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POINT OF VIEW

Vitamin D1 versus ecdysteroids: Growth effects on cell regeneration and malignant growth in insects are similar to those in humans

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Abstract. Polyhydroxylated derivatives of 6-keto,7-dehydrocholesterol (ecdysteroids) are common constituents of various plants. In 1965, they were accidentally discovered in the search for the insect moulting hormone. These biologically important natural compounds are neither insect hormones nor inducers of insect ecdysis. Due to their strong anabolic, vitamin D-like effects in insects, domestic animals and humans, I propose the use of the arbitrary term vitamin D_1. The present paper describes the effects of vitamin D_1 on the growth and regeneration of excised epidermal cells of the tobacco hornworm, *Manduca sexta* (Sphingidae). The periods of programmed cell death and cell proliferation (histolysis and histogenesis, respectively) exactly coincide in insects with endogenous peaks of increased concentration of vitamin D_1. Epidermal cells communicate with each other, creating a mutually integrated tissue, connected by mechanical, chemical, electrical, ionic or other so far incompletely known factors. After natural cell death, or after the artificial removal of some epidermal cells, the neighbouring cells that lose communication integrity, begin to divide mitotically to replace the disconnected part. Cell divisions are arrested as soon as the integrity of the living tissue is established. During insect ontogeny, the application of juvenile hormone causes regenerating epidermal cells to repeat the previous morphogenetic programme (i.e., development of patches of larval tissue on the body of a pupa, or metathetely). Conversely, the application of vitamin D_1 (20-hydroxyecdysone) caused the regenerating cells to prematurely execute a future morphogenetic programme (i.e., development of patches of pupal tissue on the body of a larva, or prothetely). Among the key features of insect regeneration, is the arrest of cell divisions when tissues resume living cell-to-cell integrity. This prevents the formation of aberrant groups of cells, or tumours. It is well established that the main physiological systems of insects (e.g., circulatory, respiratory, neuro-endocrine) are structurally and functionally similar to corresponding systems in humans. Thus the basic principles of cell regeneration and the role of vitamin D_1 in insects may also be valid for humans. The common vitamins D_2 (ergocalciferol) or D_3 (cholecalciferol), are exclusively lipid soluble secosterols, which require activation by UV irradiation and hydroxylation in the liver. By contrast, the neglected vitamin D_1 is a natural derivative of polyhydroxylated 7-dehydrocholesterol of predominantly plant origin, which is both partly a water and partly a lipid soluble vitamin. It neither requires UV irradiation, nor hydroxylation due to 6 or 7 already built-in hydroxylic groups. Like other vitamins, it enters insect or human bodies in plant food or is produced by intestinal symbionts. Vitamin D_1 causes strong anabolic, vitamin D-like effects in domestic animals and in humans. I am convinced that avitaminosis associated with a deficiency of vitamin D_1 in human blood may be responsible for certain hitherto incurable human diseases, especially those related to impaired nerve functions and somatic growth, aberrant cell regeneration or formation of malignant tumours.

INTRODUCTION

Recent progress in biology and medicine has been largely influenced by technical advances and innovations. During the twentieth century, human health care became dependent on the massive use of pharmaceutical drugs. Yet, some old medical problems remained unresolved. One example of this probably occurred approximately a century ago, in the search for the growth promoting, antirachitic (to cure or prevent rickets) action of vitamin D. In 1930, organic chemists found provitamin D in skin, which was thought to be tentatively converted into vitamin D_3 (calciferol) by UV irradiation.

I have investigated the effects of insect vitamins and hormones on tissue and cell growth for more than 50 years (Sláma et al., 1974; Sláma, 2013, 2016). The results have revealed close structural and functional similarities between the endocrine systems of insects and humans (Sláma, 1993, 2012, 2013, 2015a, 2016). It is estimated that 37% of the genes in the genome of the fly, *Drosophila*, are homologous to those in the human genome (Devillers, 2013). Somatic growth in the human body depends mostly on the action of calcified bones and associated musculature, which forms an endoskeleton. In insects, by contrast, somatic growth depends on periodic replacement of an integumental exoskeleton. The growth effects of vitamin D

in the human body can be compared with its effects on the growth of insect exoskeleton, which is manifested by the formation of a new, enlarged integumental cover. As a matter of fact, both in insects and mammals, essential growth factors with a similar function to vitamin D are structurally related to 7-dehydrocholesterol (Sláma, 1993, 2014, 2016; Sláma & Zhylitskaya, 2016).

Previous studies on the effects of vitamins and hormones in insects were made on immature larvae and pupae of the greater wax moth, *Galleria mellonella* L. (Sláma, 1975) and the carpet beetle, *Dermestes vulpinus* De Geer (Sláma et al., 1974; Sláma 1999, 2015). Similar investigations were done using the tobacco hornworm, *Manduca sexta* L., which is substantially larger. These experiments mainly involved isoprenoid analogues of insect juvenile hormone (JH) (Sláma et al., 1974; Sláma, 1999) and the polyhydroxylated derivatives of 6-keto,7-dehydrocholesterol (ecdysone, ecdysteroids; Sláma et al., 1974, 1993; Sláma, 2015), which were initially thought to be arthropod moulting hormones. During the last three decades, extensive data on the effects of ecdysteroids have been published on insects (Sláma, 2014, 2015a; Sláma et al., 1993) and also on anabolic, vitamin D-like effects of these compounds in mammals (Sláma & Lafont 1995; Dinan & Lafont, 2006). The existence of polyhydroxylated sterols was unknown to the pioneers of vitamin D research as these were discovered three decades later by Karlson (1996). Ecdysteroids, long believed by organic chemists to be the arthropod hormones stimulating insect ecdysis (Dinan et al., 2009; Kumpun et al., 2011; Lafont et al., 2011) are according to my view the erroneously assigned, true vitamin D. Since these polyhydroxylated derivatives of 6-keto,7-dehydrocholesterol are neither hormones produced by the prothoracic gland of insects nor inducers of insect ecdysis (see below) and to avoid further confusion of earlier work, I proposed earlier to use the term vitamin D_6, due to its strong anabolic, vitamin D-like effects both in insects and mammals (index 6 for the six hydroxylic groups in the molecule and the Latin "Hexapoda" for six-legged creatures, the class Insecta) (Sláma, 1993, 2015a, b, 2016; Sláma & Zhylitskaya, 2014).

A brief history of vitamin D research

A brief survey of the history of vitamin D indicates its close relations to rickets (rachitis), which is a bone disease caused by vitamin D deficiency. In 1921, it was found that the occurrence of rickets was related to seasonal variations in sunlight (Hess & Unger, 1921). Sunlight would cure rickets just as well as cod-liver oil. Irradiation of food items (e.g., milk butterfat, dietary oil) could also impart antirachitic potency. Due to this, the history of vitamin D research was closely related to UV-irradiated compounds (Windaus et al., 1931; Karlson, 1981). The story of D-vitamin research was perhaps best reviewed by Wolf (2004). Herein, I quote only a few essential points from his 2004 review.

After 1924, it was found that the antirachitic properties of certain animal fats were increased by UV irradiation. Irradiated cholesterol also shows increased biological activity and Hess et al. (1925) propose that "it would seem quite possible that cholesterol in the skin is normally activated by UV-irradiation and rendered anti-rachitic". It was assumed that the cholesterol obtained from brains of rats, contained a small amount of an impurity that could be a precursor of the vitamin. Thus, to shed more light on this problem, A.F. Hess asked the famous German steroid chemist, A. Windaus, to collaborate on the elucidation of the chemical structure of the antirachitic agent. In 1937, Windaus & Bock (1937) isolated and identified a compound related to 7-dehydrocholesterol (see Fig. 1A), from pig, rat and human skin, as well as from animal sources, such as whole milk or liver. The active compound was named vitamin D_3 (cholecalciferol, see Fig. 1B and see Wolf, 2004 for a review).

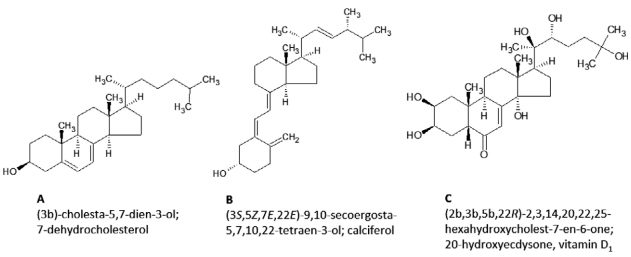

Fig. 1. Chemical structures of some of the molecules mentioned in this paper. A – 7-dehydrocholesterol; B – Vitamin D_3 or Calciferol; C – 20-hydroxyecdysone, Vitamin D_1.

Investigations on vitamin D were directed to purely lipid soluble animal sterols, because 7-dehydrocholesterol was supposed to be exclusively an animal sterol (zoosterol). The research concentrated on animal fats, such as milk, butter or codfish oils. Cholesterol and its derivatives were considered purely lipid-soluble compounds (the existence of partly water soluble, polyhydroxylated sterols was unknown at that time). The polar, water soluble fractions were thus disposed of without being tested for the antirachitic properties of vitamin D. Moreover, the most serious confusion in the research on vitamin D was the fact that, unlike other vitamins, vitamin D was not expected to occur in plants, because 7-dehydrocholesterol was erroneously not considered to be present in plants. Since that time, the biochemical status of vitamin D was defined as a purely lipid soluble animal sterol. The preparations of commercial vitamin D_3 (calciferol) are available under the name Vigantol, the more active derivative originating by metabolic hydroxylation of vitamin D_3 in liver and kidneys is known as vitamin D_3-triol (commercial name, Rocaltrol). Exact structure of vitamin D_1 was not completely assigned at that time, which could explain the absence of D_1-based pharmacological drugs.

According to Karlson (1981), the status of vitamin D_1 was not clear. It was a crystalline substance isolated by Windaus et al. (1931) from ultraviolet irradiated ergosterol. Previous investigators predicted that vitamin D might be somewhat related to 7-dehydrocholesterol (Hess et al., 1925). When Karlson (1966) isolated ecdysone, which is a derivative of 7-dehydrocholesterol, he was so impressed by Williams's (1952) idea of an "Arthropod moulting

hormone secreted by prothoracic gland", that he did not consider the possibility that the biological activities of ecdysone and vitamin D are similar.

During the past 50 years, an enormous amount of experimental data has accumulated on the presence of polyhydroxylated derivatives of 7-dehydrocholesterol in plants (Sláma et al., 1974; Sláma, 1979; Dinan & Lafont, 2006; Dinan et al., 2009) and their strong anabolic, vitamin D-like effects in mammals, including humans (Sláma & Lafont, 1955; Sláma, 1993). Moreover, these vitamin-like compounds present in plant food did not appear to conform with the definition of an animal hormone and did not stimulate insect ecdysis (Sláma 1980, 1982, 1988, 2015a, b). Thus, to avoid further terminological confusion, I propose to rename the polyhydroxylated derivatives of 6-keto,7-dehydrocholesterol (ecdysteroids) vitamin D_1.

History of ecdysteroids

The history of ecdysteroids (here vitamin D_1) has been extensively reviewed many times (Sláma et al., 1974; Volodin, 2003; Dinan et al., 2009; Lafont et al., 2011; Gilbert, 2012). As previously mentioned, the first compound named ecdysone was discovered by Karlson in his search for an insect moulting hormone (Karlson et al., 1965; Karlson, 1966). He extracted 500 kg of silkworm pupae and obtained 25 mg of a crystalline compound, which was identified by x-ray crystallographic analysis as a polyhydroxylated derivative of 6-keto, 7-dehydrocholesterol (see Fig. 1C).

In the bioassays used by Karlson, ecdysone stimulated formation of new epidermis in headless maggots of blowfly. According to the hormonal theory of Williams (1952), the effects were ascribed to a hormone secreted by insect prothoracic glands (Karlson, 1966). However, almost immediately after Karlson disclosed the crystallographic structure of ecdysone, various phytochemists reported that this compound is present in extracts of various, taxonomically unrelated species of plants (Nakanishi et al., 1966; Jizba et al., 1967; Sláma et al., 1974 for review). The most abundant derivative found in plants as well as in insects and crustaceans was 20-hydroxyecdysone, currently known as ecdysterone (Fig. 1C). Some species of plants contained enormous amounts of this compound, assumed by organic chemists to be the "Arthropod moulting hormone". For example, just one gram of the rhizomes of the fern, *Polypodium vulgare*, contain the same amount of ecdysterone es 500 kg of silkworm pupae (Jizba et al., 1967). Additional screening of plants done mainly by Japanese phytochemists revealed that these partly water soluble, polyhydroxylated derivatives of 7-dehydrocholesterol are widely distributed in the botanical world (Hikino et al., 1968). Despite the common occurrence of vitamin-like ecdysteroids in plants (Sláma et al., 1974; Sláma, 1979), organic chemists still distinguish between zooecdysteroids and phytoecdysteroids (Kumpun et al., 2011). The first controversial definition of 20-hydroxyecdysone (vitamin D_1) proposed that: "Ecdysteroids were the reserve materials for the growth of tissues in plants" (Sláma, 1979).

Extensive physiological investigations have revealed that ecdysteroids are not insect hormones (Sláma, 1983, 1993, 2014). Prothoracic glands do not stimulate insect ecdysis (Sláma, 1983, 1998) and the peaks in endogenous concentrations of vitamin D_1 in insects occur in the absence of prothoracic glands (Delbecque & Sláma, 1980).The hypothesis regarding the role of prothoracic glands proposed by Williams (1952) was later refuted by Williams (1987). Unfortunately, most biochemists did not comprehend the significance of this refutation by Williams and continued to consider the disintegrating prothoracic glands of *Bombyx* to be the source of the moulting hormone (Iga et al., 2014; Nakaoka et al., 2017). In addition, the alternative biological definition proposed that: "Ecdysteroids were the homeostatic tissue factors synchronising the growth of tissue and cells with the essential developmental features of the moulting cycle" (Sláma, 1980), was ignored. Later it was experimentally confirmed (Sláma, 1980) that ecdysteroids do not stimulate, but actually inhibit insect ecdysis in a dose-dependent way. There is strong presumptive evidence (Sláma, 1993) that ecdysteroids (here vitamin D_1) are biologically far more important natural compounds of medical importance, than just being a hormone produced by the tiny prothoracic glands (Sláma, 1993; Sláma & Lafont, 1995).

Herein, I describe the effects of vitamin D_1 (20-hydroxyecdysone) and juvenile hormone (JH) on the regeneration of epidermal tissue in the tobacco hornworm, *Manduca sexta* L. The replacement of dead cells is the most important aspect of regeneration in all animals and humans. According to H.E. Hinton (quoted in Sláma et al., 1974), "the terms of science should correspond with the facts in nature". As ecdysteroids are neither arthropod moulting hormones (Sláma, 1983) nor stimulate ecdysis (Sláma, 1980) and have strong anabolic, vitamin D-like effects, it is more appropriate to use the generic term vitamin D_1, although it was originally proposed for a structurally unidentified UV-irradiation product of ergosterol by Windaus et al. (1931).

MATERIAL AND METHODS

Larvae and pupae of *M. sexta* were obtained from stock cultures reared on artificial diets (Sláma, 1960). Procedures used for epidermal excisions or producing local lesions in epidermal cells using thermocautery are described in Sláma (1975). Cauterization was used only on fully grown larvae of the last larval instar, other experiments were based on epidermal excisions. The exact size and location of epidermal injuries are depicted in Fig. 2.

Prior to excisions, selected larvae and pupae were immobilised by submersion in water for 15 to 35 min (Sláma, 1980, 2016). Pupae in diapause, which have relatively low gaseous exchange rates, were submerged in water for two hours. The areas from which larval epidermis was excised were covered by rapidly polymerizing cyanoacryllic glue (Fig. 2A). Epidermal excisions in the rigid cuticle of pupae were covered by a strip of a broken cover slip. The margins were sealed using bee's wax melted using the platinum wire used for thermocautery (Fig. 2B). A 10% ethanolic solution of vitamin D_1 (20-hydroxyecdysone; natural compound isolated from *Leuzea carthamoides* (Willd.) (Buděšínský et al., 2009), was injected into larvae and pupae (Sláma, 1980). Local application of vitamin D_1 was made by covering the wound

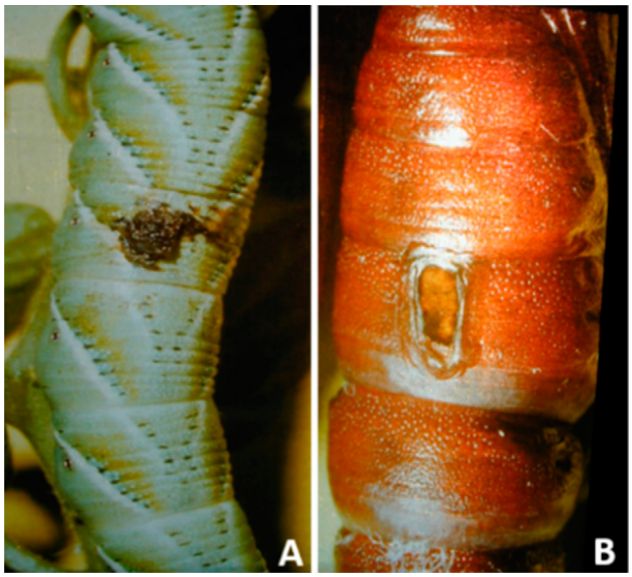

Fig. 2. A – size and location of the epidermal area excised from the last instar larva; B – window excised from the integument of a freshly ecdysed pupa of *Manduca sexta*.

with a small piece of solidified, 12% gelatine containing 2 mg/ml of vitamin D_1. The strip of gelatine was covered externally by a polymerized polyester layer produced by a small drop of cyanoacryllic glue. For topical application of a juvenile hormone analogue, we used methyl, 10,11-epoxy,7-ethyl-3,11-dimethyl-2,6-tridecadienoate (JH-I) (Sláma et al., 1974; Sláma, 1985, 1999; Paroulek & Sláma, 2014). Determination of the content of vitamin D_1 in insect bodies, expressed in 20-hydroxyecdysone equivalents, is described by Sláma (1980) and Delbecque & Sláma (1980) and in the blood of Japanese quails by (Koudela et al., 1995; Sláma et al., 1996).

The excisions or lesions in epidermal cells by thermocautery were initially done on groups of ten specimens. The results were individually evaluated, documented and stored in a personal computer database. In the case of equivocal results or increased mortality, experiments were repeated using a new group of ten specimens. When necessary, the total number of experimental specimens is reported in the text or in figure captions. The anatomical and morphological structure of the regenerated epidermal patches was evaluated after the following moult, using light (Sláma, 1975, 1980) or scanning electron microscopy (Sláma & Weyda, 1997). In exceptional circumstances, epidermal fragments were fixed in 70% ethanol for histological observations or prepared for scanning electron microscopy (Sláma & Weyda, 1997.

RESULTS

Endogenous peaks in the content of vitamin D_1 during insect development

Most insects obtain vitamin D_1 from food or symbiotic bacteria. It is used in the construction of hydro/lipophilic cell membranes by newly proliferating or regenerating cells. When the dietary supply of vitamin D_1 is superfluous to requirements, the danger of hypervitaminosis (hyperecdysonism of Williams, 1970), which is manifested in the precocious, pathophysiological secretion of new cuticle, is prevented by the excretion of vitamin D_1. During the 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 (Sláma, 1982). The schematic drawing in Fig. 3 shows the relationships between endogenous concentrations of vitamin D_1 and total body metabolism (O_2 consumption), which is valid for most endopterygote insects. It is important to note that the peaks in endogenous content of vitamin D_1 exactly coincide with minimum metabolic activity. This inverse relationship results from the low metabolic efficiency of old, disintegrating larval tissue and organs (histolysis), which is associated with the simultaneous proliferation of newly

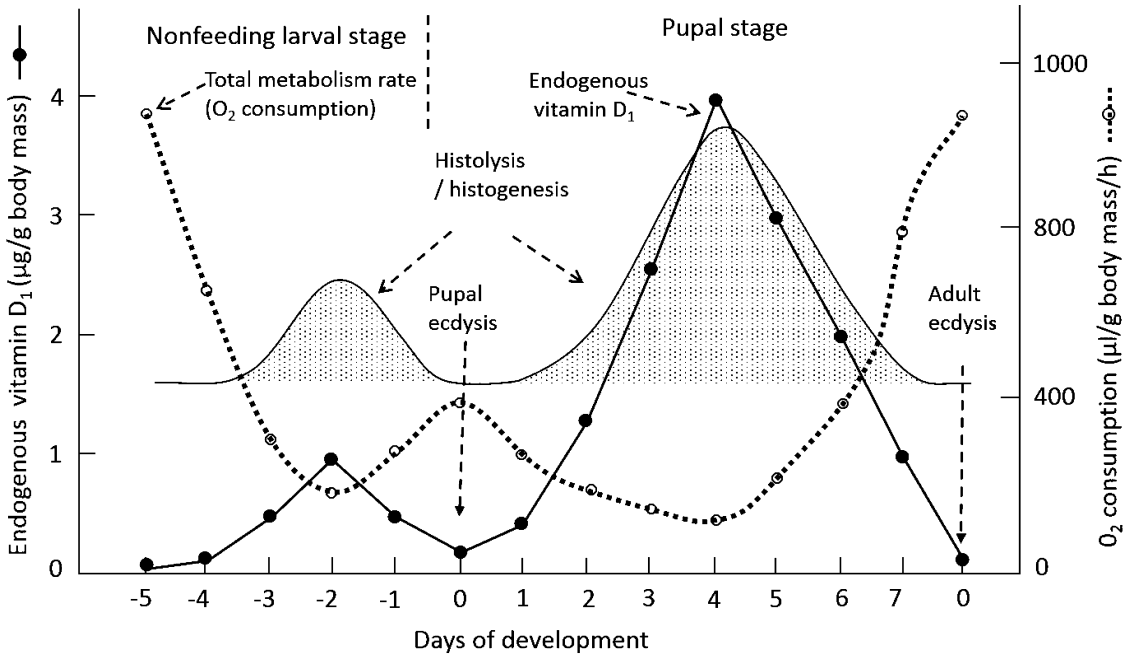

Fig. 3. Relationships between endogenous concentration of vitamin D_1 (full line), total body metabolism (dotted line) and transformation of larval-pupal and pupal-adult tissues (dotted area) during the metamorphosis of the wax moth, *Galleria mellonella* L.

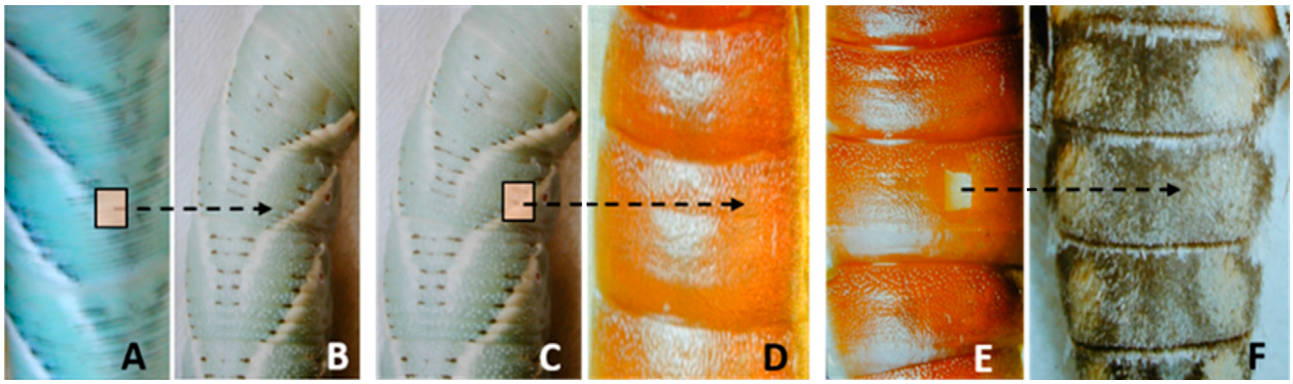

Fig. 4. Homochronic (identical) regeneration of epidermal excisions during the development of *Manduca sexta* (Linnaeus). A–B: Larval-larval moult. A – area of epidermis (3 × 3 mm) excised from a freshly ecdysed, penultimate (L_4) larva; B – regenerated excision after L_4–L_5 moult. C–D: Larval-pupal moult. C – area of epidermis excised from a last (L_5) instar larva; D – regenerated excision area after the moult to pupa. E–F: Pupal-adult moult. E – epidermal excision on a pupa. F – regenerated pupal excision after adult emergence.

developed pupal or adult tissues (histogenesis). The homeostatic turnover of vitamin D_1 is usually less pronounced during the initial larval-pupal transformation (the sterol reutilisation theory of Sláma, 1998). The pupal peaks are larger, due to the extensive transformations involved in the development of adult digestive and muscular systems (Fig. 3).

Cell-to-cell communication among living cells

The postembryonic development of insects involves distinctive moulting and reproductive cycles. The cycles are triggered by hormones of the central neuroendocrine system, which track external environmental signals (e.g., availability of food, suitable temperature and photoperiod) and transforms them into developmental instructions coded in the genome. During larval somatic growth, the body increases in size by the multiplication of cells in peripheral tissues, or increase in cell size by polyploid endomitosis, as in some Diptera. Hormones from the central neuroendocrine system instruct genes on chromosomes in the peripheral cells when to execute their inherited programmes. There are two principal types of developmental cycles regulated by centrally produced hormones:

(1) The epigenetic or "status quo" developmental cycles under the influence of JH (stationary larval-larval or pupal-pupal cycles), that take place without structural changes in the DNA.

(2) The morphogenetic developmental cycles that occur in the complete absence of JH (embryonic cycles, larval-pupal, pupal-adult, larval-adult cycles). These moulting cycles are associated with the reprogramming of genetical information. Some insects, which do not require environmental signals, exhibit autonomic (hormone independent) development (Sláma, 2015a). As reported in Fig. 3, the morphogenetic cycles are intimately associated with endogenous peaks in the concentration of vitamin D_1.

During normal development, epidermal cells excised at the beginning of an instar (before the endogenous peak of vitamin D_1) regenerated into the homochronic, one and the same population of epidermal cells, matching the structure of the integument of the host. An example of homochronic regeneration is depicted in Fig. 4, which shows that the excised patch of epidermal cells that regenerated during metamorphosis is indistinguishable from the surrounding epidermal cells. The microscope observations revealed that the excised epidermis was replaced by cells proliferating from the disconnected margins of the wound. In other words, epidermal cells removed from penultimate larvae regenerated during metamorphosis into epidermis of the last larval instar (Fig. 4A, B). Cells removed from the last larval instar regenerated into pupal epidermal structures (Fig. 4C, D). Moreover, cells removed from freshly developed pupae regenerated into adult structures with hairs and scales, indistinguishable from those of the host (Fig. 4E, F). The excisions were made at the beginning of an instar, however, the remodelling of final structures occurred later, during the endogenous peak in vitamin D_1, as can be seen in Fig. 3. The larval architecture of epidermal cells, with folded cuticle and sharply delimited, distinctly different, flat pupal cells of the heterochronic epidermal patches in *Manduc*a were monitored earlier by scanning electron microscopy (Sláma & Weyda, 1997).

The activity of JH can be artificially prolonged by applying JH analogues, which is usually followed by the development of larval-pupal intermediates or supernumerary larval instars. This type of suspended appearance of structural characters of previous, older ontogenetic stages in the next stage has been described many times as a heterochronic developmental deviation, which is called metathetely by Sir V.B. Wigglesworth (1970). Conversely, the premature appearance of structural characters of a future ontogenetic stage is called prothetely (Sláma, 1975, 1980) (usually manifested by precocious pupal wing lobes in larvae) and are associated with exogenous applications of vitamin D_1 (Williams, 1970; Sláma, 1975, 1985, 1999).

The above results have several physiological implications: (1) Disturbed cell integrity, caused by the natural death or artificial removal of a living cell, causes neighbouring cells to divide. (2) The dividing epidermal cells stop dividing as soon as the living cell-to-cell integrity is re-established. (3) This mechanism prevents the formation of tumours. (4) The regenerating epidermal cells remember

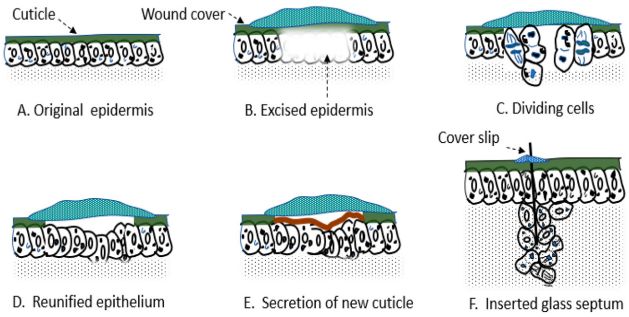

Fig. 5. Schematic outline of the regeneration of epidermis (A) excised from *Manduca sexta*. The interrupted integrity of epidermal cells (B) was repaired by mitotic divisions of the marginal cells (C) that divided until the new cells (D) reunified the living epithelium. The reconstituted epithelium remodelled extracellular stromal elements and a new cuticle externally (E). Epidermal cells separated by a cover slip divide and grow all the way around the inorganic object until they achieve living cell integrity (F).

the ontogenetic status of the neighbouring cells, e.g. larval, pupal or adult status of the host. (5) During the endogenous peak of vitamin D_1, the regenerating epidermal cells metamorphose synchronously with the epidermal cells of the host. This shows that living epidermal cells communicate and establish a unit of physiologically integrated tissue. When living cell integrity is disturbed by cell death or epidermal injury, order is re-established by multiplication of disconnected neighbouring cells.

The principles of regeneration in insects may be common to all multicellular organisms, including plants (e.g., formation of a callus), invertebrate or vertebrate animals, as well as humans (wound healing). It is very difficult to determine the exact nature of the factors responsible for the maintenance of the integrity of living cells, which arrest further cell divisions when regeneration is complete. It may be mechanical, chemical, electrical, osmotic, or ionic, among other possibilities like an undisturbed extracellular stromal sheath, which protects the external cells of each organ. A failure in resuming cell integrity can lead to aberrant cell divisions and formation of tumours.

Fig. 5 shows a schematic outline of cytological changes during regeneration of an epidermal excision in *Manduca sexta.* The excised epidermal section (Fig. 5B) is invaded by mitotically dividing cells from the margin of the wound (Fig. 5C), which replace the missing epithelium (Fig. 5D). During the endogenous peak of vitamin D_1, the newly formed cells secrete a new cuticle simultaneously with the host cells (Fig. 5E). When there is a physical obstacle to living cell continuity, such as the insertion of a cover slip into a pupa in diapause (Fig. 5F), the disconnected cells began dividing and formed a layer of cells around the object until cell-to-cell continuity was achieved.

Regeneration of epidermal cells under the influence of JH

It is pointed out in Fig. 4, that under normal developmental conditions, epidermal excisions always regenerate into homochronic structures, indistinguishable from the epidermis of the host. However, this is not the case when regenerating epidermal cells are exposed to juvenile hormone (JH) activity at the time when the host cells became JH-insensitive (see Fig. 6). The integument of fully grown last instar larvae is fully expanded and very flexible. Due to this, the patch of epidermal cells shown in Fig. 6A was not excised but cut using thermocautery. The regenerating epidermal areas developed into heterochronic, metathetelic (or previous stage), larval epidermal patches on pupae (Fig. 6B). Epidermal excisions and JH treatment of pupae resulted into the metathetelic pupal patches on the adult body (Fig. 6D). The excisions were done on 3-day-old larvae or pupae (Fig. 6A and C), because epidermal cells of uninjured specimens become insensitive to exogenous JH at this time. In contrast to normal cells, regenerating epidermal cells still retained their sensitivity to JH. They developed epidermis of the preceding ontogenetic stage (Fig. 6B), e. g. patches of larval cuticle on pupae and of pupal cuticle on adults.

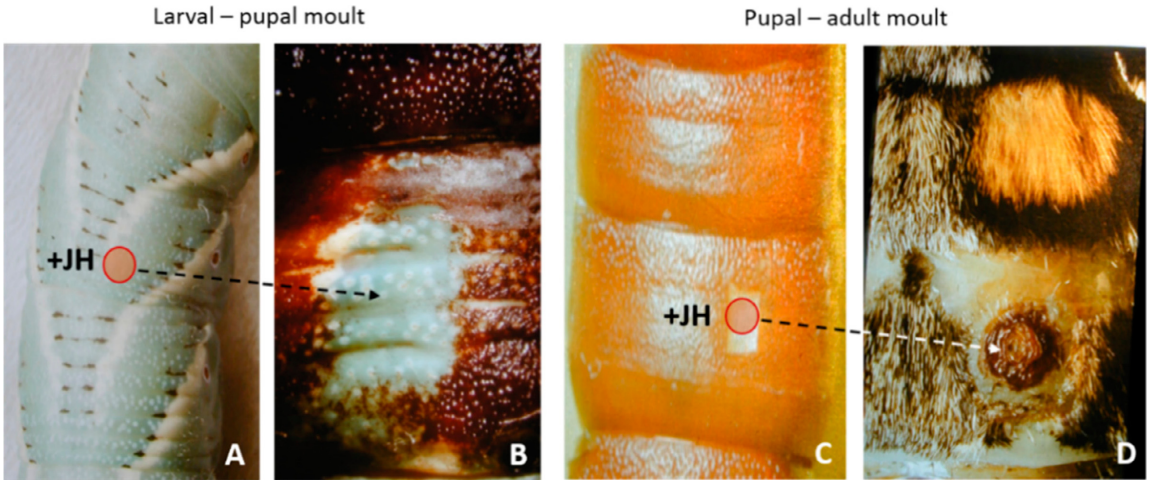

Fig. 6. Heterochronic regeneration of epidermal excisions under the effects of juvenile hormone (JH). The excisions were made on 3-day old feeding last instar larvae (A) or 3-day old developing pupae (C) of *Manduca sexta*. At this time, epidermal cells of the larvae and pupae are insensitive to JH. The dividing and regenerating cells, however, are sensitive to JH, which results in the development of metathetelic (or previous stage) larval cuticle on pupae (B) or pupal cuticle on adults (D).

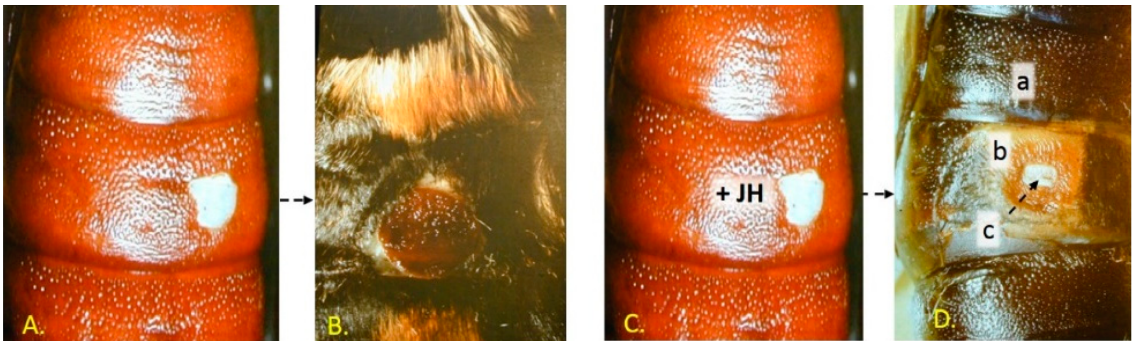

Fig. 7. A and B – metamorphosis of the metathetelic, larval epidermal patches during the pupal-adult transformation of *Manduca sexta*. C and D – heterochronic pupae treated with a JH analogue (100 µg of JH-1 per specimen) develop into secondary pupae with secondary pupal cuticle and a metathetelic larval cuticular patch in the middle (a – primary pupal cuticle, partially removed; b – secondary pupal cuticle; c – local patch of heterochronic, secondary larval cuticle).

The results in Fig. 6 perhaps represent the best examples of the local metathetelic actions of insect JH. Physiological implications of these results are: (1) Regenerating epidermal cells independently execute their own, JH conditioned, epigenetic or "status quo" morphogenetic programmes, although the surrounding host cells simultaneously follow substantially different, larval-pupal or pupal-adult morphogenesis; (2) Regenerating epidermal cells that develop into larval structures (larval metathetely) are fully compatible with the morphological more advanced surrounding pupal cells; (3) Regenerating epidermal cells of heterochronic tissues are able to cease dividing when tissue integrity is achieved.

Developmental fates of metathetelic epidermal patches

According to the long standing advocated hormonal concept of Piepho (1951) and Schneiderman & Gilbert (1964), the sequence of larval, pupal, and adult structures in insects is determined by respectively high, intermediate or low concentrations of JH. A heterochronic specimen should thus develop into the homochronic structures of either larval, pupal or adult structures, depending on the concentration of JH.

In this study, I investigated the developmental fate of 37 heterochronic patches during metamorphosis in *Manduca*. Fig. 7 shows the structural changes that occurred in the heterochronic patches during the pupal-adult transformation. The results can be briefly described as follows: (1) Larval metathetelic patches produced by the regeneration of larval epidermal cells metamorphosed into heterochronic patches of pupal cuticle on the adults (Fig. 7A and B); (2) After treatment with a juvenile hormone (JH) analogue, pupae bearing these metathetelic larval patches, developed into heterochronic patches of secondary larval cuticle on secondary pupal instars. In other words, this result manifests the "status quo" effect of JH (Fig. 7C and D). The most important aspect of this physiological feature is that the heterochronic character of the developing tissues was fully preserved. This effect is an example of the epigenetic action of JH and its 4000 bio analogues. The extant mixture of different morphogenetic structures can be influenced repeatedly many times by JH and reproduced without the morphogenetic instructions on the genome being changed.

The results in Fig. 7 indicate that there is no homochronisation of the heterochronic tissues during insect metamorphosis, contrary to what is predicted by current hormonal theories (Piepho, 1951; Gilbert, 2012). In the absence of JH, larval cells metamorphosed into pupal cells, while pupal cells simultaneously metamorphosed into adult cells. This shows that the selective physiological condition for induction of a given ontogenetic stage cannot depend simply on the concentration of juvenile hormone. This depends on the previously attained morphogenetic stage, which is associated with irreversible changes and reprogramming of genes on chromosomes in peripheral target tissues (Sláma, 1985). The epigenetic role of JH is based on informing the genes in peripheral cells when to execute their inherited genetical programmes. Fig. 7C and D demonstrate that the "reversal of metamorphosis" under the influence of high concentrations of JH (the dosages of JH analogue were 500-fold greater) is not possible. The heterochronic tissues in Fig. 7 were analyzed earlier using scanning electron microscopy (Sláma & Weyda, 1997). The cuticle of larvae is wrinkled and folded while the pupal epidermal cells are covered by a smooth, rigid cuticle. The boundaries of larval patches of epidermal cells on pupae are sharply delimited.

The effect of vitamin D_1 on regeneration of epidermal cells

Due to its partial water solubility, vitamin D_1 does not penetrate the lipid coating of insect integument. To be active, vitamin D_1 needs to be injected into the body cavity or ingested in food. Phytophagous insect larvae commonly feed on the leaves of plants containing about 0.01% of vitamin D_1 (Sláma et al., 1974; Zelený et al., 1997). Excess vitamin D_1 in their diets is eliminated by excretion (Sláma, 1985, 1999; Zelený et al., 1997). Injections of copious amounts of vitamin D_1 (20 µl of 0.5 mg/ml solution of 20-hydroxyecdysone in 10% ethanol) cause pathophysiological (hyperecdysonic) syndromes of hypervitaminosis, associated with adverse growth effects, like ceasing to feed, immobilisation due to muscular paralysis or formation of larval-pupal intermediates, which die due to failure to moult (Sláma et al., 1974; Sláma, 1975, 1980). In our

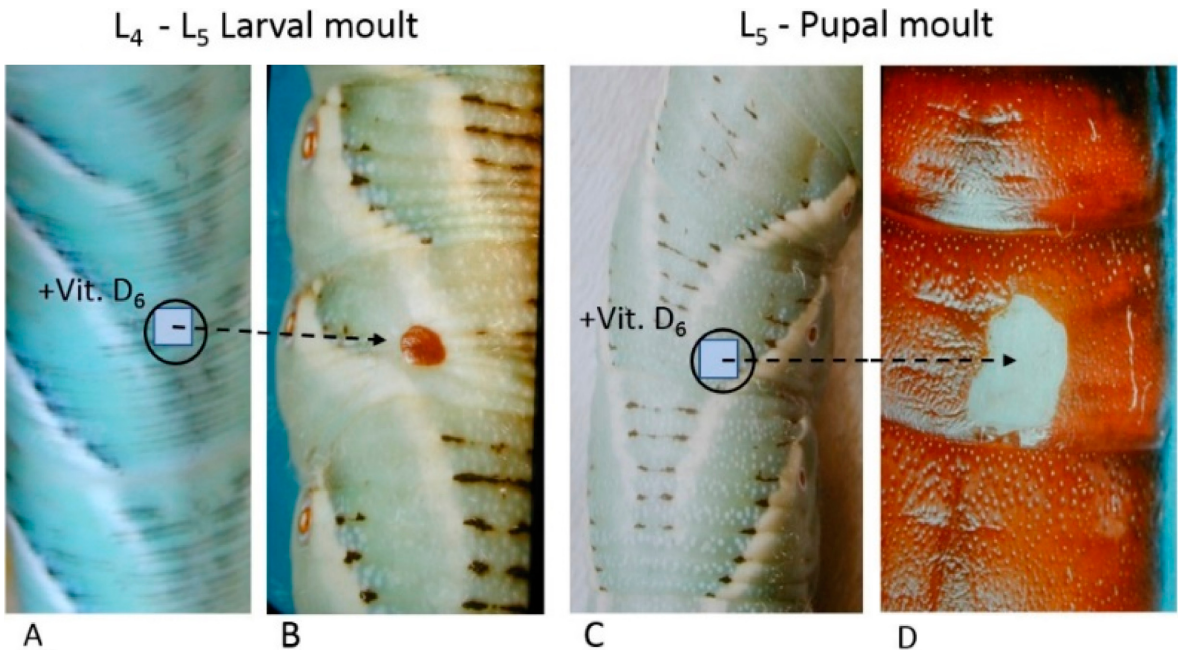

Fig. 8. A – a fourth instar larva (L_4) of *Manduca sexta* with the area of excised epidermis covered by solidified gelatine containing vitamin D_1. B – regenerated patch of prothetelic pupal cuticle on the last instar larva (L_5). C – area of epidermis excised from a last instar larva covered by gelatine containing vitamin D_1. D – local prothetelic patch of incompletely developed, scale less adult cuticle after the next moult.

experiments, the larvae of *Manduca* tolerated injections of enormous amounts (up to 1000 µg) of vitamin D_1 (20-hydroxyecdysone). In contrast, as little as 2 µg injected into a non-feeding pupa produced lethal hyperecdysonic syndromes (Sláma, 1999).

The lack of integumental penetration rules out the possibility of localized epidermal effects of vitamin D_1 (except for the dipping in methanol). To achieve the local prothetelic effects of vitamin D_1 in *Manduc*a, it was necessary to apply the vitamin selectively to regenerating epidermal cells, without increasing its content in the whole body. Finally, as shown in Fig. 8, I achieved this by supplying vitamin D_1 to the excised area of epidermis by covering it by a piece of solidified, 12% gelatine, containing 2 mg of 20-hydroxyecdysone per ml. The gelatine cover was overlain by a polyester coating consisting of a drop of quickly polymerizing cyanoacryllic glue (Fig. 8A).

The advantage of this technique was that the regenerating epidermal cells received the vitamin D_1 by diffusion from the gelatine. Unfortunately, for most (88%, n = 37) of the smaller (L_4) larvae, some physiologically active amounts of vitamin D_1 passed through the excision into the body cavity. These larvae succumbed to hypervitaminosis, such as not feeding, immobilisation and precocious secretion of new larval cuticle. Fortunately, despite these obstacles, 18 larvae survived and successfully moulted into the last larval instar. Eight of these larvae exhibited beautiful regenerated patches of sclerotized, prothetelic (precociously developed), brown pupal cuticle (Fig. 8B).

The great scientific value of prothetelic patches, shown in Fig. 8B, depends on the fact that the regenerating larval epidermal cells can be reprogrammed by vitamin D_1 prematurely to form the future, pupal epidermis. Despite the relatively high content of endogenous JH in the penultimate larval instar, the regenerated pupal cells were fully integrated with the surrounding larval cells of the previous morphogenetic stage. These experiments were more successful when done using the larger larvae of the last instar (Fig. 8C and D) and resulted in the development of prothetelic adult cuticular patches on the pupae.

The cells of the prothetelic adult epidermal patches, which were produced by the action of vitamin D_1 directly from the larval stage, actually bypassed the externally exposed pupal stage. According to my view, the regenerating larval cells were at first reprogrammed by diffusion of vitamin D_1 from the gelatine into the covert pupal stage. Then, during the subsequent transfer through the prepupal peak of vitamin D_1 in the absence of JH, the regenerated cells of the pupal ontogenetic stage developed further and realized the initial part of the pupal-adult transformation. As already seen in Fig. 3, this period is characterized by the prepupal peak in the endogenous concentration of vitamin D_1. Thus, when the larval cells of the host underwent the larval-pupal transformation, the regenerating prematurely transformed pupal cells simultaneously entered the initial stages of the pupa-adult morphogenetic programme. Therefore, pupae that successfully developed from the larvae that were operated on regenerated patches that consisted of thin, transparent adult cuticle without scales (Fig. 8D). Cytological analysis (Sláma & Weyda, 1997) revealed that the morphological structure of the prothetelic, delicate, white epidermal regeneration patches in Fig. 8D are indeed imaginal, and unlike the thick, elastic metathetelic larval patches in Fig. 7. The absence of scales and setae in the adult cuticular patches can be explained by the different time intervals needed for the larval-pupal transformation (3–5 days) and

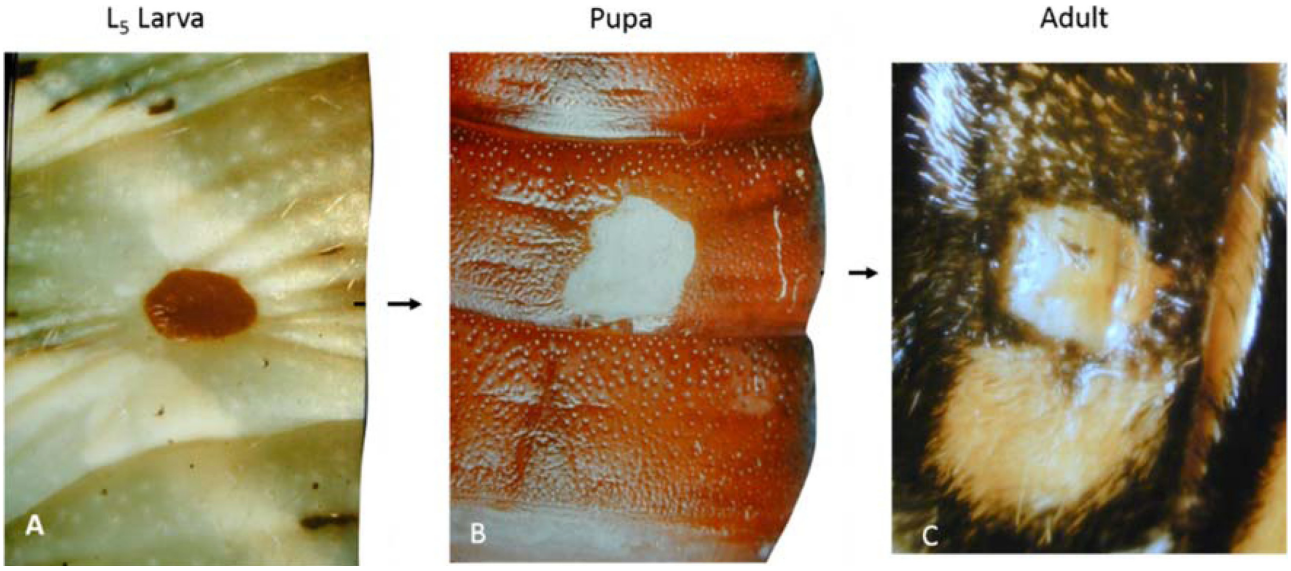

Fig. 9. Developmental fates of the prothetelic patches induced by vitamin D_1 on *Manduca sexta*. A – prothelic regeneration of pupal cuticle on a last (L_5) instar larva. B – prothetelic regeneration of adult cuticle (white patch) after the moult into pupa. Note the absence of setae ("hairs") that are abundant on adults. C – prothetelic patch of undifferentiated adult cuticle after the moult to adulthood. Note the absence of setae ("hairs").

that required for the pupal-adult transformation in *Manduca*, which is much longer (10 days). Apparently the short 3-day period of the larval-pupal transformation is insufficient for the differentiation of adult setae and scales on the heterochronic prothetelic patch.

Developmental fate of the prothetelic epidermal patches

General validity of the above is corroborated by developmental fates of the prothetelic, pupal and adult epidermal patches. The data in Fig. 9 show that the vitamin D_1 induced regeneration of prothetelic pupal patches on larvae (Fig. 9A) developed into prothetelic adult patches on pupae. As already mentioned, the adult cuticle was incompletely differentiated, without adult hairs and bristles (Fig. 9B). During the next period of the pupal-adult transformation, the prothetelic patches of incompletely differentiated adult cuticle developed into patches of thin and transparent adult cuticle, without setae and scales (Fig. 9C). This indicates that during the short (3-day) period associated with the larval-pupal moult, pupal prothetelic epidermal cells only completed the initial phase of the 10-day long pharate adult period. Obviously, there was not enough time for the complete differentiation of adult setae and scales. It is more difficult to completely develop the primitive, secondary adult cuticle. Occasionally, some heterochronic specimens exhibited adult cuticles with partly formed adult setae, but the primitive adult prothetelic epidermis was unable to develop into secondary adult cuticle with differentiated setae. I conclude that the inherited morphogenetic programme of lepidopteran insects does not contain information for repeating the development of specific adult structures.

Experience based on working with more than 4,000 JH bio analogues (Sláma et al., 1974; Sláma, 1999), revealed multiple repetitions of epidermal cells in all immature stages, except the adult stage, when treated with JH. Interestingly, it was possible to induce the local regeneration patches by using a combination of JH and vitamin D_1, and produce substantially different cuticular patterns of all immature stages on one insect body (Fig. 10).

In conclusion, heterochronic tissues, either JH-induced metathetelic or vitamin D_1 induced prothetelic, persist during insect metamorphosis and do not become homochronic with the surrounding cells of the host. The physiological reasons of this phenomenon are quite prosaic. Decisive factors are the required periods of time needed at a given tem-

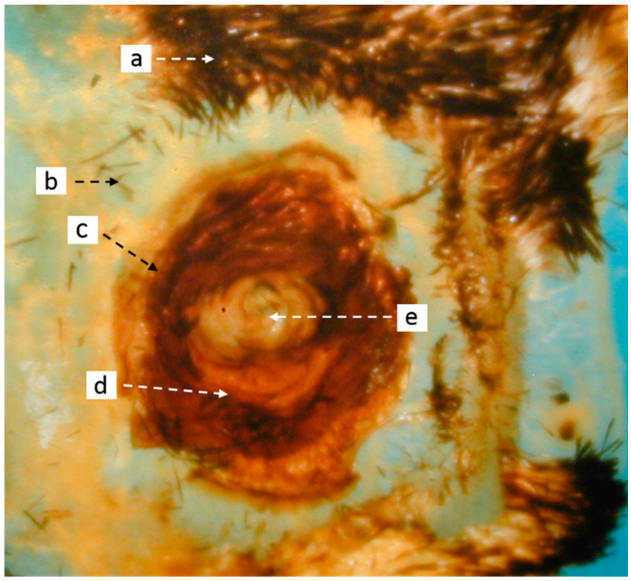

Fig. 10. Histological specimen of a multiple heterochronic patch on an adult *Manduca sexta* subjected to regeneration combined with an application of juvenile hormone (JH) and vitamin D_1 during the immature period. a – adult epidermis with setae; b – incompletely differentiated adult cuticle with minimal setation; c – primary pupal cuticle; d – secondary pupal cuticle; e – larval cuticle.

perature for the execution of cell divisions and subsequent differentiation of the structural elements. An important condition is the absence of "reversal of metamorphosis". Recognition of these rules in insect morphogenesis and understanding the actions of JH and vitamin D_1, have enabled us to obtain unprecedented combinations of heterochromic stages such as, for instance, the local cuticular patches of all immature ontogenetic stages exhibited in Fig. 10.

Unlike to vertebrate animals, avitaminosis associated with the deficiency of vitamin D_1 and malignant tumours is very difficult to be found. There are insects, like termites, feeding on pure cellulose, obtaining vitamin D_1 exclusively from intestinal symbiotic flora. In *Manduca*, the lack of vitamin D_1 occurs between the two endogenous peaks, befor the time of pupal ecdysis (see Fig. 3). At this time, specimens with excised epidermal window succumbed and died due to ecdysial failures (62.5%, n = 32). Surviving specimens showed malformed, tumour-like epidermal regenerates, such as the one marked "e" in Fig. 10.

DISCUSSION

Brief recapitulation of the role of D_1 in insects

The data presented in Figs 3–9 reveal several biologically key features: (1) The periods of extensive cell death and cell proliferation (histolysis-histogenesis) are always linked with an increase in endogenous concentrations of vitamin D_1 (Fig. 3). (2) The living cells communicate and create a mutually integrated unit of tissue. (3) Naturally dead cells or artificially removed cells disrupt the living cell integrity resulting in cell divisions associated with the reconstruction of the extant morphological structure (homochronic regeneration) (Fig. 4). (4) The disconnected cells associated with wounds divide mitotically and restore living cell integrity (Fig. 5; Sláma, 1975; Sláma & Weyda, 1997). (5) The mitotic divisions stop as soon as the cellular integrity of epidermal tissue is re-established, which is clearly manifested by the shape and size of the regenerated heterochronic epidermal patches (Figs 5–9). (6) The application of exogenous JH to regenerating larval epidermal cells produces a local "status quo" effect, manifested by local patches of previous larval cuticle on pupae (metathetely) (Fig. 6). (7) The established metathetelic character of the cuticular patterns (larval patches on pupal body) is preserved during metamorphosis, i.e. local patches of larval cuticle on pupae develop into pupal patches on adults (Fig. 7). (8) In contrast to the metathetelic action of JH, the application of vitamin D_1 to regenerating epidermal cells caused the reciprocal, premature development of structural characters of a future ontogenetic stage (prothetely), manifested by local patches of regenerated pupal cuticle on larvae (Fig. 8).

Evidently, the most important feature of regeneration is the induction of mitotic divisions in those living cells that are deprived of mutual contact within the tissue. The second most important feature is that cell division ceases immediately after tissue integrity is achieved. In insects and probably other organisms (e.g., plants, vertebrates, including humans), the final signal of cell and tissue integrity is closely related to vitamin D_1. Failure to develop functional cellular membranes due to a lack of vitamin D_1 prevents the release of the end-point signal for tissue integrity, which may result in uncontrolled cell divisions and the development of tumours. In insects, disintegrating tissues change into a polynucleated syncytia (chromatic droplets of Wigglesworth, 1970), with distorted cell membranes. These tumour-like malformations are eliminated by an invasion of phagocytotic haemocytes. In higher animals and humans this protective mechanism does not occur and tumours with polynuclear cells continue to be produced from faulty, vitamin D_1 deficient stem cells. The most crucial factor leading to the development of regenerating epidermal cells in insects is the previously attained morphogenetic stage (embryonic, larval, pupal or adult; Novák, 1966, 1975), not the concentration of JH (Sláma et al., 1974; Sláma, 1975, 1980, 1983, 1985).

Sequestration of vitamin D_1 from prothoracic glands and other organs

The results are consistent with the earlier conclusions of Novák (1975) and Sláma (1975, 1980, 1985) that once attained morphogenetic structures can be repeated by the action of JH, but they cannot develop in the opposite direction, towards a previous ontogenetic stage. Unfortunately, the controversial concepts of "reversal of metamorphosis", proposed by Schneiderman & Gilbert (1959) and Sehnal (1984) are still cited (Riddiford, 2008; Gilbert, 2012). The metathetelic regeneration patches recorded in this study on *Manduca* (Figs 6 and 7) clearly support the epigenetic role of insect JH, manifested by the repeated formation of existing morphogenetic stages, like multiple larval or pupal stages, without structural changes in the DNA. This function of JH was corroborated over the past three decades by results of experiments using 4000 synthetic bioanalogues of insect JH (Sláma et al., 1974; Sláma, 1999, 2015a, b).

The coincidence between endogenous peaks of vitamin D_1 and cell proliferation (Fig. 3) have been known for a long time (Sláma, 1975, 1982, 1988, 1998, 1999; Gilbert, 2012; Smagghe, 2009, for review). The old hormonal theories (Schneiderman & Gilbert, 1959, 1964) propose that the endogenous peaks of vitamin D_1 (ecdysteroids) are the result of the PG responding to prothoracicotropic hormone (PTTH) secreted by the brain (Gilbert & Warren, 2005). Williams who proposed the brain-PG theory (Williams, 1952) and later refuted it (Williams, 1987) after finding that disintegrating gut is a major source of ecdysteroid. According to Sláma (1999), the whole concept concerning the role of PTTH is erroneous. The more recent studies by Sláma (1982, 1983, 1988, 1998) demonstrate that the PG are under the control of JH, not PTTH. Their physiological function is the metabolic production of water from dietary lipids (Sláma & Lukáš, 2016). The release of vitamin D_1 from PG occurs, as from many other peripheral organs, only during the short period when old larval organs disintegrate during the prepupal period (Sláma, 1998). In addition, PG in species that feed on dry food in the adult stage remain functional until the end of their life.

It is difficult to challenge the biochemical, PTTH-PG theory, mainly because of the heavily branched anatomical structure of the PG. Nevertheless, I managed to remove the PG from several hundred living *Galleria* larvae (Sláma 1980, 1983, 1998). Removal of the glands had no effect on the regulation of moulting and development in this insect. In the carpet beetle, *Dermestes vulpinus*, the PG are located within the head capsule. Removal of PG by decapitation had no effect on its moulting cycles and did not affect the endogenous peaks of vitamin D_1 in its body (Delbecque & Sláma, 1980; Sláma, 2015a). These results were discredited in 1988 by Sehnal and his colleagues (Sehnal et al., 1988) who expressed doubts about whether the PG were successfully removed from the living larvae of *Galleria*. They assume that the disintegrating prepupal PG is the sole source of vitamin D_1 in this species.

A new, alternative theory proposes that the peaks in endogenous concentration of vitamin D_1 are due to the reutilisation of the built-in vitamin D_1 during the non-feeding stages in metamorphosis. The theory that vitamin D_1 is re-utilised (Sláma, 1998) proposes that structurally bound vitamin D_1, found in the cell membranes of disintegrating larval tissues, could be enzymatically hydrolysed and the free vitamin D_1, as the polar metabolite, could be reutilized for growth of the newly proliferating tissues (Sláma, 1998, 2015a, b; Sláma & Zhylitskaya, 2014). Physiological reasons for the re-utilisation of vitamin D_1 during insect metamorphosis are related to the fact that insects do not synthesize the sterol nucleus de novo (Svoboda & Thomson, 1985). They receive dietary sterols from plants or microbial symbionts and convert them into cholesterol by dealkylation (Ikekawa et al., 1993). The reports of the release of vitamin D_1 from the PG of *Galleria* (Bollenbacher et al., 1978; Sehnal et al., 1988) or *Bombyx* (Iga et al., 2014; Nakaoka et al., 2017) are only for the vitamin D_1 that is released when the prepupal PG disintegrates (*Galleria* and *Bombyx* do not feed in the adult stage; their PG disintegrate and release vitamin D_1 shortly before pupation).

Earlier investigations on the regeneration of epidermal cells, hypervitaminosis

The regeneration of insect epidermal cells was carefully investigated half a century ago by Sir Vincent B. Wigglesworth (1970) in an hemipteran species, *Rhodnius prolixus* Stål (Reduviidae). He describes the regeneration of epidermal cells after local lesions of the integument, including the regeneration of specific bristles and dermal plaques. Unfortunately, vitamin D_1 and JH analogues were not available at that time. The results of the current study (Fig. 4) on *Manduca* are fully consistent with Wigglesworth's results on epidermal regeneration in *Rhodnius*. However, these results are in direct conflict with the early conclusions of Piepho (1951), who studied the regeneration of pieces of epidermis implanted into the body cavity of *Galleria*. Essentially, Piepho proposed a hypothesis, which was used by Schneiderman & Gilbert (1959, 1964) as a corner stone of the hormonal theory, which has dominated insect endocrinology for more than 60 years (Smagghe, 2009; Gilbert, 2012; Sláma, 2015a, b). Piepho (1951) assumes that insect epidermal cells are just like a ball of hormones. He assumes that epidermal implants develop into larval, pupal, or adult structures simply in response, respectively, to high, intermediate or low concentrations of JH. According to Piepho, when the concentration of JH is high, pupal epidermal cells can dedifferentiate and then differentiate into previous, outlived morphogenetic stages. For example, pupal cells could differentiate back into larval cells under high concentrations of JH. However, this has never been independently experimentally confirmed. The concept of Piepho (1951) and the related hormonal theories (Gilbert & Warren, 2005; Riddiford, 2008) were refuted in 1997 based on the results of scanning electron-microscopy study of regenerating epidermal cells (Sláma & Weyda, 1997). The effects of JH on epidermal cells followed the all-or-none rule in terms of previous and future ontogenetic structures. Studies using JH and its 4000 bio analogues (Sláma, 1999) indicate that developmental instructions are coded in the genome. A minimum physiologically active dose of JH and a 500-million-fold higher concentration have the same effect. In contrast to this more or less qualitative action of JH, injections of vitamin D_1 cause a quantitative, dose-dependent acceleration of the moulting cycle (Sláma, 1980). Overdosage usually results in precociously formed, incompletely differentiated intermediate forms (Sláma et al., 1974, 1993; Sláma & Lafont, 1995; Sláma, 1999; Sláma & Zhylitskaya, 2014). In this respect, the difference in the effect of different physiological concentrations of vitamin D_1 (Fig. 3) and of artificially increasing the concentrations by injection is very important. Because of this, the prothetelic regeneration of patches induced by vitamin D_1 shown in Figs 7 and 8 are rather unique features observed for the first time only in *Galleria* (Sláma, 1975).

During animal evolution, vitamin D_1 acquired a special function in the regulation of insect growth, which involves periodical changes in the structure of the exoskeleton. Therefore, the main growth function of vitamin D_1 in insects does not depend on growth of bones and muscles, as in vertebrates, but on the repeated exchange and enlargement of the integument (Sláma & Zhylitskaya, 2014). During moulting in insects, epidermal cells first detach from the old cuticle (apolysis). During this pharate stage, the cells proliferate and differentiate, before forming a new epidermis and secreting a new cuticle. Moulting is controlled by endogenous peaks in the concentration of vitamin D_1 (Fig. 3). The peaks have a homeostatic function in synchronising the different stages in the moulting cycle (Sláma, 1980). Injections of vitamin D_1 made before the peak accelerate, while those made after the peak inhibit the onset of insect ecdysis. The secretion of new cuticle can only occur at a particular stage in the moulting cycle. The importance of the above was revealed by biochemists and molecular biologists investigating the effects of vitamin D_1 at the levels of receptors and genes (Gilbert & Warren, 2005; Smagghe, 2009).

The above indicate that injections of vitamin D_1 made before the endogenous peak can result in a serious developmental abnormality, manifested by pathophysiological

syndromes of D_1 hypervitaminosis. The syndromes were first described soon after the discovery of ecdysone by C.M. Williams (1970) in pupae of the Cecropia silkworm, *Hyalophora cecropia* (see Sláma et al., 1974). The pathogenicity of vitamin D_1 does not depend only on the injections of high concentrations, but also on the non-physiological, instantaneous delivery of an abundant amount. The endogenous peaks show a physiological course represented by the successive rise and decline in the concentration of vitamin D_1. Recent attempts to moderate the effects of hypervitaminosis are based on the preparation of synthetic, vitamin D_1 complexes with porphyrins (Sláma & Zhylitskaya, 2014). These complexes are enzymatically hydrolysed, slowly releasing the free vitamin D_1, thus, imitating the natural endogenous peaks.

The secretion of the new and reabsorption of the old cuticle is the most vulnerable period in the moulting cycle. These stages are usually immobile. Feeding larvae have the lowest titres of vitamin D_1 because they have high rates of metabolism and excretion. It has been assumed that high amounts of these compounds in certain plants protect them against herbivores (see below). Experimental injection of vitamin D_1 into feeding larvae produce rather dramatic results. They cause an immediate cessation of feeding, which is associated with reduced locomotion and neuromuscular paralysis ("preecdysial sleep"). In the present experiments with *Manduca*, these syndromes of D_1 hypervitaminosis were frequently encountered in association with the prothetelic effects described in Fig. 8. The affected larvae did not feed and move, quickly desiccated and died. Ignoring the hypervitaminosis features recorded in vivo can lead to misleading results related to in vitro investigations of its effects on vitamin D_1 receptors (Gilbert & Warren, 2005; Smagghe, 2009). As in insects, large hypervitaminic amounts of vitamin D_1 result in embryo toxicity in vertebrates (Košár et al., 1977) and aberrant tissue growth (Lagova & Valueva, 1981).

During the discovery of JH-active materials in plants, Sláma & Williams (1965) speculated that "by containing insect hormones and other effective compounds, plants could possibly evolve an incredibly sophisticated self-defence against insect predation". Despite criticisms (Sláma, 1979) the insects living on the vitamin D_1 rich Siberian plant, *Leuzea carthamoides*, were studied, which revealed that the caterpillars of several insect species, especially polyphagous Noctuid moths, are virtually resistant to the relatively large amounts of vitamin D_1 in their food (Zelený et al., 1997).

Distribution and sources of vitamin D_1

Due to the persistent belief in arthropod moulting hormones, the biological activity of vitamin D_1 was investigated predominantly in insects (Sláma et al., 1974, 1993; Sláma, 1993; Sláma & Zhylitskaya, 2014). Organic chemists had a golden age identifying individual hydroxylic groups in molecules in extracts prepared from numerous species of flowering plants (Hikino et al., 1968; Volodin, 2003; Dinan et al., 2009; Smagghe, 2009; Lafont et al., 2011) and various animals (Lafont & Koolman, 2009). They also discovered other structurally related polyhydroxylated sterols, such as brassinosterol phytohormones (Kripach et al., 2000). In addition to ferns (Jizba et al., 1967) and vascular plants (Dinan & Lafont, 2006; Kumpun et al., 2011; Lafont et al., 2011; Hunyadi et al., 2016), vitamin D_1 was found in mushrooms (Vokáč et al., 1998), crustaceans (Horn et al., 1968), marine arthropods (Pycnogonids) (Tomaschko & Bückman, 1992), insects (Karlson, 1966; Sláma et al., 1974, 1993), Japanese quails (Koudela et al., 1995; Sláma et al., 1996), mice (Stopka et al., 1999, Smagghe, 2009), domestic animals (Syrov, 1984; Sláma & Lafont, 1995; Košár et al., 1997; Krátký et al., 1997; Dittrich et al., 2000; Kholodova, 2001; Dinan & Lafont, 2006; Jadhav et al., 2007) and humans (Koolman & Moeller, 1986; Bhaswaid et al., 1991; Gharib et al., 1991; Sláma & Lafont, 1995; Kholodova et al., 2001).

Sláma (1979) was the first to propose that vitamin D_1 could be a reserve material for growth of tissues in plants. It is also possible that, as in the case of structurally related brassinosterols, vitamin D_1 could also act as a phytohormone. This was experimentally investigated using special bioassays, in which the effect of vitamin D_1 (20-hydroxyecdysone) was compared with the effects of certain commonly known phytohormones (Macháčková et al., 1995). The assays used were the wheat coleoptile bioassay, gibberellin bioassays using dwarf maize or rice, cytokinin bioassays using tobacco callus, brassinolide-related ethylene formation in dwarf maize and alfalfa, flowering assays using *Chenopodium* and special assays based on somatic embryogenesis in cell cultures of alfalfa. The results provide unambiguous evidence that the effects of vitamin D_1 are not similar to that of any of the above phytohormones (Macháčková et al., 1995).

I used insects as a sensitive biological assay (0.1 µg detection limits of 20-hycdroxyecdysone equivalents) (Sláma et al., 1993; Sláma & Zhylitskaya, 2014), to study the translocation of vitamin D_1 in the plant *Leuzea carthamoides*, during an entire vegetative season. The dry winter roots contain 0.5 to 2.1% of vitamin D_1 complex (95% of 20-hydroxyecdysone + 5% mixture of related ecdysteroids) and dry seeds 2.1 to 3.1%. The highest content was found in the early unfurling leaves in spring. This shows that vitamin D_1 and the related derivatives of 7-dehydrocholesterol are biosynthesized within green leaves during photosynthesis (average content of dry leaves is 0.01 to 0.05%). At the end of a season, vitamin D_1 is translocated from the shoots to the roots and seeds. Curiously enough, the seeds of *Leuzea* contain approximately 500,000-fold more vitamin D_1 (Stránský et al., 1998) than an average insect pupa (Karlson, 1966). Moreover, the content of this derivative of 7-dehydrocholesterol (zoosterol?) in the plant is 700 fold greater than that of phytosterols, ergosterol and β-sitosterol. This disproportion is due to the fact that cholesterol derivatives are preferentially hydroxylated in plants and thus disappear from the pool of free lipid soluble plant sterols (Stránský et al., 1998).

Plants of *Leuzea carthamoides* (Fig. 11) are a rich and convenient source of vitamin D_1 for pharmacological use.

Fig. 11. Crop of *Leuzea carthamoides* (Willd.) (Asteraceae) at Velký Osek, Czech Republic in 1995. The roots and seeds of this plant contain up to 3% by dry mass of vitamin D_1 (Buděšínský et al., 2009).

Pure vitamin D_1 is a white crystalline substance with a sweet taste due to its six or seven hydroxylic groups. Paradoxically, it is a partly water soluble, physiologically important "cholesterol sugar" (Sláma et al., 1996). The essential structural feature of vitamin D_1 is the conjugated, α, β-unsaturated, 6-keto,7-dehydro grouping in the B ring of cholesterol, which stabilizes the molecule and provides a strong electron accepting property (Sláma et al., 1974).

Similarities between insect and human physiological systems

It has been already pointed out in this paper that regenerating epidermal cells of *Manduca* require vitamin D_1 for the maintenance of tissue integrity and prevention of uncontrolled mitotic divisions. Similar roles in regeneration can be envisaged for vitamin D_1 in the human body. Such comparisons were generally ignored because protostomes, such as insects, and deuterostomes, like humans, have evolved separately over approximately 550 million years. However, despite such a large phylogenetic divergence, 37% of the genes in insect genomes (*Drosophila*) are homologous and used for similar morphogenetic and physiological functions both in insects and humans (Devillers, 2013). For instance, the primordial formation of insect and human hearts is orchestrated by similar sets of genes (*tinman*) (Bodmer, 1995; Sláma, 2012). In both instances the hearts perform identical, involuntary, purely myogenic contractions based on the discharge of electrical potentials from depolarized myocardial cells. In insects, as in the human heart, rhythmical beating is regulated by similar sets of special pacemaker nodi (e.g., sinoatrial, atrioventricular and Hiss bundle in the human heart; posterior nodi in the insect heart) (Sláma, 2012).

Analogous insect-human similarities were recently reported for structures and functions of their central neuroendocrine systems. For example, the neurosecretory cells in the insect brain are similar structurally and functionally to neurosecretory cells located in the human hypothalamus (Sláma, 2015a, b). Additional evidence for insect-human similarities are reported in the field of respiratory physiology. Curiously enough, insects actually breath like humans. They mechanically inspire and expire air through particular spiracles and ventilation is regulated by an autonomic, cholinergic neuroendocrine system, known as the coelopulse (Sláma & Santiago-Blay, 2017). This system is structurally and functionally like the human parasympathetic nervous system. These similarities indicate that the role and physiological functions of vitamin D_1 in regeneration and cell divisions described here for *Manduca* (Figs 4–9) could also be true for human tissues. This statement is further supported by the similar physiological role of vitamin E (γ- and δ-tocopherols) in the regulation of reproduction both in insects and humans (Jedlička et al., 2009).

The above indicated similarities between the effects of vitamin D_1 in insects and humans can be extended to other vertebrates, like Japanese quail (Koudela et al., 1995). Studies have revealed strong anabolic, vitamin D-like growth effects of vitamin D_1 (20-hydroxyecdysone), which surpass the effects of the commercial dietary additives for chickens. The quails stored the dietary vitamin D_1 in their blood depending on the amounts of the vitamin in their food (0.5, 6.0 and 8.0 ng per 100 µl of blood serum) (Sláma et al., 1996). The strong anabolic effects of vitamin D_1 in birds (Koudela et al., 1995) confirm previous reports of Ukrainian, Belorussian and Kazakhstan authors, working with pigs, cattle, and other domestic animals (Syrov, 1984; Dzukharova et al., 1984; Sláma & Lafont, 1995; Kholodova, 2001; Smagghe, 2009). Stopka et al. (1999) investigated the pharmacological effects of pure vitamin D_1 in mice, using daily peritoneal injections of from 2.8 mg/g to 10 mg/g, during their juvenile and reproductive periods. The injections of vitamin D_1 significantly enhanced the growth rate in juvenile females, but not in juvenile males. In the adult stage, the injections caused substantial increase in growth of both the male and female mice. These results are consistent with previous observations on the effects of vitamin D_1 in mice, rats and other animals, and in humans (Sláma et al., 1974; Sláma & Lafont, 1995; Kholodova, 2001). Historically, the first reports of vitamin D_1 in humans were for patients infested with parasitic worms. They excreted vitamin D_1 in their urine (Koolman et al., 1986; Baswaid et al., 1991). Later, however, it was discovered, that vitamin D_1 is also present in urine and blood samples of healthy people (Gharib et al., 1991).

The neglected role of vitamin D_1 in human health care

Clinical investigations on vitamin D_1 were hindered for a long time by the persistent belief it is an insect hormone and by the limited availability of the pure substance. In the former Soviet Union, the research was stimulated by the accidental finding that dietary additions of a drug prepared from the Siberian plant, *Leuzea* (syn. *Rhaponticum*) *carthamoides* increased the production of milk and meat in cattle (see Sláma & Lafont, 1995 for a review). They used a partly purified extract of vitamin D_1 from *Leuzea* for development of the pharmacological preparation called ECDISTEN, in 1980. Prescriptions recommend 5 mg capsules, 3-times per day. The advertisements promise a plethora of beneficial pharmacological effects like anabolic improvement of physical condition, removal of astheno-depressive

states, tonic effects, elimination of weakness, impairment of chronic intoxication, neurasthemia, neurosis, hypotension, fatigue and recovery from infectious disease (Sláma & Lafont, 1995, for review). According to Syrov (1984), the preparation was used as an anabolic drug by Soviet Olympic athletes (Syrov, pers. commun. to KS). The medication advertised a monthly increase of half a pound of muscular tissue. Due to its partial water solubility and natural occurrence, vitamin D_1 has never been classified as a prohibited anabolic steroid.

In addition to plants containing relatively substantial amounts of vitamin D_1, like for example, the fern *Polypodium vulgare* L. the daisies *Leuzea carthamoides* (Willd.) and *Serratula coronata* L. as well as the mint, *Ajuga turkestanica* (Jizba et al., 1967; Volodin, 2003; Jadhav et al., 2007; Dinan et al., 2009; Lafont et al., 2011), vitamin D_1 is a common constituent of vegetable foods like, Quinoa (*Chenopodium quinoa*; 0.03%; Kumpun et al., 2011); spinach (*Spinacia oleracea* L., 0.01%; Hunyadi et al., 2016) and Suma root (*Hebanthe eriantha*, 0.66%; Hunyadi et al., 2016). A more recent preparation, SERPISTEN, registered in Russia, uses the purified extracts of vitamin D_1 from *Serratula coronata* L. (Volodin, 2003; Ufimtsev et al., 2009). In summary, the most important pharmacological properties of vitamin D_1 in humans are the strong anabolic, vitamin D-like effects on somatic growth of bones and muscles and improvement of neuromuscular performance (Sláma & Lafont, 1995; Kholodova, 2001; Dinan & Lafont, 2006). Unfortunately, these important actions of vitamin D_1 in veterinary and human medicine have been seriously underestimated by western scientists, engaged predominantly in investigations of the tentative ecdysone receptor (Gilbert & Warren, 2005; Gilbert, 2012).

Evidently, the general role of the antirachitic vitamin D in adult stages depends mainly on the enhanced growth and regeneration of certain tissues and organs. Based on what happens in *Manduca*, vitamin D_1 should also provide functional cell membranes for preventing uncontrolled endomitotic cell divisions in human organs. So far, I have not found any reports of tests on the role of vitamin D_1 on the ratchet bone disease, using randomized controlled trials, the gold standard in medical testing. The original reports on vitamin D, completed almost 100-years ago, noted an increased antirachitic property of samples collected during summer (Hess & Unger, 1921; Hess et al., 1925). The effects were attributed to increased UV-radiation, increasing the conversion of cholesterol into the antirachitic 7-dehydrocholesterol. I feel that seasonal effects on antirachitic properties can be explained in a more prosaic way. Namely, sunshine increases the photosynthesis of vitamin D_1 in the green leaves of plants. The pioneers of vitamin D research did not know this, because they did not know that cholesterol derivatives such as vitamin D_1 are present in plants.

The credibility of the theory of UV-based activation of secosterol vitamins D_2 and D_3 in the skin (Windaus et al., 1931; Windaus & Bock, 1937), however, needs to be re-examined. Who knows where vegetarians get their essential vitamin D from, if it is an animal sterol? In addition, where do Nordic people get their vitamin D, if their skin is permanently covered and not exposed to UV radiation?

Polyhydroxylated, 6-keto, 7-dehydrocholesterol (ecdysone, vitamin D_1) was discovered in 1965 (Karlson, 1966). This is almost three decades after the end of the basic antirachitic investigations. In 1965, nobody suspected that an "insect moulting hormone" could be the true vitamin D. The early suggestion that ecdysteroids could be the neglected vitamin D (Sláma, 1993), was ignored. The respected authority on the chemistry of vitamins, Prof. P. Karlson (Karlson, 1981) and the associated group of chemists, generally known as "ecdysonists", did not comprehend the possibility of ecdysone being a vitamin and adhered strictly to the old concept of it being an insect hormone. The results reported here of the study on *Manduca* (Figs 4–10) confirm the key role of vitamin D_1 during tissue regeneration (Sláma, 1993, 2014, 2015a, b). These findings are in good agreement with the role of endogenous peaks of vitamin D_1 in the control of cell divisions in insects (Fig. 3) and it is highly likely that vitamin D_1 has a similar role in cell division and regeneration in humans. Confirmation of this will depend on the availability of a sufficient quantity of vitamin D_1 for pharmacological investigations.

As already mentioned, vitamin D_1 is present in human blood and urine (Koolman & Moeller, 1986; Gharib et al., 1991). Its concentration in human blood should reflect the concentration of vitamin D_1 in the diet. The storage of vitamin D_1 in the blood of Japanese quail (Sláma et al., 1996) indicate that humans could also store vitamin D_1 in their blood and excrete the excess in their urine. Experimental proof of the above can be easily obtained using radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA), which are well documented and widely used in studies on insects (Delbecque & Sláma, 1980; Sláma et al., 1996; Smagghe, 2009; Gilbert, 2012).

The D_1 avitaminosis theory of malignant growth

According to the data presented in previous sections, vitamin D_1 should be included in the sterolic vitamin D group with anabolic, growth stimulating and antirachitic properties. It is widely produced by microorganisms, fungi and plants, and due to its amphoteric solubility, can be placed in both the partly water soluble and lipid soluble categories of vitamins. In juvenile vertebrates, vitamin D_1 stimulates anabolic growth of bones and musculature (antirachitic activity); in the adult stage it is essential for growth and regeneration of individual tissues and cells. The structurally well-formed cellular membranes, produced in the presence of vitamin D_1 result in the resumption of living cell integrity in regenerating tissues. The resumed integrity between living cells stops further divisions of regenerating cells not only in invertebrate, but also in vertebrates. By contrast, the deficiency of vitamin D_1 during somatic growth or regeneration may lead to the formation of aberrant cell divisions (Syrov, 1984; Sláma & Lafont, 1955; Kholodova, 2001). This may finally result in the formation of polynucleated syncytia of pernicious malignant cells. In insects, the polynucleated syncytia appear in disintegrating tissues and are swallowed and destroyed by phagocytotic

haemocytes. This protective, anti-tumour mechanism does not operate in humans, where the defective polynucleated cells persist and create malignant tumours.

The chemical structure of Vitamin D_1 is derived from 7-dehydrocholesterol and includes many hydroxylic groups on the secondary or tertiary carbon atoms. Due to this, its biological activity does not depend on hydroxylation in the liver or kidneys. In addition, its biological activity equally does not depend on the conversion of cholesterol into 7-dehydrocholesterol by UV-radiation. Moreover, the most pronounced pharmacological effects of vitamin D_1 in humans are manifested straight by strong anabolic, vitamin D-like effects on the growth of the body (Sláma & Lafont, 1995). As already mentioned, there is a plethora of reports of the beneficial health effects of vitamin D_1, such as rejuvenation, increased growth, enhanced muscle performance, tonic, neurasthenic, neurogenic, anti-depressive, anti-fatigue, immuno-stimulating and similar vitamin D-like effects (review by Sláma & Lafont, 1995).

Similar to the effects in insects depicted in Figs 4–10, a deficiency of vitamin D_1 in human blood might result in defective regeneration of naturally dead cells or artificially wounded tissue. As a consequence of uncontrolled mitotic or endomitotic divisions (malignant tumour growth) can occur in vitamin D_1 deficient tissue (Sláma, 2018).

Chemotherapy, which abruptly stops all mitotic divisions, can temporarily abolish malformed, vitamin D_1 deficient regeneration. It is likely that success in restraining malignant growth will be related to the accidental presence or absence of vitamin D_1 during and after chemotherapy. When vitamin D_1 deficiency is prolonged, the oncological problems can come back again in the form of metastases. Naturally, the most vulnerable and most susceptible tissues to vitamin D_1 deficiency are the relatively large, acinose cells, whose cell membranes are adapted to secrete proteinaceous products (such as mammary glands, salivary glands, prostate cells, pancreatic cells, intestinal epithelium). External factors increasing the incidence of malignant growth (hereditary, malnutrition, adverse chemicals, senescence, obesity, repeated injury, viral infections, alcoholism, smoking, etc.), have a common denominator, a deficiency of vitamin D_1 in the blood. As far as I know, nobody has tested whether the addition of the newly defined vitamin D_1 could stop or shrink already developed malignant tumours. However, there are many lucrative research proposals for destroying malignant growth by destroying already existing, polynuclear tumour cells. According to my view, these cells are the consequences, not the cause, of the defective growth of stem cells. Solution of the problem with malignant tumours would be to eliminate the cause, not just the consequence of the malignant growth. In other words, the goal should be to prevent vitamin D_1 deficient stem cells from producing malformed tumour cells.

In addition to these oncological problems, there are other incurable diseases such as spinal muscular atrophy or neural dysfunctions (Alzheimer disease, Parkinson's disease in non-dividing nerve cells). Based on what is here presented on the role of vitamin D_1, I conclude that the hydrolipophilic properties of vitamin D_1 may be essential for the faultless electrophysiological functioning of nerve cells.

I hope sincerely that this 50-year long story of the hunt for the fictious insect hormone and 100-year neglect of vitamin D_1, may stimulate investigations by professionals working in the field of human health. Medical research was too expensive for me as it is far greater than that required for studying insects. I trust, however, that a simple analysis of the vitamin D_1 content and deficiency in blood of healthy and oncological patients might help resolve the previously neglected and now re-defined role of vitamin D_1 in the formation of pernicious malignant tumours.

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