Review Paper

Calcitox-Metamorphosis in Insects: The Calcium (Ca^{2+})-Homeostasis System as the Integrated Primordial Receptor System for both Juvenile Hormone and Ecdysteroids[1]

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Abstract: Changes in titers of Juvenile Hormone(s) [JH(s)], the "status quo" hormone, and the molting hormone 20-OH-Ecdysone (20E), since 2019 also referred to as the anabolic steroid of plant origin named Vitamin D_1, are key players in insect metamorphosis. Ecdysterone 20E is a water- and lipid soluble compound. Juvenile hormone (JH) occurs in six sesquiterpenoids isoforms, which are esters of farnesol, all highly hydrophobic compounds with high affinity for cell membranes. They are synthesized by the mevalonate biosynthetic pathway found in uni- and multicellular eukaryotes, including the evolutionarily ancient zooflagellates (Opisthokonta), which are presumed as ancestral to all animals. The mevalonate pathway of insects does not include the enzyme squalene synthase. Hence, they cannot biosynthesize cholesterol, the precursor for the biosynthesis of (ecdy)steroid hormones. Cholesterol and plant steroids are utilized as vitamins by insects. What is the mechanism that drastically reduces the titer of JH causing metamorphosis in holometabolous insects? How can the absence of JH cause drastic changes in insect development? Could this be caused by the collapse of Ca-homeostasis after the binding sites for endogenous sesquiterpenoids on Ca channels, Ca^{2+} pumps, etc. are no longer occupied? What is the exact mode of action of 20E/vitamin D_1, not only in insects but also in humans? Are JH and 20E antagonists, as was already postulated during the 1960s? Do both hormones compete for the same receptors? We argue that this is not the case and offer an alternative explanation. First, not only the nuclear, but also the membrane receptors of JH and 20E differ. Second, both hormones seem to antagonistically control Ca^{2+}-homeostasis of target cells. Farnesol, an agonist of JH-biosynthesis, is a potent inhibitor of specific voltage-gated Ca^{2+}-channels, and thus inhibits the influx of Ca^{2+} in target cells. In contrast, upon activation of its membrane GPCR, 20E facilitates the influx of Ca^{2+} into the cytoplasm. In essence, hormone-propelled changes in intracellular free Ca^{2+}-concentration, $[Ca^{2+}]i$, are the primordial driving force of complete metamorphosis. This hypothesis explains genomic and non-genomic effects that occur at high or low hormonal titers.

Key Words: Programmed cell death, development, diapause, obesity, sNPF, vitamin D, membrane integrity, choanoflagellates, apoptosis, calcitox, metamorphosis, insects: calcium (Ca^{2+}), homeostasis, juvenile hormone, ecdysteroids, integrated primordial receptor system

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1. Introduction

Metamorphosis and punctuated molts are often depicted as processes that serve a specific "goal", namely enabling growth and acquiring the necessary morphological and physiological characteristics for feeding and reproduction in the adult stage. Yet, current evolutionary theory says that, some exceptions not considered (Pookottil 2013), there is no goal whatsoever in evolution (De Loof 2017a). Changes happen from physiological necessity, for a variety of reasons. Only if the effect of the changes is that a particular problem is adequately solved, they may be hereditarily conserved. Over time, fitness-increasing mutations accumulate, eventually resulting in pronounced morphological and/or physiological changes.

The gradual but intermittent (at molts) changes during the life cycle of insects with incomplete metamorphosis (Hemimetabola), in which the young larvae closely resemble the adults seem "normal" as this type of development resembles the gradual development of many vertebrates (Figure 1). This is less the case in holometabolous insects like lepidopterans, that have a caterpillar stage as larva, a pupa, and a butterfly (or moth)-phenotype in the adult stage. These drastic changes give the impression to be deviations from "normal" development, thus, to be "abnormalities". From a (wrong) teleological viewpoint, one could argue that the changes are essential and needed for accommodating the transition from the herbivorous feeding with adapted mouthparts and digestive system, such as in most lepidopteran larvae, to a feeding regime generally dependent on nectar with adapted mouthparts and digestive system in the adult stage, in addition to enabling reproduction.

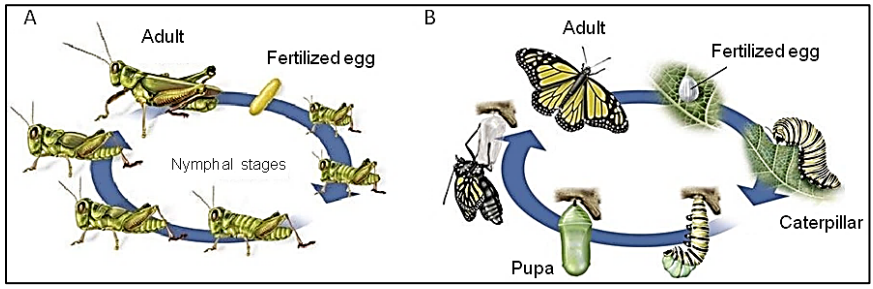

Figure 1. A. Schematic representation of insect life cycles. A. Hemimetabolous species, a locust. B. Holometabolous species, the Monarch butterfly (Lepidoptera: Nymphalidae), *Danaus plexippus*. Source: Google Images, Arizona State University - Ask a Biologist – Metamorphosis. Open Access.

Is it possible that the coming into existence of deviations from the evolutionarily ancient gradual developmental scheme has a *toxicological* cause that results from an as yet to be defined mutation? If this would be the case, which toxic substance or physiological process would cause such "malformations", giving the impression that metamorphosis is in fact a serious illness, a disease

state? Could Ca^{2+}-intoxication be the (ancient) causal agent? If Ca^{2+}, which is better known for its beneficial than for its toxic effects (De Loof 2017b), is indeed the trigger, how do the key hormones controlling metamorphosis, juvenile hormone (JH) and ecdysteroids, make use of it? We hypothesize that rather than the action of a number of nuclear transcription factors (Kayukawa et al. 2016, 2017, Li et al. 2019) and separately operating membrane receptors, the *Ca^{2+}-homeostasis system acts as the integrated primordial receptor-system for both JH and ecdysteroids.* If correct, this view introduces a drastic paradigm shift, not only regarding the principles of metamorphosis, but also regarding the way of thinking in general and comparative endocrinology and on animal development.

Upon comparing the mechanisms that govern metamorphosis in the evolutionarily ancient ancestors of all animals, the zooflagellates and the sponges (currently an active research topic, see section 2 below), with those in insects, the mechanisms which have been very well conserved over hundreds of millions of years are likely the ones that truly matter in governing metamorphosis. Maintaining and, when needed, repairing epithelial integrity and the role of Ca^{2+}-homeostasis are at the forefront in both sponge development (Nakanishi et al. 2015) and insect metamorphosis (Sláma 2019).

2. The major challenge in metamorphosis: preserving epithelial integrity at all times

The primordial task of any metamorphosing animal is to maintain its epithelial integrity. This follows from the very essence of being an animal: "An animal develops from a blastula". In other words, an animal is an organism that, in its early development, organizes itself as a blastula, thus as *tightly closed epithelium* (De Loof 1992). Loss of integrity, such as by changes in Ca^{2+}-homeostasis that affect the functioning of intercellular junctions, results in intercellular leakage of inorganic ions and of self-generated electric currents that will traverse the embryo, making it into as a higher order miniature electrophoresis chamber, "the cell" being the smallest such unit (De Loof 1986). From this concept, it follows that the focus of the mode of action of signaling molecules controlling metamorphosis should not be restricted to transcriptional mechanisms, but that it should be widened to other aspects of cell biology as well. In particular, the "electrical rewiring of cells" during metamorphosis is an important issue (De Loof et al. 2014). Such rewiring involves changes in transmembrane fluxes of selected inorganic ions with possible effects on control of transcription as a result (De Loof 2016).

3. Sponges undergo metamorphosis. The role of Ca^{2+} homeostasis is ancient

The Ca^{2+} signaling is instrumental in the induction of metamorphosis in a range of eumetazoans (Freeman and Ridgway 1990, Freeman 1993, Clare 1996, Biggers and Laufer1999). Already in sponges the earliest phyletic lineage(s) to branch off the metazoan tree, the elevation of the intracellular Ca^{2+} levels in the

sensory secretory flask cells, is required for the initiation of metamorphosis (Nakanishi et al. 2015). Such flask cells (Figure 2) in a range of sponge and eumetazoan larvae, translate environmental information into internal developmental signals, thereby using Ca^{2+} signaling. This suggests that Ca^{2+}-signaling is a deeply conserved system that coordinates larval settlement and metamorphosis. In eumetazoans, a neurosecretory mode of signal transmission at metamorphosis appears to be largely conserved. The increase in intracellular Ca^{2+} triggers exocytosis and the release of neurotransmitters/neurohormones, propagating the metamorphic signal from the nervous system to the effector cells. Metamorphic signals may also spread through epithelial conduction of Ca^{2+} via gap junctions, as has been proposed for the hydrozoan cnidarian, *Mitrocomella polydiademata* (Freeman and Ridgway1990).

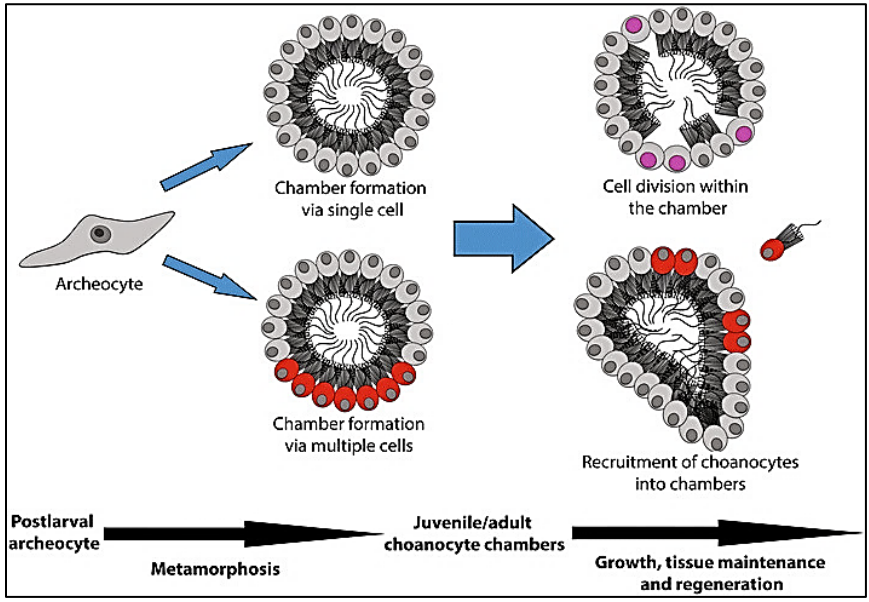

Figure 2. The ontogeny of choanocyte chambers during metamorphosis in the demosponge (Porifera), *Amphimedon queenslandica*. From Sogabe et al. (2016) Figure 9. Available in Open Access via license CC BY 4.0.

The Ca^{2+}-homeostasis and signaling have been shaped to near perfection during billions of years of evolution. The system can concurrently control various cellular processes with a few rather simple molecular mechanisms. It is very flexible. The main phases of life, juvenile-, adult-, and aged states reflect substantial changes in hormonally controlled Ca^{2+}-homeostasis (De Loof et al. 2014, 2015a, 2015b; De Loof 2017b). In insects, the juvenile state ends when upon changing hormonal conditions, the drastic drop in Juvenile Hormone (JH) levels and the rising ecdysteroid titre elevate substantially the influx of Ca^{2+} in

selected cells/tissues (De Loof and Schoofs 2019a). In the present paper we cover several physiological aspects in order to provide arguments for the theory that the Ca^{2+} homeostasis system is the primordial receptor system for juvenile hormones and ecdysteroids.

4. Insect metamorphosis in a historical perspective

4.1. Insects: From a life in iodine-rich seawater to an iodine-poor terrestrial environment

According to recent developments in comparative genome analysis, the eumetazoan from which all animals evolved may have been an early descendant of a choanoflagellate-like protist. This zoophyte opisthokont[4] managed to organize itself *into a closed epithelium thereby giving rise to the first animals*, sessile ancestral sponges with dispersive ciliary filter feeding larvae (Cavalier-Smith 2017, Degnan and Degnan 2006). The choanoflagellate cell type is still part of the internal lining of contemporary sponges.

Many originally marine species eventually left the sea with its high concentrations of NaCl, Ca^{2+} and iodine (that is incorporated in some hormones) and started a (permanent) terrestrial life. The ancient marine origin of vertebrates is reflected by the requirement of iodine for the biological activity of thyroid hormones in chordates. While many crustaceans (i.e., crabs, lobsters, shrimps, crayfish, krill, isopods, barnacles, copepods, ostracods, and many others) still live in the sea, very few insects ever fully adapted to a marine environment (Cheng 1976). Apart from some beach dwellers, they all became fully terrestrial, sometimes with part of their life cycle in fresh water. They do not use evolutionarily ancient iodine-rich hormones [Thyroxin (T4) and Triiodothyronine (T3)] like chordates continue to do.

4.2. Archaeology of the mevalonate biosynthetic pathway

Juvenile hormone(s) and ecdysteroids, along with a complex system of peptide hormones (Žitňan et al. 2007) are the key hormones controlling metamorphosis in arthropods. All six identified isoforms of JH are esters of farnesol, an endogenous sesquiterpenoid that is biosynthesized in the mevalonate biosynthetic pathway in all eukaryotes, as well as in some Archaea and Bacteria. Farnesol is still a "noble unknown" to many researchers in the biomedical sciences, mainly because hardly anyone recognized the importance of the experimental evidence provided by the electrophysiologists Roullet et al. (1999) and by Luft et al. (1999) that farnesol is a potent endogenous blocker of particular types of voltage-gated Ca^{2+}-channels. For review, see De Loof and Schoofs (2019a). Figure 2 summarizes the main features of this pathway.

[4] Opisthokonts, such as animals and the spores of chytrids (Fungi), have cells with flagella located posteriorly.

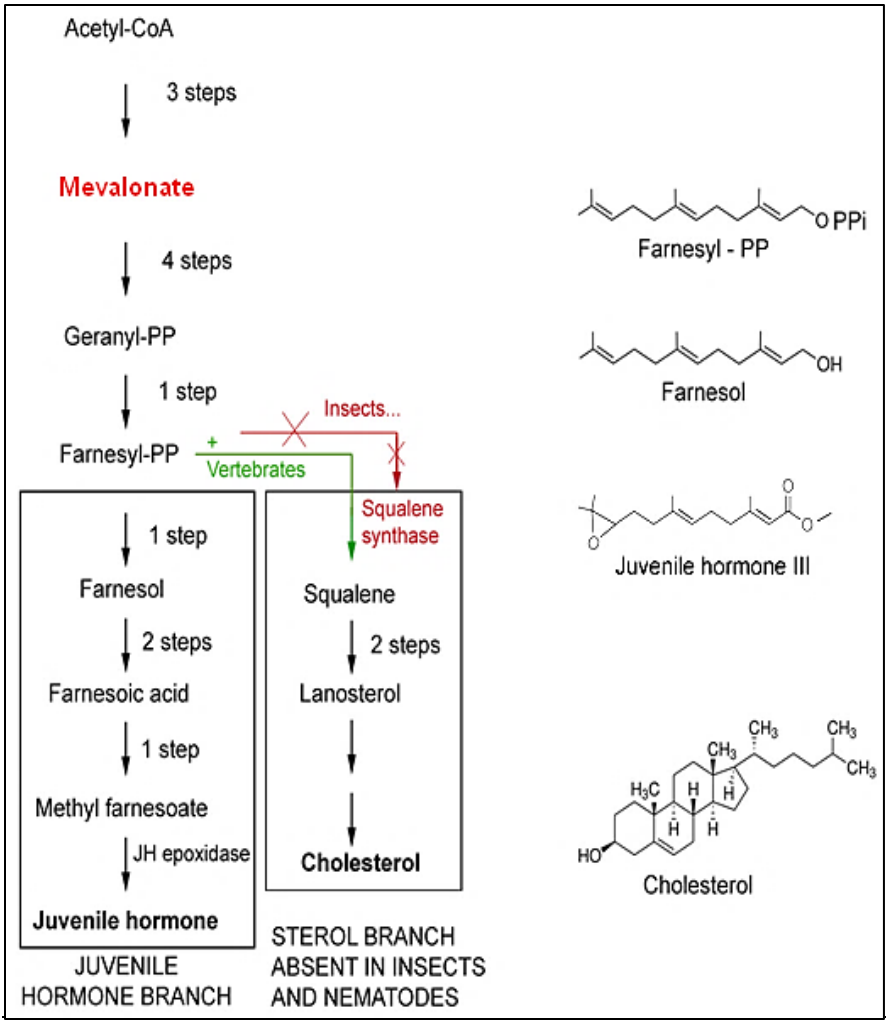

Figure 3. The mevalonate biosynthetic pathway. It operates in all eukaryotes, mammals and humans inclusive, but with some "branch-specific "variability that is mainly linked to the presence or absence of the gene coding for the enzyme squalene synthase that is needed for the synthesis of squalene. Squalene is one of the precursors of cholesterol, which has many functions, such as in the synthesis of sterols and "vertebrate-type steroid hormones". Organisms placed in the group of protostomates animals known as Ecdysozoa (i.e., the phyla Arthropoda, Onychophora, Tardigrada, Kinorhyncha, Priapulida, Loricifera, Nematoda, and Nematomorpha) cannot synthesize squalene. Hence, for them, cholesterol is a vitamin. Juvenile hormones are esters of farnesol. Their juvenile hormone activity is much higher than the mild one of farnesol itself. Borrowed from De Loof and Schoofs (2019a). Own work and Open Access.

4.3. The classical model on the mode of action of JH and ecdysteroids is more and more challenged

Karlson and Sekeris (1966) advanced the hypothesis that the moulting hormone ecdysone could pass the plasma membrane of target cells "unhindered" or "unnoticed", next diffuse through the watery environment of the cytoplasm, and finally (in)activate specific genes in the nucleus. This hypothesis was immediately challenged by Markus Lezzi, and Heinrich Kroeger and others (for references see De Loof et al. 2014), who found that so-called "ecdysone or JH-specific puffs on polytene chromosomes of some dipteran insects" could be induced by just changing the ionic composition of the culture medium in which salivary glands of some model larval dipterans were incubated *in the absence of any hormone*. At that time, puffing was considered to be indicative for changes in chromatin structure, an essential element in control of gene expression. For a figure of "puffing", see De Loof and Schoofs (2019a). The hypothesis was (partially) supported by Ashburner et al. (1974) and Dworniczak et al. (1983), amongst others, but for a variety of reasons it did not make it into the mainstream of thinking in on the mode of action of hormones (references in De Loof et al. 2014). The main reason was the unsuccessful struggle for identifying membrane receptors. It is only recently that Okamoto et al. (2018) could prove that a membrane transporter, namely Ecdysone Importer (EcI) is required for steroid hormone uptake in *Drosophila*. Via another approach, the comparison of the activity of 42 farnesol-JH compounds in the reduviid hemipteran, *Rhodnius*, JH-bioassay by Wigglesworth (1969) confirmed his earlier hypothesis (Wigglesworth 1957). He had proposed that JH, a lipophilic hormone, influences the gene-controlled enzyme system within the cells by regulating permeability in cellular membranes. Although the hormone-receptor concept was not yet well developed at that time, the cited authors initiated the question through which type of receptors, membrane- and/or nuclear, hormones act. Indirectly they raised the question whether or not the lipophilic JH and the more hydrophilic 20E act through different types of receptors. As will be discussed later, the receptors are different.

5. Complete metamorphosis

5.1. The gross events

An early last instar larva eats voraciously and increases substantially in weight. The alimentary canal becomes very voluminous, and the inhibition of gonadal development continues. The duration of the voracious feeding is species-specific. At a given moment, food intake stops. Other aspects of behavior also change. In most species of insects, the larva leaves the food source (wandering stage) and begins searching for a hiding place where it will pupate. Before pupation, the larva empties its alimentary canal. In some species, in particular in many lepidopterans and in some other insect orders as well, the salivary glands are also emptied, for example, by secreting the stockpiled silk proteins or the "glue

proteins" as in the fly genus *Drosophila* (Poels et al. 1971, Lane et al. 1972). Next, the larvae become almost completely immobile giving the impression of going into a coma, that in a species-specific way can last long, such as the whole winter. Their rhythm pattern changes drastically. The larval cuticle is shed, and a new pupal cuticle is deposited. Inside the body a most drastic rebuilding takes place. Many tissues undergo programmed cell death. The fat body, the midgut, the salivary glands and some larval muscles are obvious examples. Some are replaced by adult-type tissues. The nervous system, the Malpighian tubules, the hearth and the nascent gonads develop and differentiate further. Adult muscles also develop. At the end of the pupal life, the pupal cuticle is shed, and an adult cuticle is deposited. The adult will next eclose (Figure 1). The hormonal regulation of programmed cell death is species-specific. In some species, absence of JH induces programmed cell death, in others it does the opposite (Manaboom et al. 2012) while 20E is more important (Manaboom et al. 2009).

5.2. Anthropomorphic-medical interpretation of the phenomenon "metamorphosis": an "unbiased observer's" view

In anthropomorphic-medical (medical) wording, an unbiased observer might describe this process as follows: "Overeating makes sick, and it takes time to recover". In holometabolous species, early last instar larvae, no matter what their larval diet was, all tend to eat so much (med: bulimia) that in a short time they become obese (sickly obesity), and give the impression to first feel satisfied (satiety) but next uncomfortable (indications of "hang over/feeling bad" because of overeating). They leave the food and wander away to defecate. They hide somewhere in a protected place (usually away from light) to let their body recover (coma, vegetative life state), or to die. The larvae are pricked in too small a cuticle. They shed and replace it by a new one that is stronger and more rigid. Internally, some tissues become functionless and are lysed, as it happens during programmed cell death of some muscles (sarcopenia in medicine), fat bodies, etc. Meanwhile, other tissues regenerate from stem cells. During remodeling of the epidermis, the animals do not bleed to death, indicating that its epithelial integrity remains unaltered. When metamorphosis is completed, the insect lives in a new body (plastic surgery/modeling completed in medicine). The advantage, for both research and teaching purposes, of incorporating some anthropomorphic medical vocabulary (which may be disliked by some) resides in the fact that it encourages comparative research into the fundamental mechanisms involved.

5.3. Two views: Complete metamorphosis as normal development versus as a disease state?

When studying the mechanisms instrumental to metamorphosis, one can follow the reductionist method and study it piece by piece. The more pieces, the harder it is to conjure up the coherent image hidden in the puzzle. To understand the stepwise construction of our model, one should keep in mind that we started

from asking the following basic question: Is metamorphosis a normal aspect of development? Or, given that hemimetabolous insects develop gradually, could it be that complete metamorphosis reflects an *abnormal way of development* due to an ancient mutation that caused a very severe *illness state*? The cause of the illness? The link between the drastic increase in $[Ca^{2+}]i$ and the induction of programmed cell death/apoptosis (Orrenius et al. 2003), a most important process in complete metamorphosis is well documented. Is the excess of intracellular Ca^{2+} the sick maker?

6. Metamorphosis: an intoxication process as a result from excess Ca^{2+}?

6.1. The basics of Ca^{2+} homeostasis

The inorganic ions H^+ and Ca^{2+}, which are well known as key secondary messengers, were probably the evolutionarily most ancient regulators of gene expression in the prokaryotic common ancestor(s) of all contemporary cells on earth. Superfluous to say that inorganic ions cannot be (enzymatically) degraded like the evolutionarily more recent organic control factors such as hormones and neurotransmitters. Hence the only way to differentially control cellular processes by inorganic ions is to vary their intracellular concentration. Exactly this ancient principle still goes on in all living cells.

6.2 Calcitox: Ca^{2+} as Janus-faced inorganic ion.

The term "Calcitox was introduced by De Loof (2017b), in particular for teaching purposes. It intended to condense into a single term the fact that Ca^{2+} which is best known for its numerous beneficial effects (e.g., as a secondary messenger and in the construction of some types of skeletons), can also generate toxic effects. Ca^{2+} can be categorized as a "Janus-faced inorganic ion" which can represent both beneficial and toxic effects. A paper by Orrenius et al. (2003: "Regulation of cell death: The calcium-apoptosis link" formed the basis for the formulation of the "Calcitox concept" as particularly important for a better understanding of the endocrine control mechanisms of metamorphosis. At rising concentrations Ca^{2+} can make the 3D conformation of various macromolecules, in particular proteins, change. This results in changes in activity of various enzymes, of cytoskeletal proteins (e.g., muscle contraction), etc. Thus, in fact even at sublethal increases in concentration Ca^{2+} is toxic, but we experience the resulting effects as beneficial. However, when its cytoplasmic concentrations rise above a critical value for too long, Ca^{2+} induces cell death.

Our approach may give the impression that we (unintentionally) overestimate the role of Ca^{2+} in metamorphosis and undervalue the complexity of the numerous other control mechanisms of development and metamorphosis. This is not so: we fully support the broad view of one of the anonymous reviewers of this paper who formulated the complexity as follows: "*It is generally or widely accepted that nuclear receptors as transcription factors or entire transcriptional cascades are known to perform crucial developmental decisions and implement developmental*

transitions, generally named as genomic effects. This crucial and decisive action does not appear to be delegated to ions, neurotransmitters or their membrane receptors. Effects of membrane-anchored receptors via which majority if not all transmitters and peptidic hormones act lead to fast and immediate responses at non-genomic level which may include also various changes in the redistribution and or release of ions, including calcium. In contrast, longer time requiring developmental processes like morphogenesis (incl. metamorphosis) rely on coordinated and still very specific action of multimolecular complexes implementing genetically-encoded program and cannot be decided by such a simple and non-specific molecules as ions. Ions, of cause may have very specific local functions, notably due to cellular compartmentalization, but they are not known to drive entire developmental transitions at genomic scale, although may take a part in it." The reason why we limit ourselves to a focus on Ca^{2+} is that we feel that the role attributed to Ca^{2+} in endocrine control of metamorphosis deserves a substantial upgrade.

6.3. Four key mechanisms

We argue that both JH and ecdysteroids employ the Ca^{2+}-homeostasis system to control developmental effects. To understand this argumentation, one should be familiar with the principles of Ca^{2+}-homeostasis (Figure 4). In physiology, Ca^{2+} is best known for its beneficial functions, in the skeleton, in muscle contraction, in the functioning of neurons, as secondary messenger in many signaling systems [(e.g., in G Protein-coupled Receptors (GPCRs)], etc. This contrasts with the counterintuitive fact that *Ca^{2+} is the most abundant toxin on earth,* and that it is exactly its toxicity at rising cytoplasmic concentrations that forms the basis for the mentioned beneficial effects (De Loof 2017b). The toxicity of Ca^{2+} is inherent to the fact that at increasing concentrations, Ca^{2+}, like H^{+}, causes changes in the 3D structure and activity of a variety of macromolecules, in particular of proteins/enzymes. For example, muscle contraction is based upon this principle. Ca^{2+}-induced apoptosis is another example. For readers who are not very familiar with the principles of Ca^{2+}-homeostasis, a concise, well-illustrated introduction has recently been published (De Loof 2017b). In this paper, it is explained that in order *to keep the cytoplasmic Ca^{2+} concentration*, $[Ca^{2+}]i$, *below the toxicity level (= very low, in the order of 100 nanomolar)*, cells have several possible mechanisms at hand. Briefly, as outlined elsewhere (De Loof 2017b) these are:

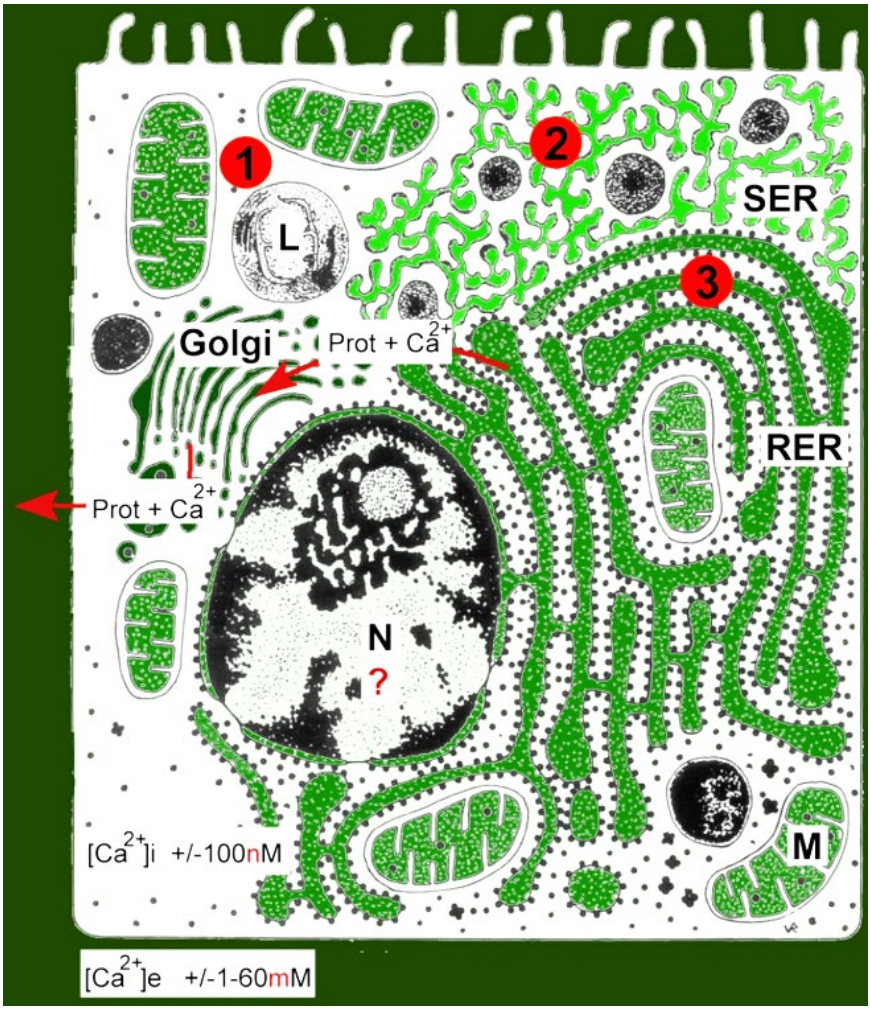

Figure 4. Schematic representation of the Ca^{2+} gradients (adapted from De Loof et al. 2014 and 2017b) The different shades of green are not meant to give an exact representation of differences in Ca^{2+}-concentration. L: lysosome, N = nucleus, M = mitochondrion, RER = rough endoplasmic reticulum, SER = smooth endoplasmic reticulum. By looking at the ultrastructure of cells and by evaluating how abundant the SER and RER are, one can make plausible guesses about the gross outline of their Ca^{2+}-homeostasis system, as well as of some of its non-genomic effects on some enzyme systems, such as the ones involved in lipid, steroid- and protein synthesis. This is because the SER and RER are membrane systems in which numerous enzymes of which the activity is (partially) controlled by the Ca^{2+} gradient over their membrane are anchored. The red dots with 1, 2, and 3 correspond to the mechanisms 1-3 for keeping $[Ca^{2+}]i$ low (see text). Copyright permission: From Open Access sources.

Mechanism 1: Make the plasma membrane of cells as impermeable to Ca^{2+} as possible (red dot with number 1): keep the number of Ca^{2+} channels low, or/and restrict the time they are open by controlling their gating mechanism(s). In case some excess Ca^{2+} enters the cell, Plasma Membrane Ca^{2+}-ATPases (PMCAs) will be activated.

Mechanism 2: When the influx of Ca^{2+} exceeds the out-pumping activity of the PMCAs, rescuing activity is needed. One possibility is to store a limited amount of excess Ca^{2+} in storage sites. These are the mitochondria and the lumina of the endoplasmic reticulum, in particular of the smooth ER (SER) (red dot number 2). Muscle cells use the release and the reuptake of part of the stored Ca^{2+} for controlling their contractility. Some of the enzymes of the biosynthetic pathways of lipids, phospholipids and steroids reside in the cisternae. *The intraluminal Ca^{2+}-concentration in the cisternae influences their activity*. The temporary release of Ca^{2+} may not last long, otherwise cell damage will occur. The duration of a heartbeat gives an indication of what "long" means.

Mechanism 3: In some cell types, the influx of Ca^{2+} can be high, e.g. under the influence of some steroid hormones, e.g. the female sex steroids, which increase the permeability of the plasma membrane by gating membrane G-protein coupled estrogen receptors for Ca^{2+} (Prossnitz et al., 2007). As a result, the combined efforts of the Ca^{2+}-ATPases in the plasma membrane and the temporary storage of $[Ca^{2+}]i$ in the SER and mitochondria do not suffice for neutralizing the increasing $[Ca^{2+}]i$. In that case the RER gets involved (red dot number 3). This $[Ca^{2+}]i$ rise seems to cause the development of an abundant RER. This requires synthesis of new lipids, ribosomes, and proteins. The RER, in concert with the Golgi system, secretes proteins *with a role outside the cell, referred to as "cargo proteins"*. A problem emerges: How can this excess Ca^{2+} be removed before it will induce apoptosis? The answer: the only way to do so is to remove Ca^{2+} along with the proteins that are secreted by the RER-Golgi system. In our opinion, *the largely overlooked universal role of the RER is not just the secretion of any type of (cargo) proteins, but specifically of Ca^{2+}-binding/transporting cargo proteins.* Thus, cells with an abundant RER, are cells with a major Ca^{2+}-homeostasis problem. The Golgi system is sensitive to presence or absence of JH, with drastic changes in its activity as a result (see section 10.1).

Mechanism 4: If all three aforementioned mechanisms fail, the only option left is to activate Ca^{2+}-induced apoptosis (Orrenius et al., 2003). For introductory figures on this topic, see (Multiple authors a, 2019).

During metamorphosis, all four mechanisms are at work to various degrees in different tissues. This contributes to the tissue-specificity in remodeling the body. Some tissues continue to further differentiate during metamorphosis, others undergo programmed cell death, and are rebuilt from stem cells. Researchers should be aware of the fact that homogenization of cells and tissues destroys all Ca^{2+}-gradients. Some effects of hormonal treatments may become unobservable after such treatment.

7. The links of the mode of action of 20E and JHs with Ca^{2+}-homeostasis

7.1 Introductory remark: the concept that the corpora allata (CA) are the only site of synthesis of JHs is wrong

The view that insects have only one type of gland that produces JH, namely the corpora allata, is almost generally accepted. Yet, it is wrong. It is often forgotten that JH I was identified for the first time in extracts of abdomens of males of the moth *Hyalophora cecropia* (Lepidoptera: Saturniidae). The active substance was present in the male accessory glands (MAGs), in which the JH-active substance accumulated during metamorphosis, thus in the phase of development in which the CA, the supposed unique site of synthesis, are completely inactive. The search for the site of synthesis of MAG-JH took several decades. First, Borovsky et al. (1994a) demonstrated that the MAGs of four *adult* species of mosquitoes (Diptera: Culicidae: *Aedes aegypti, Culex nigripalpus, Anopheles rangeli, and Anopheles trinkae*) synthesize JH III de novo. Moreover, Borovsky et al. (1994b) also reported that the ovaries of the adult mosquito *Aedes aegypti* can synthesize (10*R*)-JH III from farnesoic acid, and that they can also synthesize JH III-like molecules from L-methionine and acetate. However, the international community remained skeptical, despite support for Borovsky's pioneering work was presented by other researchers (refs. in De Loof and Schoofs 2019a). It took another twenty years before Paroulek and Sláma (2014) presented part of the explanation for the *Cecropia* MAG-JH enigma. It says that MAG-JH-I, which is *synthesized during metamorphosis*, thus prior to adult emergence, is not synthesized by the CA, but by the MAGs themselves, MAG-JH is not released into the haemolymph, but it is transported along with MAG secretions into the female during mating. This shows that JH is not simply transported by random diffusion, but *in a polarized way*. This probably implies a hitherto unrecognized role of the Golgi system in all farnesol/JH-secreting cell types, the corpora allata inclusive (De Loof and Schoofs, in preparation). Thus, MAG-JH is "*exocrine JH*", while CA-JH is "*endocrine JH*" (Paroulek and Sláma 2014).

De Loof and Schoofs (2019a) suggested that MAG-JHs may function in protecting the spermatozoa against toxic Ca^{2+} concentrations, and in enabling their flagellum to undulate. If the flagellar function was indeed important, it may, perhaps, have been conserved in all eukaryotic cells with a motile or a primary (non-motile) cilium, making the functioning of the centrioles a possible but overlooked target of endogenous farnesol-like sesquiterpenoids.

7.2. Identifying the membrane receptors for JHs and 20E turned out to be challenging

It is known since long (Prestwich et al. 1999) that JH binds to various proteins, JH binding proteins (JHBPs), a nuclear JH receptor(s), JH esterases (JHEs), JH epoxide hydrolases (JHEHs), and methyl farnesoate binding proteins (MFBPs) etc. Yet, binding as such is an insufficient criterion for functioning as receptor: JH even binds to glass and stainless steel. Proving that binding of a hormonal ligand induces a signaling cascade that starts at the level of the plasma membrane can be tedious. The major reason is that the identification of membrane receptors, the main actors in involving the Ca^{2+} homeostasis system in the mode of action of 20E and JHs, is much more difficult than of nuclear receptors.

A reviewer made the following pertinent remark: "Yes, JH is highly hydrophobic, and of course during various purification procedures and even *in vivo* (mainly at higher doses) can bind non-specifically to highly lipophilic membranes. But would anybody expect that this non-specific binding that can be observed for even water-insoluble vitamins and hundreds of other lipophilic molecules, could elicit such specific, coordinated and programmed action as influencing the metamorphosis?" Our answer is that this is indeed possible if the conditions are right. It is a matter of matching hydrophobicity and of the presence of matching binding pockets in transmembrane proteins. The lipid bilayer part of membranes acts as a good solvent (which is not the same as a "genuine receptor") for molecules with a matching hydrophobicity profile. Many different types of hydrophobic molecules enter the membrane system this way. The fact that biomembranes are fluid makes that such compounds can rapidly diffuse throughout the whole membrane system: the more fluid the membrane, the faster the diffusion. However, only when the diffusing molecules meet a matching binding pocket on a transmembrane macromolecule, e.g. on a Ca^{2+} channel as is the case for farnesol, this may generate an appropriate physiological effect. As an example: Vertebrate sex steroids are, like farnesol and JHs, hydrophobic. The solubility in water for estradiol is 3.9 mg/L, and that of testosterone is 23.4 mg/L. Sex steroids affect all cells of the body just like JH does during larval life of insects. Apparently, all cells have receptors with matching binding pockets for such steroids. This whole-body effect of vertebrate sex steroids also acts through the Ca^{2+}-homeostasis system. It has been named "Calcigender" (De Loof, 2015, 2019). Such whole-body effect is also possible with some hydrophobic peptide hormones. An appealing example is the whole-body melanization effect of the undecapeptide [His7] corazonin that plays a key role in phase transition (solitarious-gregarious) in locusts. When this colorless peptide is injected into albino locusts, melanization only occurs if the peptide is dissolved in an oil. No effect when injected in the form of a watery emulsion (Tawfik et al. 1999).

7.3. A GPCR-type membrane receptor for 20E with a link to Ca^{2+} signaling

The nuclear receptor-mediated genomic pathways of the animal steroid hormones, both ecdysteroids (Koelle et al. 1991; Riddiford et al. 2000) and vertebrate-type steroids, are well documented. 20-hydroxyecdysone (20E) regulates gene transcription via a genomic pathway by forming a transcription complex that binds to DNA with the help of the chaperone proteins, heat shock proteins (Hsps) Hsc70 and Hsp90 (Arbeitman and Hogness 2000, Liu et al. 2014, Cai et al. 2014b). However, the cell membrane receptor-mediated nongenomic pathways of the animal steroid hormones in general are much less well understood. Substantial progress has been made in the recent decade not only regarding the receptors of vertebrate steroids (e.g., Leung and Sadar 2017) and Thyroid hormone (TRH) (e.g., Kalyanaraman et al. 2014, Hammes and Davies 2015), but also on the membrane receptor of the main ecdysteroid hormone (20E) of insects.

In insects, Srivastava et al. (2005) were the first to report rapid, nongenomic responses to ecdysteroids and catecholamines mediated by a novel *Drosophila* G-protein-coupled receptor. This DmDopEcR showed sequence homology with vertebrate beta-adrenergic receptors and was activated by dopamine to increase cAMP levels and to activate the phosphoinositide 3-kinase pathway. Additional data on this receptor have been published by Ishimoto et al. (2013), Abrieux et al. (2013 2014), Evans et al. (2014), Petrucelli et al. (2016), and Lark et al. (2017).

In *Helicoverpa armigera* (Lepidoptera: Noctuidae) the 20E receptor in the plasma membrane is a G-protein-coupled receptor, named ErGPCR-2 (Wang et al. 2015; Cai et al. 2014a). It transmits steroid hormone 20E signaling, and it controls 20E entrance into cells. The binding of 20E to this receptor causes a rise in $[Ca^{2+}]i$ (Cai et al. 2014a, Wang et al. 2015, 2016; Jing et al. 2015). This receptor is internalized by 20E induction (Wang et al. 2015). Li et al. (2017) showed that in accordance with the general rule that animal steroid hormones stimulate extracellular Ca^{2+} influx into cells, such influx that follows stimulation by 20E is modulated by the Ca^{2+} release-activator calcium channel modulator 1 (CRACM/Oral1). This is causal to the induction of apoptosis in *Helicoverpa* midgut cells. ErGPCR-2 GPCR kinase 2 participates in 20E-induced ErGPCR-2 phosphorylation and internalization. The internalized ErGPCR-2 is degraded by proteases to desensitize 20E signaling. ErGPCR-2 knockdown suppresses the entrance of 20E analog, tritiated ponasterone (also abbreviated as ^{3}H-Pon, [(3)H] ponasterone A, [(3)H]Pon A) into the cells. ErGPCR-2 overexpression or blocking of ErGPCR-2 internalization increases the entrance of [(3)H]Pon A into the cells. However, [(3)H]Pon A does not bind to ErGPCR-2.

According to Ren et al. (2014) a G-protein alpha q (Gαq)(1) subunit participates in the 20E nongenomic pathway in the cell membrane, and contributes to regulating gene expression during moulting and metamorphosis. Ecdysterone 20E-induced phosphorylation of Gαq was detected. RNAi knockdown of Gαq suppresses the development of larvae, delays metamorphosis,

and inhibits 20E-induced gene expression. Gαq is distributed throughout the cell and migrates toward the plasma membrane upon 20E induction.

7.4. The membrane Ca^{2+}-channel type receptor of Farnesol/FLS

Farnesol was the first discovered endogenous compound (family) with Juvenile Hormone activity (Schmialek 1961). It is a sesquiterpenoid. It is the precursor of all known isomeric Juvenile Hormones, which are esters of farnesol (FLS: Farnesol-like Substances) (Sláma 2013, 2015; Qu et al. 2018; De Loof and Schoofs 2019a). According to Cheong et al. (2015) and Schenk et al. (2016) sesquiterpenoids are ancient animal hormones present in many, if not all, invertebrate and chordate species. Farnesol and its ester JH III is not only biosynthesized by the mevalonate pathway in animals (section 4.1), but also by the very same pathway in some plants, such that the sedge, *Cyperus iria* (Bede and Tobe 2000, Bede et al. 2001).

Because the test system used by Roullet et al. (1999) and Luft et al. (1999) was a vascular smooth muscle system of rodents, the farnesol membrane receptor they identified apparently escaped the attention of insect endocrinologists. Farnesol itself is bioactive when tested in assays designed to detect JH bioactivity (see Figure 2 in De Loof et al. 2014). Therefore, it is likely that the membrane receptor(s) of JH and farnesol belong to the same receptor family. Yamamoto et al. (1988), studying the functioning in an *in vitro* system for the *Drosophila melanogaster* male accessory gland, were the first to suggest a membrane - protein-mediated effect of juvenile hormone that involves calcium and kinase C. The membrane receptor involved remained to be identified biochemically until to date.

Albeit less active that JHs, the trans,trans farnesol isomer was reported to be by far the most active farnesol-like substance in the *Rhodnius* bioassay that detects JH activity (Wigglesworth 1969). This isomer occurs in the brain of rodents and humans at physiologically relevant concentrations of 100-800pmol/g wet weight, and it acts as a relatively non-discriminatory rapid open channel blocker of all types of high voltage-activated channels, with a mild specificity for L-type (= Long Lasting) channels. Open channel block by farnesol may require partition of farnesol into the lipid phase (of the membrane). Apparently, the block of the L-type channels is almost fully developed prior to channel opening (resting block). A schematic representation of the different subunits of an L-type high voltage gated Ca^{2+} channel is given in Figure 5. According to the same authors, farnesol blocks the L-type Ca^{2+} channel by targeting the pore forming α_1 subunit, in particular the α_{1c} subunit. Administration of 250nM trans,trans farnesol induced an N-type (= Neuron/Non-L)-specific hyperpolarizing shift in channel availability. The data provided by the cited authors constituted the first description of a selective blockade of these channels by a small endogenous organic molecule that is omnipresent in all eukaryotic cells. It suggests a novel mechanism not only for the precise regulation of brain Ca^{2+} homeostasis and neurotransmitter release

implicating the mevalonate pathway and farnesol production in the brain, but also for a better understanding of juvenile hormones in development and aging (De Loof 2017b, De Loof and Schoofs 2019a, and this paper).

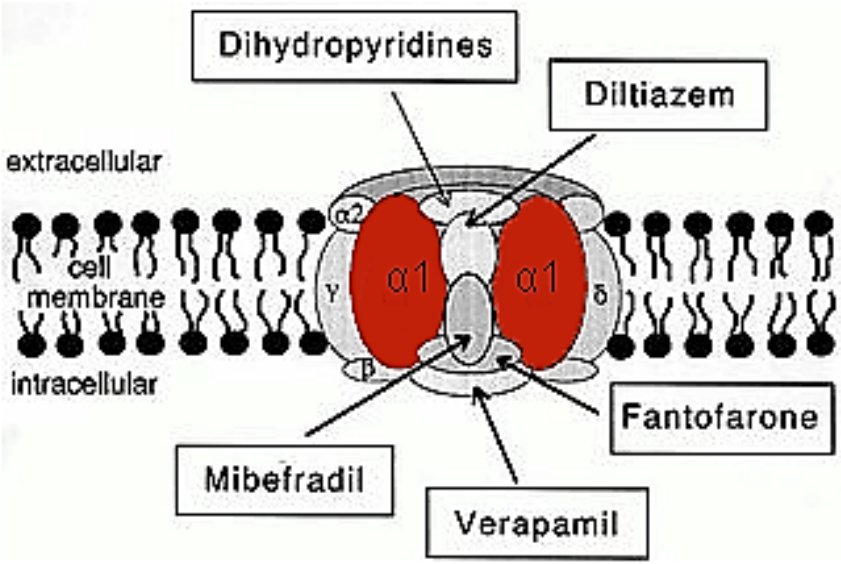

Figure 5. Depiction of binding sites of various antagonistic drugs in the L-type calcium channel. According to Roullet et al. (1999) and Luft et al. (1999) farnesol inhibits voltage-gated channels by interfering with the α1 subunit (in red). This blocking mechanism is different from those of classic synthetic L-type channel blockers. Adapted from Multiple authors b (2019). Open Access.

7.5. Some G Protein coupled receptors (GPCRs) are also (indirect) receptors for farnesol/FLS

Many G-protein coupled receptors use Ca^{2+} as a secondary messenger. A variety of hormones, such as the numerous neuropeptidic hormones (Caers et al. 2012, Schoofs et al. 2017), use such receptors to mediate their effects. Out of the five GPCR families, the rhodopsin-type is most widespread in nature, and comprises about 80 percent of all known GPCRs. It is not sure whether all GPCRs are the progeny of a common ancestral rhodopsin, that acted as an H^+-pump, and that did not make use of G-proteins. Its serpentine 7TM structure has, however, been well conserved during evolution. There is still uncertainty how the change in 3D conformation that occurs when a ligand binds to the receptor-pocket leads to an intracellular effect that is mediated by changes in $[Ca^{2+}]i$.

De Loof and Schoofs (2019b) outlined a model for the functioning of GPCRs in which *prenylated* α and γ subunits of G-proteins and trans molecular ion fluxes (mainly Ca^{2+}) play a role (to be published elsewhere). Prenylation (Vögler et al.

2008) is a chemical reaction by which, for instance, a farnesyl group (= precursor of juvenile hormone) or a geranylgeranyl group is attached to a protein, in order to facilitate protein-protein interactions (Multiple authors 2019 d). The essence of the model is that the farnesyl/prenyl-group, which is *covalently* bound to a G protein, upon being attached to a GPCR, might function as a hydrophobic flexible (rotatable bond count of 7) flip-flopping horseshoe-shaped valve for restricting - though not fully inhibiting - the untimely intramolecular passage of Ca^{2+} through the GPCR, like retinal does for the passage of H^{+} in microbial rhodopsins that are ancestral to many GPCRs.

7.6 The SERCA-pump: a possible target for farnesol/FLS? Other non-genomic effects as well

The observation that *absence of JH* induces Ca^{2+}-induced apoptosis in all tissues, which actively secrete proteins through their RER-Golgi systems, resulted in the hypothesis that at the cell-membrane level, JH is thought to bind to a specific pocket on the SERCA-Ca^{2+}-ATPase. That binding pocket, probably the same as the one for thapsigargin binding [a potent SERCA-pump blocker and also, like JH, a sesquiterpenoid (De Loof et al. 2014)], requires a horseshoe-shape to functionally link the three transmembrane helices of the SERCA pump which are actively involved in the transmembrane transport of Ca^{2+}, namely TM3, TM5 and TM7 in such a way that *JH can act as a molecular spring and valve*. Its probable function is to bring back these helices into their "relaxed position" after each pumping cycle of a Ca^{2+}-ion.

Whether this also applies to the PMCAs (Plasma Membrane Ca^{2+} ATPases) is unknown. Anyhow, Ca^{2+}-pumps and Ca^{2+}-channels have to act in a coordinated way in order to effectuate homeostasis. When dealing with the 4 key mechanisms that operate in Ca^{2+} homeostasis (section 6.2), we mentioned that the activity of various enzyme systems which reside in the membranes of the SER, RER etc. *may depend upon the height of the transmembrane Ca^{2+} gradients*. These are nongenomic effects. This does not mean that the synthesis of the enzymes does not depend on the central dogma, but that the enzymes are already in place in the membranes. They had been synthesized before the changes in Ca^{2+}-gradients take place. Other possible targets of JH action may be the Golgi system and motile-nonmotile cilia with their connection with the centrioles, as mentioned before.

8. Nuclear receptors for JHs: Ca^{2+}-dependent transcription factors?

8.1. Methoprene-tolerant and Gce: genuine JH "receptors" or rather JH "targets"?

In the literature, Methoprene-tolerant (Met) and Germ cell-expressed (Gce), which are bHLH-PAS transcription factors and, in *Drosophila,* products of two paralogous genes, have been categorized as "genuine" nuclear receptors for JH (Charles et al. 2011; Abdou et al. 2011; Jindra et al. 2013, 2015a, 2015b). This resulted from the combination of genetic experiments, *in vitro* ligand binding

studies, and computer modelling of the interaction of JH with the Met-PAS-B domain of the beetle *Tribolium castaneum* (Coleoptera: Tenebrionidae) The authors have assumed that their interpretation based on *in vitro* experiments could be validly extrapolated unchanged to the *in vivo* situation. However, they have overlooked the fact that for their model to be valid *in vivo*, JH has to end up in the nucleus one way or another. To date (December 5, 2019), this proof is still missing.

If JH(s) would be readily water-soluble, the classical model of ecdysteroid signaling could be used without problem. However, instead JH(s) are highly hydrophobic and scarcely soluble in water. For example, the solubility of the precursor of JHs, farnesol, in water is 1.7 mg/L at 25 °C (Pubchem), that of the JH analog methoprene is 1.4 mg/L (PubChem), and that of the C18 Juvenile hormone is 5 mg/L Tris-buffer (Kramer et al. 1974). The reported solubility in water for other JHs is lower than 5 mg/L. All JHs have a high affinity for membranes. Upon contacting the plasma membrane, it will start diffusing through the entire integrated membrane system of cells. Keeping in mind that many enzymes with roles in lipid-, steroid-, protein- etc. synthesis, and transmembrane proteins with a role in, for instance, ionic regulation, Ca^{2+}-homeostasis, GPCR signaling etc. are anchored in specific parts of the membrane system, and that (trans)membrane proteins have hydrophobic domains by definition, the chances that the diffusing JH will meet matching binding pockets in macromolecules anchored in membranes, is substantial. This is illustrated by the numerous non-genomic effects that manifest themselves by the fall to zero of the JH titre at the onset of metamorphosis. In our opinion, categorizing Met/Gce as being Ca^{2+}-sensitive transcription factors (De Loof and Schoofs 2019a) is compatible with the fact that not only the presence, but the absence of JH can differentially signal. Yet, in theory, it is not *a priori* excluded that JH would end up in the nucleus. This could be achieved if it could bind to a lipophilic carrier protein that could diffuse through the cytoplasm, and that is small enough to transit the nuclear pores. Such protein has not yet been found. Hence, to date's (2019) situation is that there is no experimental evidence whatsoever that, *in vivo,* JH enters the nucleus. Thus, as long as such entry is not proven, Met should not be categorized as a genuine nuclear receptor, and certainly not as *the* JH receptor, but as one of the various (already known) target molecules of JH. If Met/Gce are indeed Ca^{2+}-sensitive transcription factors, there is no absolute need for JH to enter the nucleus for influencing transcription.

The complexity of JH signaling, both in the nucleus and in integrating membrane and intranuclear effects, keeps expanding. E. g. Met can form a complex with Taiman (Tai) (Lozano et al. 2014). Liu et al. (2015) and by Ojani et al. (2016) postulated that a membrane-initiated signaling pathway modifies the DNA-binding activity of MET via phosphorylation and thus facilitates the genomic responses to JH. Thus, there is an interplay of genomic and nongenomic signaling mechanisms of JH. A lot of additional research is needed.

8.2. Ca^{2+} affects chromatin structure, the cytoskeleton and the nuclear matrix

Long before the central dogma (Crick 1970) and transcription factors were known, studies on the control of puff formation in polytene chromosomes of dipteran insects revealed that changes in chromatin structure are an important player in the control of gene expression. Puffs are not the result of the (in)activation of one single gene, but of clusters of genes. The modern methods of molecular biology enable more detailed studies with respect to the quantification of such "clusters of genes". For example, in T cells the cooperation between calcium and kinase signaling influences a chromatin landscape that comprises around 2100 chromatin regions. They typically function as inducible enhancers in regulating T cell activation (Brignall et al. 2017). According to Lee et al. (2015) chromatin decondensation in T cells can be initiated by mobilization of Ca^{2+} from intracellular stores by itself, thus without the need of influx of calcium and independent of the activity of the downstream factor NFAT. Lai et al. (2009) and Lee et al. (2015) reported that upon toll-like receptor 4 (TLR4) signaling in macrophages, the mammalian Swi/Snf-like BAF chromatin remodeling complex is recruited to many TLR4 target genes where it remodels their chromatin to promote transcription. By itself this is insufficient. Indeed, the binding of Ca^{2+}/calmodulin to the HMG domain of the BAF57 subunit within the BAF complex is also needed to realize transcription.

Especially when analyzing the mode of action of a given hormone, such data urge for caution when attributing (or not) a role to Ca^{2+} itself or to a (additional) transcription factor such as the early JH-inducible gene Krüppel homolog 1 (*Kr-h1*) that plays a key role in the repression of metamorphosis as a mediator of JH action, namely via direct transcriptional repression of Broad-complex (*BR-C*), a pupal specifier gene (Kayukawa et al. 2016, 2017; Minakuchi et al. 2009).

Changes in $[Ca^{2+}]i$ itself can provoke changes in the protein synthesis cascade. Thus, if a given hormone like the *hydrophobic* JH influences transcription, part of this effect may be brought about by its effects on the Ca^{2+}-homeostasis system of which the key players (pumps and channels) are anchored in the cellular membranes, the nuclear envelope inclusive. Not only selected transcription factors can have a binding site for endogenous (farnesol-like) sesquiterpenoids, but this is also the case for some types of Ca^{2+}-channels, Ca^{2+}-pumps, G-proteins etc. (De Loof and Schoofs 2019b). For the moment being, the hypothesis that JH itself binds to the transcription factor Met/Tai complex, the reported nuclear receptor of JHs inside the nucleus, is more favored by researchers. However, we think that the role of farnesol-like endogenous sesquiterpenoids in cellular physiology can only be understood in full if *all their binding sites* are concurrently considered, thus not only the intranuclear ones.

It is not clear at this moment how hydrophobic hormones like the JHs could migrate from the hemolymph into the nucleus. It is not because in *in vitro* assays JH binds to some protein(s) that this results *in vivo* in gene (in)activation (Prestwich et al. 1996). Finally, the role of hormones in general, JH and

ecdysteroids inclusive, and in particular of changes in $[Ca^{2+}]i$, on the cytoskeleton should not be undervalued (De Loof et al. 1996).

9. Not the presence of JH, but the drop to zero of the JH titre triggers the onset of complete metamorphosis

9.1. Opposite fluctuations in JH and ecdysteroid titres occur during metamorphosis

Using the very sensitive *Galleria* (Lepidoptera: Pyralidae) bioassay for detecting JH, De Loof and Van Loon (1979) demonstrated the JH drop to zero phenomenon in *Galleria* (Peferoen and De Loof 1980) (Figure 6A). More detailed qualitative and quantitative analyses of JH and ecdysteroids from egg to the pupal molt were carried out in the noctuid (Lepidoptera), *Trichoplusia ni* (Grossniclaus-Bürgin and Lanzrein 1990).

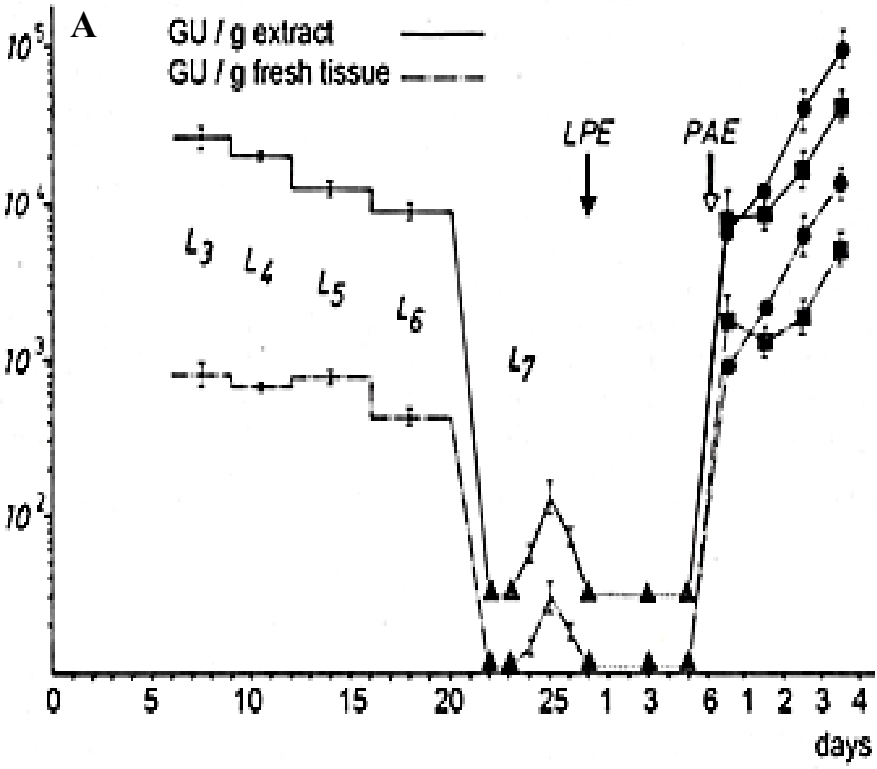

Figure 6. A. JH concentrations in extracts of the wax moth *Galleria mellonella* as measured by the *Galleria* bioassay (De Loof and Van Loon 1979). GU: *Galleria* units; L_3-L_7: larval instars; LPE: mean time of larval to pupal ecdysis; PAE: mean time of pupal-adult ecdysis. Circles represent females, squares males. Triangles: no JH activity found. Day 0: eclosion of first instar larvae. From Peferoen and De Loof (1980).

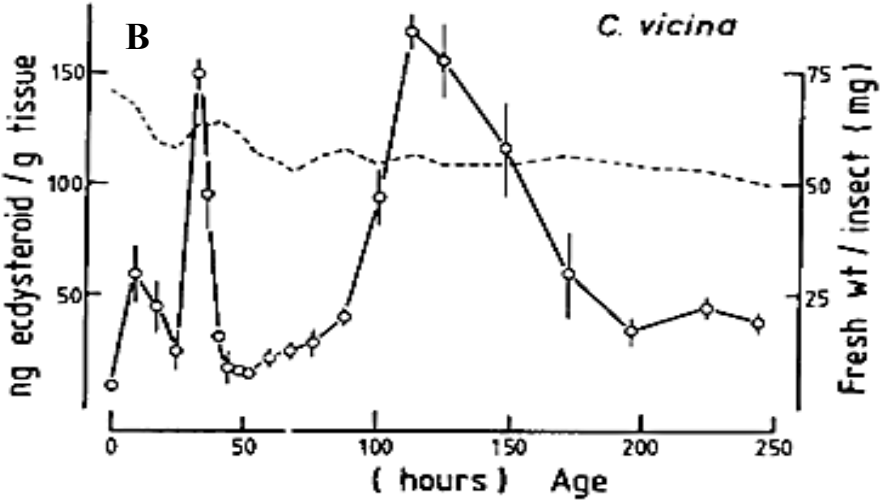

Figure 6 (continuation). B. Ecdysteroid titre during metamorphosis of the fly *Calliphora vicina*. Fresh weight of insects (---), SD less than the size of the data points are not shown. From Briers et al. (1983). Copyright permission: own work and Open Access.

9.2. The cause of the drop in JH titre? Allatotropins, allatostatins, sNPFs, others?

Because the corpora allata (CA) can neither accumulate JH nor stop releasing the hormone once it has been synthesized, the cause of the JH drop must be situated at the level of its synthesis and/or degradation.

Mechanisms include the cessation of expression of JH biosynthetic genes (e.g., JHAMT, Minakuchi et al. 2008) and activation of JH esterase to clear residual JH. Causal evidence has also been provided by, for example, RNAi-mediated knockdown of JHAMT gene (*Tribolium*, Minakuchi et al. 2008) or KO (*Bombyx*, Daimon et al. 2015), or by JH gain of function experiments in *Bombyx* (Lepidoptera).

JH synthesis in both the CA and the male accessory glands or ovaries is regulated by neuropeptides. Neuropeptides that stimulate or inhibit JH synthesis have been respectively designated as allatotropins and allatostatins (Stay and Tobe 2007), usually based on their *in vitro* activity on isolated CA. Synthesis can decrease because of diminished allatotropic hormone activity (Stay and Tobe 2007). Other possibilities are an increased allatostatic activity, or/and increased degradation of circulating JH, or other yet unknown causes, such as the nature of "nutritional status" (Oeh et al. 2000, Duve et al. 2003, Noriega 2004, Audsley et al. 2008, Verlinden et al. 2015). *In vivo* studies confirming the *in vitro* effects of all reported allatostatin and allatotropin neuropeptides are scarce, indicating the importance of developmental timing. RNAi knockdown analysis of neuropeptide precursors in *Spodoptera*

frugiperda (Lepidoptera: Noctuidae) showed that allatatostatin C and allatotropin-2 regulate the synthesis and accumulation of JH I and JH II in the MAG of unmated males and also the synthesis of JH III in the CA of adult males (Hassanien et al. 2014). Other neuropeptides that based on their *in vitro* effects on the CA, were designated as allatoregulatory, including allatostatin A and allatotropin-1, had only minor effects. AST-C and AST-CC stimulate *in vivo* JH biosynthesis in larvae, while inhibiting it in female adults of the notodontid lepidopteran, *Clostera anastomosis* (Dong et al. 2017). Also, dsRNAi analysis in the Colorado potato beetle, the chrysomelid, *Leptinotarsa decemlineata*, showed that AST-C inhibits JH biosynthesis (Meng et al. 2015). In contrast to AST-C, JH-levels remained unaffected when the AST-A neuropeptide precursor is silenced, as in all age groups of the cockroach *Blatella germanica* (Maestro and Bellés 2006). Also, in *D. melanogaster,* AST-A does not regulate JH biosynthesis (Wang et al. 2012).

Ecdysis Triggering Hormone (ETH), an important peptide hormone in insects (Röller et al. 2000, Kingan et al. 2000; Žitňan et al. 2007) seems to function as an obligatory allatotropin to promote JH synthesis in the mosquito *Aedes aegypti* (Azeira et al. 2014) and in fruit flies (Meiselman et al. 2017), as ETH signaling deficits alter it. Also, mutations in the insulin signaling pathway alter JH production and significant interactions between the regulation of JH production, energy balance, and insulin signaling have been reported (Tu et al. 2005). Also short Neuropeptide F (sNPF), which is implicated in the regulation of food consumption (Fadda et al. 2019), body size, stimulation of ovarian development in locusts (Cerstiaens et al. 1999) and energy balance, possibly through mediation of insulin signaling, may regulate JH III production in adult *Drosophila*, as shown by sNPF-receptor RNAi (Wang et al. 2012). The sNPFs should not be confused with NPFs, which are phylogenetically unrelated (Nässel and Wegener 2011, Fadda et al. 2019). In last instar larvae of *Bombyx mori* the cessation of JH biosynthesis is the key event for initiating pupal metamorphosis in which the neuropeptides AST-C and sNPF are key factors (Kaneko and Hiruma 2014, 2015).

There is probably a connection between overeating and obesity on one hand, and the inactivation of the CA. Feeding is continuous during the larval stages of insects and ceases only in late instar larvae when the wandering phase sets in. sNPF-signaling stimulates food intake and growth during larval development in *Drosophila* (Hong et al. 2012, Lee et al. 2004) and also affects the release of insulin-like peptides (Carlson et al. 2013). Therefore, one of the factors that should be causal to shutting down the CA, is probably not an allatostatin, but a factor that causes the *clearance of the CA-stimulating neuropeptide sNPF*. It has been shown that sNPF disappears from the body in prediapausing Colorado potato beetles (Huybrechts et al. 2004). During prediapause the body is cleared from JH. sNPF is also absent in the larval -

but not adult - tsetse fly, where it displays an unusual development inside the uterus of the mother and immediately pupates upon birth (Caers et al. 2016).

This raises the questions: How does the voracious feeding in last instar larvae, that is coordinated by several neuropeptides (Audsley and Weaver 2009) ends abruptly, and comes to a complete stop? One possibility is that at the onset of the last larval instar, several neuronal and hormonal factors enable a bulimia-type of feeding. Possibly, after some time, stretch receptors in the gut and the body, will feedback to the CNS in order to inhibit the bulimia-type behaviour. Satiety signaling can be triggered, such as by a cholecystokinin-like peptide (Nässel and Williams 2014). If this would lead, directly or indirectly, to a total absence of the feeding stimulating factor such as sNPF, NPF or another neuropeptide, the effects of "having overeaten" would start manifesting themselves.

9.3. The ecdysteroid titre (repeatedly) peaks during metamorphosis

While the JH titre drops to zero, the ecdysteroid titre starts peaking (Figure 10B). Briers et al. (1983) compared the fluctuations in ecdysteroid titre during metamorphosis in three blowfly species. The highest values found were about 200-250 ng/ g tissue in all species. Although in the calliphorid dipteran, *Calliphora vicina*, ecdysteroids peak three times, in *Lucilia caesar* and in *Phormia terraenovae*, which are also calliphorids, only two peaks were observed. Ecdysteroid peaks may be causal to the synthesis and secretion of cuticular proteins, according to mechanism 3 of Ca^{2+}-homeostasis. The most widely accepted view on the cause of the rise in E-titre is that the release of a Prothoracicotropic Hormone (PTTH)-like molecule from the brain stimulates the synthesis in ecdysteroids in the supposedly (not everybody agrees: see Sláma 2019) major production site, the prothoracic glands (PGs), or a functionally related tissue. However, an alternative explanation has been forwarded by De Loof et al. (2015b). They think that the drop to very low (Hemimetabola) values or even to zero (Holometabola) of the JH titre is the universal primordial trigger of the sharply rising ecdysteroid titres. Furthermore, they observed that other tissues than the PGs (of locusts), in particular the immature gonads are also very active sites of ecdysteroid synthesis. The occurrence of more than one E-peak can either be due to an oscillating pattern of PTTH-(like) neuropeptides, or to a differential timing of programmed cell death of different tissues, with causal consequences to the appearance of an ecdysteroid peak(s). Finally, the authors advanced the view that the PGs may be the functional equivalent of the thymus gland of mammals, with a key function in immunology. Sláma (2019) also adheres the view that the PGs are not the primordial site of synthesis of ecdysteroids. He also explains where 20E/vitamin D_1 comes from (see section 9.4.1). This paper does not elaborate on nuclear receptors of ecdysteroids.

9.4. Ecdysterone 20E = Vitamin D1: a truly challenging novel approach

9.4.1. Ecdysteroids as anabolic growth factors that do not use the nuclear EcR system: an overlooked mechanism?

Nearly all textbooks on insect physiology list 20-hydroxyecdysone (20E) as the moulting hormone of insects, but since the discovery of the peptide hormones that also play a role (Röller et al. 2000, Kingan et al. 2000, Zitnan et al. 2007), the complexity of the system has become apparent. In a recent paper, Sláma (2019) described the results of the effects of 20E, that he renamed as Vitamin D_1, on the growth and regeneration of excised epidermal cells of the tobacco hornworm (Lepidoptera), *Manduca sexta*, on programmed cell death, and on proliferation. Based on these, and on former experimental data (Sláma et al. 1996), he advanced convincing arguments for reconsidering the status of "hormone" for 20E. A major argument is that if 20E is injected into a mid-larval instar, no induction of moulting ensues. The counterargument is that 20E is only active if injected at the "right time" of development. What the mechanisms underlying this timing-issue are is seldom explained. Apparently the "status of the epithelial integrity" of the epidermis, however that may be achieved, matters. As he already did long before, Sláma (2019) accentuates several undervalued aspects of ecdysteroids, but in particular that 20E is a potent anabolic steroid, partly water-soluble and partly lipid soluble. Some additional data follows:

(i) Ecdysteroids are polyhydroxylated derivatives of 6-keto,7 dehydrocholesterol. Ecdysozoa cannot synthesize cholesterol by themselves.

(ii) They are common constituents of various plant species, sometimes occurring in high amounts. Their role in plants is largely unknown. A single gram of rhizomes of the true fern *Polypodium vulgare* contains 25 mg 20E (Jizba et al. 1967), which equals the amount of 20E that was isolated as the moulting hormone of insects from 500 kg of silkworm pupae (Karlson 1966, 1996). Plant leaves on which phytophagous insect larvae commonly feed contain about 0.01 percent of vitamin D_1.

(iii) 20E enters insect or human bodies through plant food or is produced by intestinal symbionts.

(iv) Upon storage in the body, 20E/vitamin D1 can be converted to cholesterol or biologically active ecdysteroids. According to Sláma (2019), during non-feeding stages vitamin D_1 is retrieved from disintegrating tissues and reutilized for the construction of cell membranes in proliferating pupal or adult tissues.

(v) 20E shares characteristics with vitamin D and causes strong anabolic vitamin D-like effects in domestic animals and in humans.

(vi) In contrast to classical vitamin D, it does not need UV radiation for activity, nor hydroxylation due to 6 or 7 already built-in hydroxylic groups.

Vertebrates do not synthesize ecdysteroids, but some, such as 20E, have anabolic effects in humans. Tóth et al. (2008) reported that 20E increases fiber size in a muscle-specific fashion in rats. Gorelick-Feldman et al. (2008, 2010) demonstrated that in both murine and human skeletal muscle cells 20E elicits a rapid elevation in intracellular Ca^{2+}, followed by sustained Akt serine/threonine-specific protein kinase 1 activation and increased protein synthesis. They also showed that the effect is mediated by a G-protein coupled receptor. Some of these effects resemble those of testosterone and its anabolic derivatives (Báthori et al. 2008). Ecdysterone 20E has been legally used as water soluble anabolic steroids for improving muscular strength of older people (Sláma 2019).

These results show that mammals do have a phytoecdysteroid-responsive membrane receptor(s) and signaling pathways. In mammals ecdysteroids do not bind to cytosolic steroid receptors (Báthori et al. 2008). Whether in insects 20E-stimulated muscle cell growth can also be achieved without binding of 20E to nuclear receptors, remains to be verified. Thus, in addition to their role as sex steroids (De Loof and Huybrechts 1998) and their role in moulting, ecdysteroids may also act as growth hormones, at least for muscle cells, thereby acting through membrane receptors.

9.4.2. Physical training and anabolic steroids mimic each other's effects: an explanation based upon the principles of Ca^{2+}-homeostasis

In some species of insect, the flight (and other) muscles develop to great strength during metamorphosis, despite the fact that they cannot be intensely trained as long as the animal is immobilized during metamorphosis. Such insects can start flying as soon as their wings are spread (e.g., flies). How can this be achieved? In vertebrates, muscle development requires repeated contraction activity. When one breaks an arm that is next immobilized for several weeks in a plaster cast, the arm muscles start atrophying due to the forced inactivity. After the plaster is removed, it takes at least several weeks of training to make the muscles "get stronger" again. How does muscle/body training make the muscle mass increase thereby mimicking the effect of anabolic steroids, and vice versa? Which is the common denominator of physical training and (administration of) anabolic steroids? At each contraction of a muscle, Ca^{2+} is released from the lumina of the SER. Reuptake of Ca^{2+} restores equilibrium. Some enzymes needed for steroid biosynthesis

reside in the membranes of the SER, others in the mitochondria. In resting conditions, the lumen of the SER is loaded with Ca^{2+} for which it acts as a storage site. Probably high concentrations of intraluminal Ca^{2+} inhibit some of the enzymes involved in lipid- or/and steroid biosynthesis. However, in case of muscle contraction during training the Ca^{2+} gradient decreases. Maybe that short-lived decrease in intraluminal Ca^{2+} suffices for allowing the synthesis of a tiny amount of steroids. Such steroids could next activate the synthesis of muscle proteins (actin, myosin, etc.) in one way or another.

10. Major physiological changes causally related to absence of JH and/or the peaking of ecdysteroids

During metamorphosis the whole body is remodeled. That requires both a lot of energy and building blocks. Some tissues are first lysed and next replaced by novel adult ones, starting from stem cells. Others are gradually transformed and electrically rewired (De Loof et al. 2014).

10.1. Metamorphosis starts with excessive feeding (med: bulimia) as if the larvae are preparing for diapause: the trigger?

The Colorado potato beetle, *Leptinotarsa decemlineata*, is a species in which the relationship between changes in JH titre and morphology-physiology of the fat body has been well studied. The most obvious feature is that only when JH is *absent*, a massive accumulation of not only lipid droplets and glycogen, but as well of large protein vesicles takes place. An ultrastructural analysis showed that this seems to be due to malfunctioning of the Golgi system (De Loof and Lagasse 1970) in the fat body cells (Figure 7).

Apparently, when JH is absent, the Golgi apparatus can no longer complete the secretion of protein vesicles into the hemolymph. Consequently, the small vesicles remain in the cytoplasm, they fuse with each other and form large protein vesicles. This way, a protein reserve can be built to be used during the months-lasting diapause. Similarly, the very same process takes place in the fat body of pre-metamorphosing beetle larvae which are not triggered to enter diapause (De Loof 1972). A second physiological change, namely the huge accumulation of "storage proteins" takes place when JH disappears from the body, thus in both premetamorphosing larvae and in prediapausing adults. A third similarity is the high rise of the ecdysteroid titre in both prediapausing (Briers et al. 1982) and metamorphosing beetles. These similarities, among others, yielded the statement that in *Leptinotarsa*, metamorphosis displays typical "diapause phenomena" although the larvae will not enter (long lasting) diapause.

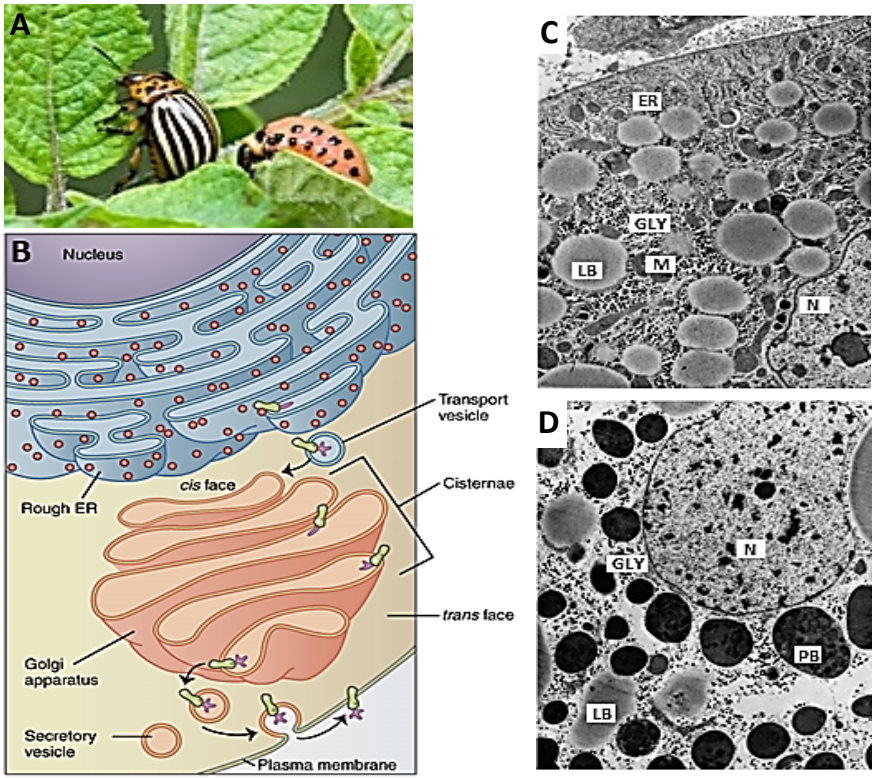

Figure 7. Comparison of the ultrastructure of fat body cells of two adult females of the Colorado potato beetle that were forced to undergo hypertrophy of the whole fat body under two different regimes. A. Adult and larva that were forced to undergo hypertrophy of the whole fat body under two different regimes. B. Golgi system, or GERL, consisting of the Golgi apparatus, endoplasmic reticulum (ER) and secretory vesicles (e.g., lysosomes, L). C. Juvenile hormone present. This beetle was reared under long-day conditions which activate corpora allata. In addition, the beetle was ovariectomized. Thus, the hypertrophy happened in the presence of Juvenile hormone. D. Juvenile hormone present. This beetle was reared under short-day conditions which inactivate the corpora allata. Thus, here the hypertrophy happened in the absence of Juvenile hormone. The comparison shows that in the absence of JH, there is an enormous accumulation of protein bodies (PB) containing "storage or diapause proteins" which result from the fact that the normal processing of secretory vesicles in the Golgi apparatus (B) gets jeopardized. How the Golgi is a target for JH is not known. In the absence of JH, very few mitochondria are present. The fat body of last instar larvae (A) in which JH disappears from the body undergoes similar changes. This leads to the conclusion that some of the changes in the physiology of metamorphosing animals are "diapause-like". Abbreviations: ER: rough Endoplasmic reticulum; GLY, glycogen; M, mitochondrion; N, nucleus; LB, lipid body; and PB, protein body. Copyright: A from Google images, Open Access. B: From Multiple authors (2019c) Open Access. C and D: Slightly modified from figures 70 and 71 in "Aggregaatsthesis" of De Loof (1972) (Open Access). Additional information can be found in De Loof and Lagasse (1970).

10.2. Massive but selective programmed cell death during metamorphosis. Ca^{2+}-induced apoptosis, in particular in cells rich in RER

The most drastic change takes place in all tissues that are very active in protein synthesis for secretion (Mechanisms 3 and 4: this paper section 6.2) such as the fat body, the midgut, the salivary glands etc. (De Loof et al. 2014) Their cells enter the Programmed Cell Death Program. They have in common that their RER-Golgi system is very abundant. From the Ca^{2+}-induced apoptosis principle forwarded by Orrenius et al. (2003), it can be inferred that after premetamorphosing insects stop feeding, the $[Ca^{2+}]i$ in the cells of tissues that actively secrete (cargo) proteins somehow reach the threshold above which programmed cell death in this type of cells is induced. An obvious cause is the drop to zero of the JH titre. In the absence of JH, JH-sensitive Ca^{2+} channels are no longer blocked by JH. As a result, Ca^{2+} enters the cells. When the 20E titre starts rising, the influx of Ca^{2+} is additionally stimulated. Ca^{2+} apoptosis will be induced. The affected cells will die. They will be degraded in lysosomal pathways. Although absence of JH is the primordial trigger of metamorphosis, the role of 20E in inducing programmed cell death during metamorphic development is better documented. Jiang et al. (1997) showed that histolysis of the larval midgut and salivary glands is a stage-specific programmed cell death response triggered by pulses of 20E. More recently, Cai et al. (2014a) and Wang et al. (2016a, 2016b) showed that this hypothesis was correct: the steroid hormone 20E promotes higher calcium mobilization to induce apoptosis in *Helicoverpa armigera* (Lepidoptera: Noctuidae). Dong et al. (2015) described how 20E and the serine/threonine Ste20-like kinase Hippo signal promotes programmed cell death during metamorphic development. Ecdysterone 20E up-regulates Hippo.

10.3. Maintaining epithelial integrity: How do epidermal cells, the heart etc. escape programmed cell death? Synthesis of cuticular proteins

Preventing bleeding to death during remodeling of the cuticle during metamorphosis is of utmost importance. Moulting is a complex process, which is well described in textbooks of insect physiology. Sedlack and Gilbert (1976) reported on changes in the ultrastructure of epidermal cells of pupae of *Hyalophora cecropia* upon application or either JH or of 20E. The effects were rapid, cell specific and dose dependent. Large doses of 20E elicited both precocious cuticle deposition and premature autophagic vacuole formation (= an indication of programmed cell death and of high $[Ca^{2+}]i$). JH prevented the formation of autophagic vacuoles (= anti-apoptosis effect and low $[Ca^{2+}]i$). Some results led the authors to the conclusion that JH may act at the membrane level. It took until Roullet et al. (1999) and Luft et al. (1999) to point at some types of Ca^{2+} channels as membrane receptors of farnesol, and probably of its JH esters as well.

As can be seen in Figure 6, at least two peaks of ecdysteroids occur during metamorphosis of dipteran insects. According to Cai et al. (2014a) 20E promotes the entry of Ca^{2+} in target cells with the help of a GPCR. The absence of JH also

promotes the influx of Ca^{2+} into epidermal cells. If more Ca^{2+} flows in epidermal cells than can be removed by Ca^{2+}-pumps in their plasma membrane, mechanism 3 of Ca^{2+} homeostasis is activated. The cells will engage in protein synthesis for secretion. Secreting cuticular proteins (Charles 2010) is thus not a means "invented to protect the weak body", simply because such view erroneously implies a goal in evolution, but to get rid of excess toxic Ca^{2+}. Cuticular proteins are in fact not more than *coincidental cargo* proteins that were conserved in evolution because they protected the weak body indeed. A role of secreting cuticular proteins as a means for protecting against excess Ca^{2+} may sound "exotic" in insects, but it is normal for many crustaceans with a thick Ca^{2+}-rich exoskeleton, such as crabs and lobsters.

Some vital tissues escape from undergoing programmed cell death: the nervous system, the heart, the Malpighian tubule system etc., They continue their development during metamorphosis. This indicates that the permeability for Ca^{2+} in their cells is such that the threshold for Ca^{2+}-induced apoptosis/programmed cell death is never exceeded.

11. Discussion

The goal of the Calcitox-metamorphosis paradigm is to outline a conceptual framework in which numerous data obtained by reductionist experimentation are aligned in a system governed by few basic physiologic principles. The undervalued Ca^{2+}-homeostasis system, and in particular the fact that it has binding sites for endogenous farnesol/JH sesquiterpenoids (De Loof and Schoofs 2019a) represents its very heart. Through 3 universal and evolutionarily well conserved mechanisms, all based upon their effects to keep $[Ca^{2+}]i$ as low as possible in order to prevent fast aging (De Loof 2017b) and Ca^{2+}-induced apoptosis (= mechanism 4), several types of cellular activities regarding lipid-, steroid-, rRNA-, protein metabolism etc. can be differentially (in)activated. This paradigm emphasizes that in order to better understand the mode of action of JH and ecdysteroids as well as the full nature of metamorphosis, the role of the well-studied nuclear hormone receptors has to be complemented with a relatively large number of additional actors/parameters. Some of them are undervalued in other models: the differences in modes of action of water- and soluble hormones; (farnesol-like) sesquiterpenoid hormone receptor sites on, for example, Ca^{2+} channels and Ca^{2+} pumps which are anchored in intracellular membranes; the fact that all hormones first have to act at the level of the plasma membrane before they are able to transmit their message into the cytoplasm and to the nucleus; the RER-Golgi as a crucial means to remove excess Ca^{2+} by secreting Ca^{2+}-binding/transporting (cargo) proteins, thus not of any protein-type; non-genomic effects elicited by direct (in)activation of some enzymes by Ca^{2+} (De Loof 2017b); 20E = Vitamin D_1 (Sláma 2019) as an anabolic steroid growth factor; direct effects of Ca^{2+} on the induction of RER-Golgi and its activities; direct effects of inorganic ions, in particular of Ca^{2+} and H^+, on control of gene expression (the Lezzi-Kroeger model

from 50 years ago: references in De Loof et al. 2014); the significance of the rotatable count number in signaling isoprenoid molecules for a better understanding of the relationship between a particular 3D configuration of a given hormone and its mode of action; the ionic compartmentalization of the nucleus with the possibility that, perhaps, Ca^{2+} can by itself (= without the help of Ca^{2+}-sensitive nuclear receptors and the chromatin remodeling system) control gene expression in a selective way.

The long-standing question whether or not JHs and ecdysteroids are each other's antagonists, can by now be answered, at least partially. With respect to their role in Ca^{2+}-homeostasis, they are certainly antagonists. Farnesol/JHs keep $[Ca^{2+}]i$ low by acting as inhibitors of particular voltage gated Ca^{2+} channels as well as by keeping the Ca^{2+} concentration high in the lumen of the SER and low in the cytoplasm. A role for the sesquiterpenoid binding site in the SERCA pump has been proposed (De Loof et al. 2014). Alternatively, the team of Zhao and collaborators (many references in the reference list) has shown that 20E "facilitates" the entry of Ca^{2+} into cells by acting through the GPCR2 membrane receptor. This duality suggests that, after all, a rather simple mechanism underlies the drastic changes in the body during metamorphosis. It all boils down to levels of $[Ca^{2+}]i$ to which a plethora of enzymes and signaling molecules, by altering their 3D conformation, respond. Cell physiological processes involve coordinated actions of various pathways. They seldom involve an effect of a hormone on one single molecular target. In addition, these processes have to be well coordinated. In this respect Cheng et al. (2014) found that in *Tribolium*, like in vertebrates, a POU factor [Ventral veins lacking (Vvl)/Drifter] coordinates both JH and ecdysteroid biosynthesis as well as moulting behaviour to influence moulting and the timing of metamorphosis. Finally, with respect to the interaction with their nuclear receptors, both hormones act through different nuclear receptors. Thus, in this respect, they are not antagonists.

The concept introduced here may also stimulate further research into the relationship between sNPF-like peptides and the stimulation of JH biosynthesis in the CA, and of ecdysteroids in some other tissues. It may direct the identification of membrane receptors for JH towards refined patch clamp techniques. In addition, the game changing CRISPR-Cas9 methodology (Jinek et al. 2012, Jiang and Doudna 2017) may also facilitate the search for membrane receptors for JH and ecdysteroids and their physiological effects. The non-genomic effects of JH and ecdysteroids need further exploration, such as its effects on selected microRNAs, an emerging relatively new field (Qu et al. 2018). And the last word is not yet said about the primordial cause of the cessation of feeding just prior to the onset of metamorphic changes, neither about the nature of which protein(s)/peptide the Golgi systems present in the corpora allata (Bradley and Evans 1979) may secrete into the haemolymph.

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