cytoplasmic Ca\(^{2+}\) concentration is the (often overlooked) absolute requirement for Ca\(^{2+}\)-based secondary messenger systems to become functional. This formed the basis for the view of De Loof and Schoofs 2019a, b) that not a single membrane- or nuclear target molecule, but the integrated whole Ca\(^{2+}\)-homeostasis system acts as the primordial receptor system not only for JH, but for ecdysteroids as well.
• In 2013 Sláma reported that about 4,000 different compounds, most of them synthetic ones, have been shown to be active in JH-bioassays. Many are farnesol-like, some are peptides. It is highly unlikely, not to say impossible, that all these compounds would only bind to a single receptor (Met) in the nucleus. Bittova et al. (2019) reported that the exquisite ligand stereoselectivity of the Drosophila Gce transcription factor contrasts sharply with its broad agonist repertoire (including synthetic Gce agonists of disparate chemistries). This is an argument in favor of the existence - in JH-target cells - of multiple JH binding sites.

• In 2018 Borovsky et al. (2018) reported that JH affects the splicing of Culex quinquefasciatus Say, 1823 (Diptera: Culicidae) early trypsin messenger RNA. How this effect is achieved is not yet known, neither whether there is a role for Ca^{2+}.

• All this led to the view that JH not only can act as a hormone, but also as an “inbrome” acting from within the cell (De Loof et al. 2015) in ways, different from the transcription-dependent mode (De Loof and Schoofs 2019b, 2020).

This time table is far from exhaustive, but it suffices to support our claim that, given JH’s hydrophobic properties and its role in the complex Ca^{2+}-homeostasis system, there must be many binding sites/targets for JH.

6.4. Met needs companion transcription factors. Programmed cell death

The research field of insect nuclear receptors for JHs and 20E keeps expanding as do the insights into the complexity of the Met system. Met does not stand on its own. Indeed, in Drosophila, Methoprene-tolerant (Met) and Germ cell-expressed (Gce) bHLH-PAS transcription factors are products of two paralogous genes as already cited in section 6.2. According to Liu et al. (2009) Met and Gce redundantly transduce JH action to prevent 20E-induced caspase-dependent Programmed Cell Death (PCD) during larval molts by induction of Krüppel homolog1 (Kr-h1) expression. Kr-H1 then inhibits the appearance of broad transcription in the early larva. With respect to PCD, an important issue in metamorphosis, one should not overlook the fact that not only transcription factors, but the rise of the intracellular Ca^{2+}-concentration is also causally linked to PCD/apoptosis (Orrenius et al. 2003). The important role of 20E in PCD will not be discussed here.

Krüppel homolog1 (Kr-h1) is a transcription factor that plays key roles in molting and metamorphosis and in modulating JH action. Bellès (2020) refers to Kr-h1 and Early ecdysone response gene (E93) as the doorkeeper and the key to insect metamorphosis. In the bed bug Cimex lectularius Linnaeus, 1758 (Hemiptera: Cimicidae) high levels of Kr-h1 mRNA occur in the penultimate nymphal stage. They drop to low values in the last nymphal stage. Knockdown of
Kr-h1 in the penultimate stage results in precocious development of adult structures. According to Gujar et al. (2016) Kr-h1 maintains the immature stage by suppressing E93 (early ecdysone response gene). Knockdown of E93 in the last nymphal stage results in the formation of supernumerary nymphs. Topical application of methoprene to last instar nymphs induces Kr-h1, it suppresses E93, and induces the formation of supernumerary nymphs. This sequence of events is rather similar to that described in the cockroach Blattella germanica Linnaeus, 1767 (Blattodea: Ectobiidae) by Santos et al. (2016).

6.5. MicroRNAs

A novel insight concerns the involvement of microRNAs (miRNAs) in metamorphosis (Tsang et al. 2020, Bellés 2017). In hemimetabolans metamorphosis, miR-2 controls Kr-h1 transcripts, which determines adult morphogenesis. This mechanism is quite exceptional and was apparently lost during the transition from hemimetaboloy to holometaboloy (Bellés 2017). According to Song and Zhou (2020) miRNA target genes are involved in JH and 20E signaling pathways and both hormones reciprocally regulate miRNA expression, forming regulatory loops of miRNA with JH- and 20 signaling cascades. The alternative splicing of genes in JH and 20E-signaling pathways has distinct functions in insect metamorphosis and oogenesis. The authors focused on alternative splicing of JH receptor dimer gene Taiman.

6.6. The Colorado potato beetle is again showing up as a valuable model.

Fifty to sixty years ago, the Colorado potato beetle, Leptinotarsa decemlineata, served as a model to elucidate the role of JH as a master hormone in development, induction of diapause and control of reproduction. Some of the effects resulting from induced absence of JH resemble those occurring in the brain of late Alzheimer’s disease patients (De Loof and Schoofs 2019d). In the recent past (2017-), L. decemlineata has again become attractive as a model to study the role of transcription factors linked to the signaling of JH and 20E (Xu et al. 2019 a-d, 2020). In this insect, the transcription factor Taiman is involved in the mediation of both JH and 20-hydroxyecdysone signaling. According to Xu et al. (2019b, c) silencing Taiman impairs larval development. Continuous ingestion of dsTai for three days by their (penultimate) instar larvae caused approximately 20% larval mortality and 80% pupation failure. Broad (Xu et al. 2019d) and ecdysone receptor isoforms (Xu et al. 2020) also play roles in metamorphosis in Leptonotarsa.

This beetle might be a good experimental model to address a most challenging issue in the mode of action of JH at the level of transcription. Indeed, as already mentioned before, when in adults the JH titer is high, the synthesis of “reproduction related-proteins” (yolk proteins in females and male-specific protein in males) is triggered, while concurrently the transcription of the genes coding of the “diapause-related proteins” is inhibited. Upon allatectomy, this
pattern is reversed: the synthesis of “reproduction-related proteins” will be terminated, while the synthesis of the three major diapause proteins is stimulated. These proteins accumulate to high levels in the hemolymph. Absence of JH in adult beetles coincides with a sharp rise in ecdysteroid titer (Briers et al. 1982). It is thus far unknown which transcription factor(s) are involved, nor how the switch is made. Maybe, the synthesis of diapause proteins is driven by ecdysteroids and their receptors while the synthesis of reproduction-related proteins (vitellogenins in particular) may require both hormones. See also section 7.2 for the occurrence of the ecdysone receptor (EcR) as homo- or heterodimer (Truman 2019).

The synthesis of diapause proteins is also induced by the drop in JH titer in the early last larval instar of *Leptinotarsa*. This made De Loof (1972) suggest that diapause phenomena not only occur in adults raised under short photoperiod (which inactivates the CA), but also in last instar larvae of *Leptinotarsa*, independent of the photoregime (long-day or short day) under which they were reared.

7. Two often overlooked issues

7.1. The flexibility/plasticity of cell membranes

It follows from the high content in (specific) lipids that cell membranes are flexible. This property is so self-evident that in cell biology, this property is given little attention, in particular with respect to the fact that hormones might be “influencers”. The plasticity of biological membranes is very outspoken in cellular processes such as during contraction-relaxation of muscle cells, in adhesion and sliding (Gori et al. 2011), and in particular in various forms of membrane folding such as endocytosis (pinocytosis and phagocytosis, Roth and Porter 1964) and exocytosis (i.e., the formation of secretory vesicles in hormone- and neurotransmitter producing cells or of microvilli, etc.). Enabling membrane fluidity/plasticity is a complex process. It needs to be stressed that lipids, their interaction and involvement of inorganic ions are absolutely insufficient to perform or drive this process. More than two dozens of vesicular and cell membrane specific lipid-anchored proteins (such as NSF ATPase, SNAPs and SNAREs, Rabs etc.) are crucial to this process, to its individual steps and for complete implementation of priming, targeting and fusion.

De Loof and Lagasse (1970) have shown that when the titer of JH drops to zero in early last instar larvae of the Colorado potato beetle, the normal secretion of secretory vesicles by fat body cells is drastically disturbed. Thus, JH plays an important role in membrane plasticity, this being one of its non-genomic effects. JHs are esters of farnesol: they are synthesized from isoprene as basic building block. Thus, they belong to the large family of polyisoprenoid alcohols that occur from bacteria to plants and mammals (Swiezewska and Danikiewicz 2005, Surmacz and Swiezewska 2011). In animals, polyisoprenoid alcohols are synthesized by the mevalonate pathway, and commonly occur not only in the liver
of vertebrates, but also in other tissues (Murgolo et al. 1989, Swiezewska and Danikiewicz 2005). In some plants, a second biosynthetic pathway occurs (Swiezewska and Danikiewicz 2005). The chemical properties of polyisoprenoid alcohols (dolichol is the best studied molecule (Murgolo et al. 1989)) enable a membrane fluidizing/plasticizing effect. Dolichol, farnesol as well as many other related substances have a horseshoe-shape. This also holds true for the very well documented application of phthalates as plasticizers in the PVC (Polyvinylchloride) industry (Figure 4). According to Murgolo et al. (1989) the activity of such compounds (e.g., of dolichol, its derivatives, and smaller polymers of isoprene - farnesol inclusive) is intimately linked to this conformation. This might be particularly true in fusion processes in secretory cells and in transport in organelles. Two effects are important: membrane fluidization (i.e., lowering viscosity and increasing plasticity), and protein glycosylation (Swiezewska and Danikiewicz 2005, Walski et al. 2017), the latter also being an important activity of the Golgi system (Figure 3 in De Loof and Schoofs 2020).

Figure 4. A. Phthalate: From Multiple Authors (2021) Wikipedia: Phthalate. B. All trans farnesol from PubChem. Both sources are Open Access.

The best studied model on the role of farnesol on membrane plasticity is the pathogenic (to humans) yeast, Candida albicans (C.-P. Robin) Berkhout (1923) (Fungi: Ascomycota). Not only does farnesol influence the hypha-to-yeast morphogenetic transition of this fungus (Lindsay et al. 2012), it also acts a quorum sensing molecule, the first discovered one in eukaryotes (Leontardt et al. 2015). In addition, farnesol activates innate immune cells, but suppresses cellular adaptive immunity as a virulence factor in various ways (e.g., by modulating cytokine release, Leontardt et al. 2015).

7.2. Gaining muscle strength despite forced immobility during the pupal stage.

In holometabolic insects, some muscles, in particular flight muscles, are underdeveloped at the onset of metamorphosis. By contrast, in some species, the
adults start flying very soon after adult emergence. This raises the question how developing muscles can gain strength in the absence of physical exercising. During the pupal stage ecdysteroids initiate and orchestrate the development of the flight muscles. According to Truman (2019): “Depending on insect group, (the ecdysone receptor) EcR can either act as a homodimer or heterodimerize with the retinoid X receptor (RXR). RXR binds ligands such as 9-cis-retinoic acid (9-cis-RA) but there are variants that lack the ligand binding domain or bind a constitutive structural lipid. The latter form is called Ultraspiracle (USP)”. EcR and USP are two separate proteins encoded by different genes.

Maybe, the relatively novel idea about the mode of action of 20-hydroxyecdysone (20E), which has been advanced by Sláma et al. (1996) and Sláma 2019) also provides a (partial?) answer. It says that ecdysteroids, in addition to their signaling through a nuclear receptor (Koelle et al. 1991) that occurs in two isoforms, (the EcR protein and Ultraspiracle protein (USP) as a non-covalent heterodimer, can also act as a provitamin D1 that stimulates muscle development and physical performance. Remarkably, this water-soluble anabolic (ecdys)teroid that is legally used by sportspeople and in geriatry, does not act through the same nuclear (ecdysteroid) receptors in mammals and insects, but through the Akt serine/threonine kinase 1 activation pathway (Gorelick-Feldman et al. 2008, 2010; Isenmann et al. 2019), a pathway that is not only present in insects but in humans as well. Humans do not have the classical EcR. However, according to Parr et al. (2014), 20E seems to act through the Estrogen Receptor ERβ. Remarkably, in females of some fly species 20E acts as the counterpart of estradiol as female sex hormone as cited before (Huybrechts and De Loof 1977, De Loof and Huybrechts 1998, De Loof 2006).

8. Is entering the nucleus an absolute requirement for JH’s effect on transcription?

In to date’s almost generally accepted view, this is indeed the case. However, this has not always been the case. Indeed, some 50 years ago, there was an animated discussion whether small hormones like ecdysone and JH have to enter the nucleus in order to generate effects at the level of transcription. While Karlson and Sekeris (1966) thought that entering was necessary, others working with isolated polytene chromosomes from dipteran insects, thought it was not. They showed that by changing the inorganic ion composition of the incubating medium, they could induce changes in puffing pattern similar to those of E/20E or JH in the absence of any hormone (refs in Lezzi 1970). However, as one did not see which mechanism could explain such results, the hypothesis met with skepticism (e.g., Ashburner and Cherbas 1976), and the inorganic ion-hypothesis fell into oblivion.

However, recent experiments by Maczewsky et al. (2017) may revive it. These authors studied the mode of activation of the liver X receptor (LXR). It is closely related to other nuclear transcription factor receptors such as the farnesoid
X receptor (FXR) and the retinoid X receptor. These transcription factor receptors are important regulators of lipid and glucose homeostasis. LXR acts as a glucose sensor. 15 mM glucose induces oscillations of the cytosolic Ca\(^{2+}\) concentration, and these effects were completely suppressed by 10 µmol T0901317 (Sigma), a non-polar compound, due to inhibition of mitochondrial metabolism, thus inhibiting ATP production and reducing the cytosolic ATP concentration. The compound eventually inhibited glucose-induced insulin secretion. These effects were rapid in onset and not compatible with the activation of a nuclear receptor. Remarkably, MT0901317 never entered the nucleus (fide indirect personal communication), and acted on the mitochondrial membrane or other targets including increase in Ca\(^{2+}\) influx, etc.

This mode of action is appealing to us as it suggests that, perhaps, a similar mechanism may also function during JH signaling. Perhaps, JH does not have to enter the nucleus, but may act from the nuclear envelope in order to activate the “Met JH receptor”. Such a hypothetical mechanism of JH signaling, although not proven, is compatible with published reports. The sequence of events might be:

a. The hydrophobicity of JH enables it, upon release from the CA, to bind a lipoprotein carrier in the hemolymph. Upon contact with a target cell plasma membrane, JH leaves its lipoprotein carrier and binds the cell plasma membrane, most likely because of its higher (hydrophobic) affinity to the cell membrane than to the lipoprotein carrier.

b. After binding, JH diffuses throughout the integrated endomembrane system of cells, including the endoplasmic reticulum-Golgi system, and the nuclear envelope.

c. JH encounters Ca\(^{2+}\)-ATPases that are anchored in membranes, such as the SERCA-Ca\(^{2+}\) pumps that have a binding site for thapsigargin, a common blocker of Ca\(^{2+}\)-pumps in insects and probably all eukaryotes. The outer membrane of the nuclear envelope is continuous with the RER, thus it can harbor SERCAs, with their binding site for thapsigargin.

d. Thapsigargin, a plant toxin, is a sesquiterpenoid, a rarely mentioned characteristic which it shares with farnesol/JHs that are also sesquiterpenoids.

e. De Loof et al. (2014) proposed that the thapsigargin binding site in Ca\(^{2+}\)-pumps is probably well conserved in evolution and serves as a binding site for a thus far still uncharacterized endogenous ligand that acts as an agonist for particular Ca\(^{2+}\)-ATPases such as the SERCA Ca\(^{2+}\) pumps. Circumstantial evidence indicates that the unknown ligand might be an endogenous farnesol-like sesquiterpenoid.
f. Thapsigargin is very toxic because it has higher affinity for the ligand’s site of Ca\(^{2+}\) -ATPases resulting in displacing natural (farnesol-like?) ligands and thereby stopping Ca\(^{2+}\)-pumping.

g. If farnesol-like endogenous sesquiterpenoids are indeed the natural ligand(s), it follows that Ca\(^{2+}\)-pumps, need the presence of an agonist for active pumping. Such pumping of free Ca\(^{2+}\) is directed from the cytoplasm into membrane-limited storage sites, in particular the lumen of the ER and the mitochondria. The result is that the concentration of free Ca\(^{2+}\) in the cytoplasm can be kept low: in insects this is essential for being in the juvenile state, but it could also be true for vertebrates (De Loof et al. 2015).

h. At maximal concentration of Ca\(^{2+}\) inside the lumen, some membrane-bound enzymes are inactive. Lowering the Ca\(^{2+}\)-load (e.g., by Ca\(^{2+}\)-induced Ca\(^{2+}\) releases from storage sites), temporarily activates some enzymes. This, as well as the reuptake mechanisms are well documented (e.g., in muscle- and in steroid hormone producing cells).

i. Because the nuclear envelope – as element of the RER system – can also harbor SERCA-type Ca\(^{2+}\) pumps, it is able to participate in Ca\(^{2+}\)-homeostasis of the nuclear compartment, with effects on transcription of specific sets of genes as well as on the Ca\(^{2+}\)-sensitive chromatin structure. Thus, it may act as an additional level of control mechanisms during hormonal stimulation.

9. Summary of arguments For and Against Met as the JH receptor

In classical experiments involving allatectomy and reimplantation of active CA in various insect species that were carried out mainly in the 1960ties and 1970ties, JH emerged as being a key hormone in controlling many cell functions. To name a few: protein-, lipid-, and glycogen synthesis, mitochondrial functions, inhibition of metamorphosis and of programmed cell death, functioning of the Golgi system, membrane fluidity, architecture of the cyto- and nuclear skeleton, behavioral and growth aspects to list the major ones. JH was shown to act in concert with a variety of other hormones. It acquired the status of “master hormone”. JH signals dually: when present in a high titer (in young larvae and in reproducing adults), it stimulates some cellular mechanisms and concurrently inhibits others. When JH disappears from the body, stimulation stops, and inhibition is lifted: this initiates metamorphosis.

To date, it is believed that Met/Gce is the JH (nuclear) receptor, and authors regularly title their papers following this notion (e.g., Jindra and Bittova 2020). However, there are also counterarguments (Table 1).
Table 1. Arguments for and against Met as the JH nuclear receptor. There are at least two types of receptors, one that focuses on receptors/targets for control of intranuclear activity, and the second type that mainly comprises membrane receptors/targets.

<table>
<thead>
<tr>
<th>Arguments in favor</th>
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<th>Arguments against</th>
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<tr>
<td><em>Met</em> and <em>gce</em> <em>Drosophila</em> mutants display resistance to the JH-analog Methoprene.</td>
<td>Wilson and Fabian (1986), Baumann et al. (2010), Jindra et al. (2003)</td>
<td><em>JH</em> is a master hormone as shown by classical experiments (e.g., allatectomy). <em>Met/Gce</em> silencing does not mimic all allatectomy effects.</td>
<td>De Loof and Lagasse (1970)</td>
</tr>
<tr>
<td>The loss of Met in <em>Tribolium</em> by RNAi prevents normal metamorphic responses to JH.</td>
<td>Konopova and Jindra (2007), Parthasarathy and Palli (2009)</td>
<td><em>JH</em> is a hydrophobic molecule and can end up in the membrane systems of all cells, including the ER, the nuclear envelope etc.</td>
<td></td>
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<td><em>Met</em> has been isolated from many different insects and found to be more similar to <em>Drosophila gce</em> than to <em>Drosophila Met</em>.</td>
<td>Jindra et al. (2003), Wang et al. (2007), Konopova et al. (2011)</td>
<td>Some functions of JH are controlled by enzymes anchored in endomembranes (i.e., the ER, a system with a high transmembrane-lumen Ca(^{2+}) gradient).</td>
<td></td>
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<td>The loss of both <em>Met</em> and <em>gce</em> prevents the normal metamorphic responses to JH.</td>
<td>Baumann et al. (2010), Jindra et al. (2003), Konopova et al. (2011)</td>
<td><em>JH</em> has been shown to act via so far unknown transmembrane receptors.</td>
<td>Li et al. (2019), Davey (2000), Ilenchuk and Davey (1983)</td>
</tr>
<tr>
<td>Gce binds <em>Drosophila JH</em>, its MF precursor, and several synthetic JH mimics <em>in vitro</em>.</td>
<td>Jindra et al. (2003)</td>
<td><em>JH</em> signaling is since long thought to at the plasma membrane. Changes in nuclear activity are a secondary effect.</td>
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<td><strong>Taiman forms a heterodimer with Met in response to JH and then activates transcription of downstream genes.</strong></td>
<td>Jindra et al. (2003), Konopova et al. (2011)</td>
<td>JH affects the cell’s electric system – changes in membrane potential – in seconds.</td>
<td>Telfer (1980)</td>
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<td><strong>Like in Met, Tai mediates effects of JH on metamorphosis.</strong></td>
<td>Lozano et al. (2014), Xu et al. (2019c)</td>
<td>Changes in the inorganic ion composition of the incubation medium are sufficient to mimic typical hormone-dependent effects as shown by puff induction experiments in larval salivary gland chromosomes (Diptera).</td>
<td>Lezzi (1970), Ashburner and Cherbas (1976)</td>
</tr>
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<td><strong>Daphnia Met responds to methyl farnesoate in a way insect Met responds to JH.</strong></td>
<td>Miyakawa et al. (2014)</td>
<td>Effects of JH on follicular patency mediated by the Na⁺/K⁺-ATPase (e.g., by phosphorylation).</td>
<td>Ilenchuk and Davey (1983), Ling et al. (2018)</td>
</tr>
<tr>
<td><strong>Some JH effects (e.g., related to metamorphosis), can be attributed to Met/Gce</strong></td>
<td></td>
<td>Both Ca²⁺ and protein kinase C activity are required for the JH III-induced stimulation of protein synthesis in the accessory glands of virgin <em>Drosophila</em>.</td>
<td>Yamamoto et al. (1988), Wilson et al. (2003)</td>
</tr>
<tr>
<td><strong>It is assumed that JH enters the nucleus through a nuclear pore.</strong></td>
<td></td>
<td>A Ca²⁺ channel-type acts as a membrane receptor for farnesol in mammals. Because Ca²⁺ plays a role in JH signaling, it can be hypothesized that the Ca²⁺-homeostasis</td>
<td>Roulet et al. (1997), Roulet et al. (1999), Luft et al. (1999)</td>
</tr>
</tbody>
</table>
a. Nuclear: Met/Gce which is the receptor for its direct actions on the promotors of specific genes such as Kr-h1 which has a JHR response element that binds the JH-Met:Tai complex in its promotor.

b. Extranuclear: One or more to-be-identified membrane receptors which signal rapid responses to JH via phospholipid and/or calcium signaling or possibly even just modulation of the sodium pump ATPase activity as may be the case for the regulation of the intercellular spaces in the follicular cell epithelium.

### Suggestions for Further Research

**The molecular physiology of juvenile hormone**

1. **Many activating compounds vs. “exquisite ligand stereoselectivity”**

   According to Sláma (2013), there are some 4,000 compounds, some with widely different chemical properties that have JH activity. But, according to Bittova et al. (2019), there is “exquisite ligand stereoselectivity of a *Drosophila*
juvenile hormone receptor that contrasts with its broad agonist repertoire”. How can these two views be reconciled?

2. How is the synthesis of Met/Gce controlled?

3. Identification of the proteins under control of Met/Gce.
   How can JH concurrently be needed for activating selected genes while others are inhibited? The Colorado potato beetle, *Leptinotarsa decemlineata* Say, 1824 is a good example: JH is needed for activation of reproduction-related proteins (vitellogenin e.g., De Loof and de Wilde 1970), while it concurrently inhibits the synthesis of “diapause proteins” (De Loof 1972).

4. During evolution, the CA got company from other sites of JH synthesis.
   The CA are still thought to be the master site of synthesis of JHs. Are other sites of synthesis, e.g., gonads, dependent or independent of the CA? Might there be a hierarchy among them?

5. The gland cells of the CA have Golgi systems (Scharrer 1964). Their functions?
   Golgi systems are multifunctional. Do they secrete a protein(s)/peptide(s) that remains to be identified? If they do: a role in Ca$^{2+}$ secretion? Is the unidirectionality of JH transport inside the JH-producing cells linked to the unidirectionality of transport which is typical for Golgi secretions?

6. Farnesol
   What is the exact role of (farnesol-)prenylation in the functioning of some G-Protein-Coupled Receptors (GPCRs)? Does it also have a role in Ca$^{2+}$-homeostasis?

7. Where does JH ultimately dock in the nuclear compartment?
   Does JH - being hydrophobic - end up inside the nucleus? Or does it end up in the nuclear envelope from where it might play a role in controlling the Ca$^{2+}$ concentration in different hormonal conditions?

8. How many targets does JH have?
   Is Met/Gce the only JH receptor or only one of JH’s many targets?

9. Juvenile hormone, calcium concentration, and ecdysteroid biosynthesis
   Does the fall in JH titers pave the way to increased ecdysteroid biosynthesis? If so, is there a role for the collapse of intracellular Ca$^{2+}$ gradients?

10. Short neuropeptide F and the corpora allata
    Is absence of short NPF during metamorphosis (in some species at least) an undervalued key player in inactivating the CA at the onset of metamorphosis?
The role of calcium ($Ca^{2+}$)
11. $Ca^{2+}$ regulation in the plasmalemma and in the sarcoplasmic/endoplasmic reticulum

Like in all eukaryotic cells, keeping the free cytoplasmic $Ca^{2+}$ concentration low in unstimulated insect cells is a vital activity. The universal key mechanism is: keep, in controlled ways, $Ca^{2+}$-channels closed as much as possible, and activate $Ca^{2+}$ pumps when needed. This activity is poorly documented in animal endocrinology in general, insects inclusive. Are some insect $Ca^{2+}$-channels controlled by farnesol-like endogenous sesquiterpenoids, like is the case in mammals (Luft et al. 1999; Roullet et al. 1997, 1999)?

Do sesquiterpenoids also play a role in controlling SERCA-pump activity as first suggested by De Loof et al. (2014), who advanced the following argument. Both the presence of the (SERCA) $Ca^{2+}$ pump blocker thapsigargin and the falling to zero of the JH-titer (in early metamorphosis) induce cell death in particular tissues. Both JH and thapsigargin are sesquiterpenoids: thus, the endoplasmic reticulum has a binding site for some sesquiterpenoids. Could it be that thapsigargin is toxic because it displaces a still unidentified natural, endogenous farnesol-like ligand that might be needed for keeping the SERCAs pumping? Link with $Ca^{2+}$ (c.f., Orrenius et al. 2003).

Cellular membranes
12. Endogenous sesquiterpenoids and cellular membranes

Do endogenous sesquiterpenoids have a role in the fluidity-plasticity of cellular membranes? If “yes”, what is their exact role? One can imagine the possibility that membrane lipids facilitating binding or transport of JH act in concert with membrane-anchored protein(s) some of which can be putative or potential JH receptors transmitting its nongenomic action.

Discussion

The main conclusion of this paper says that, in our opinion, insect Juvenile Hormone, the master hormone in development, acts as “status quo hormone” in larvae, mainly because at high titer it keeps the free cytosolic $Ca^{2+}$ concentration low in JH-target cells. Hitherto this truly vital activity in cells, namely preventing the cytoplasmic free $Ca^{2+}$ concentration from rising for too long above its toxicity limit, undeservedly received little attention in (insect) endocrinology. In unstimulated cells, it is either taken for granted, or it is thought to be “strictly genetic”, thus not requiring specific hormones. Next, the reason why such a low free $Ca^{2+}$ concentration in the cytoplasm is vital is also seldom addressed. The explanation is rather simple: Without keeping the cytoplasmic free $Ca^{2+}$ concentration below the toxicity limit of $Ca^{2+}$ (i.e., when rising above 100 nanomolar for a longer period of time), the secondary signaling systems that make use of increases in $[Ca^{2+}]$ above low background values such as “$Ca^{2+}$ as a secondary messenger”, “$Ca^{2+}$-induced $Ca^{2+}$-release from intracellular storage sites
(e.g., the endoplasmic reticulum), and “Control of the activity of some enzyme systems which are anchored in endomembranes and which can be inhibited by a high intraluminal Ca\textsuperscript{2+} concentration” could not become functional. This means that short-lived small increases in Ca\textsuperscript{2+} influxes could not be experienced.

The role of Ca\textsuperscript{2+} should not be over-extrapolated. There is no doubt that functions of some inorganic ions, in particular Ca\textsuperscript{2+} and H\textsuperscript{+} in control of gene expression and in bringing about effects of hormones have become undervalued in some studies. Yey, fluctuation in intracellular Ca\textsuperscript{2+} should be implemented with a good knowledge of the principles of Ca\textsuperscript{2+}-homeostasis, because most effects manifest themselves at a very generalized level of calcium involvement (on Ca\textsuperscript{2+}-channels (e.g., Roullet et al. 1999), and mostly, but not always (farnesol, e.g., Roullet et al. 1999) without any specific or clear link to particular hormone action or effect. It should be emphasized that changes in local microenvironment of inorganic ions at chromatin level are certainly involved in transcriptional regulation of many genes. This means hundreds or thousands of genes that can be simultaneously or concomitantly transcribed; transcription initiation as well as elongation complexes will require rapidly changing ion microenvironments at the particular transcription units, at each of them independently.

The neglect of the importance of low cytoplasmic Ca\textsuperscript{2+} concentration in resting cells continues to cause different interpretations and debate on the relative importance of nuclear receptors, in particular of Met/Gce, and of the Ca\textsuperscript{2+}-homeostasis system in enabling JH-activity. Everybody agrees that both are necessary. But what is causing the difference in interpretation of the published data? Molecular biologists focusing on transcription processes work on cells that developed/matured far enough to have functional transcription factors, and they assume that homogenization of cells - which destroys intracellular ionic/Ca\textsuperscript{2+} gradients - is an acceptable procedure. They (inadvertedly) assume that the Ca\textsuperscript{2+} homeostasis-system is tuned to “near perfection” without needing any additional input from JHs. However, this is erroneous. A functional Ca\textsuperscript{2+} homeostasis system requires the controlled activity of Ca\textsuperscript{2+}-channels and pumps, lifelong from birth to death. In particular research in vertebrates revealed that some Ca\textsuperscript{2+} channels act as receptors for the sesquiterpenoid farnesol (Roullet et al. 2017, 2019; Luft et al. 2019). The fact that the sesquiterpenoid thapsigargin binds to a binding site on the endoplasmic reticulum shows that the ER has a binding sited for some sesquiterpenoids. Could farnesol, perhaps, be the natural endogenous sesquiterpenoid that acts as a universal agonist of the SERCA-Ca\textsuperscript{2+} pump?

If one superimposes all this, the integrating view emerges that endogenous sesquiterpenoids contribute to bring the Ca\textsuperscript{2+} homeostasis system in such shape that the next order control system of JH action that involves nuclear activity (transcription factors) can start. Thus, in our model, extranuclear (membrane-) and intranuclear nuclear targets/receptors (e.g., transcription factors) are necessarily complementary: it is not an “either – or” but an “and – and” story.
It has been logically deduced (De Loof and Schoofs 2019a, b) that a high JH titer inhibits the production of ecdysteroids in the SER through keeping the intraluminal Ca\(^{2+}\) concentration high, this being one of the non-genomic effects of JH. To form a next larval instar, the accompanying molt generates a thin larval cuticle. This requires a short-lasting drop in JH titer which lifts the block to ecdysteroid synthesis, enabling a short-lived ecdysteroid peak. In its turn, this increased E peak enables the synthesis of larval cuticular proteins. When the JH titer drops drastically early in the last larval instar of holometabolous insects, the larval phenotype gets lost as is marked by the transformation of the larva into a pupa. The long-lasting absence of JH is permissive for the long-lasting increase in ecdysteroid titer, characterized in one broad or several sharp peaks. According to De Loof et al. (2015) the appearance of an ecdysteroid peak signals that somewhere in the body, a tissue is undergoing programmed cell death/apoptosis, as during metamorphosis. If different tissues undergo programmed cell death at different times, multiple ecdysteroid peaks will occur (Briers et al. 1983). This reflects multiple possible sites of synthesis. Absence of JH is permissive for an increase of the Ca\(^{2+}\) concentration in the cytoplasm, due to no longer acting as blocker of Ca\(^{2+}\) channels (Rouillet et al. 1997, 1999; Luft et al. 1999), and probably through arrest of pumping of Ca\(^{2+}\) by SERCA pumps (De Loof and Schoofs 2019a,b; 2020). One of the drastic effects of JH’s clearing from the body is the onset of programmed cell death, in particular of those larval tissues in which massive synthesis of proteins for secretion, accompanied by secretion of Ca\(^{2+}\) as well (De Loof et al. 2015; De Loof and Schoofs 2019a, 2020) through the RER-Golgi apparatus takes place. Programmed cell death/apoptosis is causally linked to an increase in intracellular Ca\(^{2+}\) above a toxicity level, as outlined by Orrenius et al. (2003). It also involves the transcription of specific apoptosis-linked genes. Finally, drastic lowering of the JH titer also affects the activity of the Golgi system, in particular in cells with an active protein secretory activity. Recently, De Loof and Schoofs (2020) argued that farnesol/JHs are likely synthesized in the Golgi apparatus in various cell types in a development-dependent way. Furthermore, the fluidizing effect on membranes of polyisoprenoid alcohols which comprise many different compounds, FLS/JHs inclusive, and by extension perhaps the large family of polyisoprenoids to which they belong, is most relevant for vesicle formation and fusion processes in secretory cells (Murgolo et al. 1989). In our opinion, FLS may be the overlooked biological functional counterpart of phthalates as used as plasticizers in the plastic industry (De Loof and Schoofs 2020, and this paper). They may contribute to a higher flexibility of the body of young organisms.

A still difficult problem to solve is how can it be explained that at high JH levels, specific genes are transcribed, and that the fall in JH titer causes a switch in transcription: some larval genes are no longer transcribed, while genes which are sensitive to JH’s absence are triggered for transcription. What does change? Is there a switch in transcription factors? Does the stereo-conformation of the
larval transcription factor(s) change so that it now recognizes other genes? Or (and) does the chromatin undergo conformational changes so that novel genes become transcribable? And what is the role of Ca\(^{2+}\) herein? Do intranuclear changes in Ca\(^{2+}\) concentration suffice for bringing about the switch or not?

JH was initially thought to be the “youth- and anti-aging hormone”. At a certain moment in development, the JH titer starts sinking. One of the effects is that the biosynthesis of ecdysteroids is no longer almost fully inhibited. The rise of the ecdysteroid titer in the hemolymph makes the plasma membrane more permeable for Ca\(^{2+}\). In vertebrates, a similar situation seems to occur, but the difference is that here farnesol seems to act as an “inbrome”, thus as a signaling molecule that mainly acts from within the membrane (De Loof et al. 2015). From this moment on, JH and (ecdy)steroids become antagonists. In insects this induces the onset of metamorphosis, and in vertebrates/mammals, it marks the onset of puberty (De Loof et al. 2014, 2015). Ca\(^{2+}\) which is best known for its beneficial effects is, in our opinion, a major agent in causing aging phenomena. It is both beneficial and toxic (beneficial Calcitox), like O\(_2\).

Another still unanswered question is why cells have to be exposed for a longer time to JH to become responsive. This effect was already described by Wigglesworth (1969). Application of JH to a very thin undamaged cuticle of *Rhodnius* did not yield an effect, probably because JH penetrated the cuticle very fast, and was next degraded in the hemolymph. Injection or use of wetting agents was also ineffective in bringing about JH effects in the *Rhodnius* bioassay. Wigglesworth found that mixing JH with an oil was an effective means of application. This system behaves as a slow-release formula. The probable explanation is that farnesol-like endogenous sesquiterpenoids (JHs inclusive) have to be continuously present to keep the Ca\(^{2+}\) channels that act as receptors for such compounds, closed. This suggests that FLS/JH binds with low affinity to the membrane receptor. As soon as a FLS disappears from the hemolymph, the influx of Ca\(^{2+}\) into JH-target cells will start. A more or less similar situation has been encountered during the isolation and identification of the neuropeptide corazonin, which is also hydrophobic. It is only active in the albino locust assay if it is mixed with an oil (Tawfik et al. 1999).

In conclusion, the insights gained from classical experiments carried out in the pioneering days of insect endocrinology, in particular through using bioassays, have fallen too much into oblivion. They deserve a reappraisal. In particular, the framing of some reductionistic results obtained with modern molecular biological methods overshadow too much the plentitude of results obtained by classical bioassays. One of the results is that some of the functions of some inorganic ions, in particular Ca\(^{2+}\) and H\(^{+}\), which can both act as secondary messengers, in control of gene expression and in bringing about effects of hormones have become undervalued in some studies. A reason is that such inorganic-ion dependent effects have to be evaluated with other methods than those in use for studying the molecular genetic ones.
One main conclusion is that given JH’s hydrophobic properties and its role in the complex Ca^{2+}-homeostasis system, there must be many binding sites/targets for JH, some of them being genuine receptors. Furthermore, in some respects, JH and 20E have since long been depicted as each other’s antagonists, and concurrently, mutually dependent in some of their functional aspects. Part of the explanation might be rather simple: in target cells, a high titer of JH tries to keep [Ca^{2+}] low, while rising concentrations of 20E allow the entry of Ca^{2+}. This is causal to numerous secondary effects.

The challenge for the future is: how to make holistic thinking in endocrinology compatible with the reductionistic contemporary approaches? That requires including some historical-retrospect searching the literature that may date back to the pioneering days of insect endocrinology, namely the 1950-70ties. So far, understanding the full mode of action of JH has been a real challenge. This may continue to be so for the near future.

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Both authors jointly conceived and wrote the paper.

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