The all-or-none rule in morphogenetic action of juvenile hormone on insect epidermal cells

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SUMMARY

We have investigated ultrastructural changes in the integuments of larval–adult and larval–pupal intermediates produced by exogenous application of juvenile hormone (JH) analogues in *Pyrhocris apterus* (Hemiptera), and *Galleria mellonella* and *Manduca sexta* (Lepidoptera). Ultrastructural analysis of the epidermis of these intermediates always revealed the presence of only two types of epidermal cell, which produced morphologically perfect cuticles of the previous and future developmental stages. There were no intermediate cuticles at the level of individual cells. It has been determined that a single epidermal cell constitutes the lowest elementary unit in the perception and realization of the developmental messages conveyed by JH to its target tissues. Further investigations revealed that the responses of individual epidermal cells to JH were strictly autonomous and qualitative, i.e. they were executed according to the ‘yes-or-no’ or ‘all-or-none’ rule. The neighbouring epidermal cells could realize independently, side-by-side, the quite dissimilar +JH (somatic growth) or –JH (metamorphosis) developmental programmes, although each of them formed biochemically, functionally, and ontogenetically different structures. The qualitative on-and-off signal given by JH for induction of the stationary (+JH) developmental cycle was limited to relatively short, genetically determined, and stage-specific developmental periods of cellular susceptibility to JH. The mosaic mixtures of the heterochronic, larval–pupal or adult epidermal cells, which we found in different proportions on the bodies of the intermediates, revealed two variable, development-related factors: (i) the presence or absence of a minimum effective concentration of JH, and (ii) positive or negative sensitivity of a particular epidermal cell to JH.

1. INTRODUCTION

The inhibitory function of juvenile hormone (JH) in insect metamorphosis was first recognized by Sir Vincent Wigglesworth in 1936. He concluded (Wigglesworth 1940) that tissues and cells of an exopterygote insect (*Rhodnius prolixus*) could develop under two different programmes: (i) the stationary nymphal–nymphal programme which occurs under the influence of JH, and (ii) the nymphal–adult programme which occurs in the absence of JH. Later on, in 1951, a more elaborate concept of concentration-dependent JH action was created by Piepho and his co-workers for endopterygote insects (*Galleria mellonella*). They assumed that insect epidermis was just a *toy* of the moult-inducing hormones (‘Die Epidermis ist also weitgehend ein Spielball der Häutungen auslösenden Stoffe...’; Piepho 1951). According to Piepho (1951), large concentrations of JH caused a cell to form larval structures, while intermediate concentrations elicited pupal structures, and a complete absence of JH resulted in formation of adult epidermal structures. This theory, which explained the developmental polymorphism simply on the basis of different quantities of JH, was quickly accepted by leading authorities on insect

endocrinology. It is still considered to be the leading theoretical account of insect biochemistry and molecular biology (Wigglesworth 1954, 1970; see Sláma (1995) for references).

The high–intermediate–low JH concentration theory of Piepho always suffered from a lack of supporting experimental evidence. Its credibility increased, however, when Willis *et al.* (1982) found concentration-dependent, gradual responses of single epidermal cells to JH in several insect species. We have now re-investigated the developmental responses of individual epidermal cells to JH in another series of ‘juvenilized’ larval–adult or larval–pupal intermediates. The effects of JH have been evaluated on the basis of the stage-specific cuticular products of individual cells using the advanced technique of scanning electron microscopy (SEM).

2. MATERIALS AND METHODS

For induction of the metathetic intermediates in *Pyrhocris apterus* L. (Heteroptera) larvae were treated topically with 10 µg of ethyl-11, chloro-3,7,11-trimethyl-2-dodecenoate in a 1 µl drop of acetone; the ID-50 effective dose was 0.05 µg per specimen. In order to cause the best larval–adult mosaic

Figure 1. Macroscopic distribution of integumental patches with transparent larval cuticle (L) and melanized dark adult cuticle (A) on abdominal sternites of two adultoids produced by exogenous application of juvenile hormone (JH) analogue in *Pyrrhocoris apterus*. These and similar specimens have been used for further investigations by scanning electron microscopy (SEM). Scale bar = 2.5 mm.

mixtures of cells on the wings, the juvenoid was applied within 24–48 h after the last larval ecdysis, at 27 °C. For induction of larval–adult epidermal patches on the abdomen, the treatment was delayed for 12–18 h (see figure 1). In *Galleria mellonella* L. (Lepidoptera), the selected juvenoid was methyl, 2,7-dimethyl-9-(2-oxolanyl)-2,4-nonadienoate, with the ID-50 effective dose being 0.005 µg per larva. Topical treatment with 10 µg of the juvenoid was applied just at the beginning of spinning, at 30 °C (see figure 2). In *Manduca sexta* (Lepidoptera), we used the common juvenoid isopropyl, 11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate (methoprene). Here we employed a local treatment using lanoline lotion of the juvenoid (10 mg of methoprene per 1 ml of lanoline) at day 5 of the last larval instar. The lotion was applied to the dorsolateral sides of the third abdominal tergite. In order to postpone local sensitivity of epidermal cells to JH, the corresponding areas of the third abdominal segment were gently cauterized by thermocautery at day 2 or 3 of the last larval instar (see figure 3).

A fixative was made using a mixture of 2.5% paraformaldehyde (EM grade) with 2.0% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). The tissue was stored in the fixative for several hours at room temperature, and then washed in 0.1 M cacodylate buffer and dehydrated through 50%, 75%, 96% and 100% ethanol. The dehydrated specimens were transferred into acetone and dried by a critical point drying method involving a POLARON dryer. The dry specimens were coated with gold in a POLARON sputter coater. Observations and photography were conducted on a JEOL 6000 scanning electron microscope.

3. RESULTS

Figures 1–3 show the macroscopic appearance of the integuments, including local patches of heterochronic cuticles on the bodies of those intermediates that were used for further SEM analysis. Figures 4–7 show differ-

ences between larval and adult cuticular patterns on the third abdominal tergite in *Pyrrhocoris apterus*. A characteristic feature of this integumental surface is that each larval epidermal cell secretes an elastic, wrinkled, cuticle with numerous folds and invaginations (figure 4) which enable identification of the boundaries between individual cells (see area 'L' in figure 4). In contrast, the adult epidermal cells of this area produce a quite different, smooth cuticle, with conical denticles (figure 5). The half-larval, half-adult intermediates ('adultoids') which are produced by JH treatment, show localized epidermal patches that have the wrinkled larval cuticle (see 'L' in figure 6) surrounded by the smooth adult cuticle (see 'A' in figure 6). When this area is magnified (see figure 7), we can see that the described heterochronic integument is composed exclusively from a mosaic of only two distinguishable types of cells: true larval cells with wrinkled larval cuticles and true adult cells with smooth adult cuticles and denticles. We did not find any ambivalent cell type in a mixture of both cuticular patterns in one cell, although we screened rather large abdominal areas in seven adultoids of *P. apterus*. We conclude, therefore, that individual epidermal cells of abdominal tergites respond to JH only in the qualitative, 'yes-or-no' manner.

The next series of figures (figures 8–11) has been related to the effects of JH on epidermal cells of the upper side of the forewing (hemicythra) in *P. apterus*. The larval wing lobes are covered by a sclerotized cuticle bearing numerous ridges and invaginations (figure 8). The territories occupied by individual larval cells are here poorly discernible (see area 'L' in figure 8). After metamorphosis, however, the slightly larger epidermal cells of an adult wing produce a very special flat cuticle containing numerous densely packed microtrichia (figure 9) which completely obscure the profile of individual adult cells (see 'A' in figure 9). With higher magnification (figure 10), we can see that the microtrichia of each cell appear as small white dots or small denticles (preventing adhesion and sticking of the wing). Figure 11 shows the cuticular structure of the aberrant, usually incompletely stretched, wing of an adultoid. Here we can see once again a mosaic of easily discernible true adult cells, with microtrichia among larval cells that possess the smooth cuticle (figure 11). Occasionally, we have been puzzled by some adult cells that contain only a few microtrichia, or by adult cells occupying a smaller cell territory than usual. This does not indicate a mixed product of some half-larval, half-adult cell, because these structures, with their less abundant microtrichia, normally occur on various parts of an adult wing.

Figures 12–16 relate to the effects of JH on the integumental ultrastructure of thoracic or abdominal tergites in the greater waxmoth, *Galleria mellonella*. Each larval epidermal cell (see 'L' in figure 12) is covered by an elastic, convex-shaped or mushroom-like cuticle, with rather thin and delicate intercellular membranes. In contrast, the pupal epidermal cells of this species produce very rigid, inverted, or concave cuticles (see 'P' in figure 13), with very thick and sclerotized intercellular membranes. However, the area occupied by the convex

Figure 2. Macroscopic distribution of the pupal (P) and larval (L) integumental cuticular structures on the dorsal side of a larval-pupal intermediate produced by juvenoid application of JH in *Galleria mellonella*. The larval-pupal intermediates of this integumental type have been used for further investigations by SEM. Scale bar = 2 mm.

Figure 3. Macroscopic distribution of the pupal (P) and larval (L) integumental cuticular structures on the third abdominal tergite of a larval-pupal intermediate produced by JH application in *Manduca sexta*. Intermediates of this and similar structures on the third abdominal segment have been used for further SEM investigations. Scale bar = 1 mm.

Figures 4–11. Figure 4. Ultrastructural architecture of the fully stretched, wrinkled cuticular structure on the third abdominal tergite of an untreated, three-day-old, fifth instar larva of *P. apterus*. The pattern reveals a common cuticular structure on larval tergites. The area indicated by white dots and labelled L shows the size and territory of one single larval epidermal cell. Scale bar = 10 μ m. Figure 5. Ultrastructural architecture of the cuticle on the third abdominal tergite (the same spot as in figure 4) after metamorphosis, i.e. in a normal male adult of *P. apterus*. Each adult epidermal cell (A) produces a smooth cuticle with one (or twinned) denticle(s), which is substantially different from the wrinkled cuticle of larval cells in figure 4. Scale bar = 5 μ m. Figure 6. Low power SEM showing mosaic-like distribution of localized patches with the wrinkled larval cuticle (L) and the smooth adult cuticle with denticles (A) on the third abdominal tergite of an adultoid produced by juvenoid treatment in *P. apterus* (see figure 1). Note the similar shape and size of bristles on larval and adult patches. The large elliptical structure is the orifice of an exocrine gland. Epidermis of this adultoid contains only the true larval (as in figure 4) and the true pupal (as in figure 5) cuticular patterns. Scale bar = 100 μ m. Figure 7. A highly magnified section from figure 6 showing the mosaic composition of the heterochronic mixture of larval cells with the true wrinkled larval cuticle (L) surrounding adult cells with the true adult cuticle with small denticles (A). (After feeding and stretching of the integument, the larval cells indicated by L acquired exactly the same size and cuticular pattern as shown in figure 4). Scale bar = 10 μ m. Figure 8. Ultrastructure of larval cuticular pattern on the upper site of the forewing lobe in an untreated fifth instar larva of *P. apterus*. White dots in the upper right corner indicate the territory of one larval cell (L). This larval cuticular pattern is

larval and the concave pupal cells is almost identical (cf. figures 12 and 13). A characteristic example of the heterochronic integumental structure which we have commonly found in several larval–pupal intermediates of *Galleria* is presented in figure 14. Here we can easily recognize the more or less randomly dispersed, convex, true larval cells (area 'L' in figure 14) among the concave true pupal cells (area 'P' in figure 14). Additional evidence that exogenous application of JH produces only a mosaic mixture of true larval and true pupal cells is provided by figures 15 and 16.

Due to easy identification of the cuticles produced by larval and pupal epidermal cells of *Galleria* (figures 15 and 16), it has been possible to survey several thousand epidermal cells containing heterochronic integumental patches of the larval–pupal intermediates. We have not encountered aberrant cells that could produce a mixture of partly convex, partly concave, or partly elastic, partly rigid cuticular products. This shows that an individual larval epidermal cell has been genetically programmed to develop according to two distinctive developmental pathways. The first pathway drives a cell towards repetition and multiplication of the extant ontogenetic stage (+JH), the second directs a cell towards differentiation into the future ontogenetic stage (–JH). The concept of two genetically programmed developmental pathways in each immature insect cell, with no intermediate and JH functioning as a switch, may conform well with the 'all-or-none' distribution of cells exhibiting the true larval and true pupal cuticles that are shown in figures 15 and 16.

Ultrastructural features found in the epidermis of larval–pupal intermediates of *Galleria* (figures 14–16) reveal some further endocrinological implications: (a) an individual epidermal cell constitutes the lowest elementary unit in the perception and realization of hormonal signals given by JH to the target tissue; (b) an individual epidermal cell selects one of the two intrinsic developmental programmes autonomously, without respect for 'decisions' made by the surrounding sister cells; (c) accordingly, the neighbouring epidermal cells can independently secrete quite dissimilar cuticles and perform fundamentally different biochemical or physiological functions without disturbing the integrity of the tissue or organ.

Finally, figures 17 and 18 show the effect of JH on epidermal cells in larvae of the tobacco hornworm, *Manduca sexta*. We have found that the ultrastructural composition of larval–pupal epidermal patches induced by JH in this species is basically similar to that found in *G. mellonella*. This may be documented by a mosaic distribution of the true pupal cells with the smooth pupal cuticle (area 'P' in figure 17) among the

true larval cells, with dense cuticular evaginations and incompletely visible cell boundaries (area 'L' in figure 17). These boundaries are better delimited, however, in figure 18 (area 'P'), which shows, conversely, a few larval cells on the pupal background. Also, in this species we have never encountered the presence of ambivalent structures that could indicate gradual responses of single cells to JH.

4. DISCUSSION

The JH of insects is the most investigated and perhaps also the most important invertebrate hormone. Elucidation of its mode of action may be crucial for the understanding of animal organogenesis and cell differentiation in general. In this work we have determined that the developmental responses of single epidermal cells to JH are qualitative, 'yes-or-no', not quantitative or gradual as it is generally assumed. These findings support the original views of Wigglesworth (1940), and they are also consistent with our previous results obtained by artificial induction of heterochronic deviations (Sláma 1975).

The developmental 'yes-or-no' responses of epidermal cells to JH (see figures 4, 8, 9, 14–18) with a virtual absence of intermediate cells suggest that a single immature insect epidermal cell has only two developmental options: (i) execution of the stationary, larval–larval or status quo developmental cycle in the presence of a minimum physiologically effective concentration of JH; or (ii) ignorance of the JH signal by cells that have already started to follow the next morphogenetic instructions, and thus passed over the JH-sensitive period. We have also firmly established that individual epidermal target cells of JH make their own, absolutely independent selection between the two developmental alternatives (see figures 14–16 for illustration).

The qualitative 'on' and 'off' function of JH, with respect to inhibition of morphogenesis, has been personified and compared with that of an electrical switch (Sláma 1985). In this comparison, the nature of the developmental consequences of turning the cellular JH-switch 'on' or 'off' did not depend on the switch itself (i.e. large-to-intermediate-to-small concentrations of JH), but primarily on the kind of device that had been plugged into the circuitry (i.e. morphogenetic instructions coded on the genome). Presenting JH functions in the form of a simple hormonal switch was very helpful in comprehending why there existed more than 4000 synthetic molecules that can mimic the action of JH on insect epidermal cells (Sláma 1985). However, the above-described story of JH is still not complete. There

Figures 4–11 (*continued*) essential for comparisons with the respective adult pattern in figures 9 and 10. Scale bar = 10 µm. Figure 9. Ultrastructure of the cuticle on the upper site of the fully stretched wing of an untreated adult of *P. apterus*. Epidermal cells of the wing (A) secrete a cuticle that is covered in numerous microtrichia, which appear as small white dots. The boundaries between individual cells cannot be discerned easily (see the two circles in the lower right-hand corner), although adult cells with microtrichia can be distinguished easily from larval cells with smooth cuticle (compare figures 8 and 9). Scale bar = 10 µm. Figure 10. Magnified detail from figure 9, showing the microtrichia of adult wing cells in more detail. Scale bar = 2 µm. Figure 11. Ultrastructure of the incompletely stretched, aberrant wing of an adultoid produced by juvenoid treatment in *P. apterus*. The larger true adult wing cells with microtrichia are scattered among the juvenile, smaller larval wing-lobe cells (L) producing only a smooth cuticle without microtrichia. Scale bar = 5 µm.

Figures 12–17. Figure 12. Characteristic ultrastructural architecture of the convex-shaped, mushroom-like, cuticles produced by individual larval epidermal cells (L) on abdominal tergites of a normal, untreated last instar larva of *G. mellonella*. The larval cells (L) are joined together by very thin intercellular membranes. Scale bar = 5 μ m. Figure 13. Surface architecture of the cuticles produced by individual pupal epidermal cells (P) on abdominal tergites of a normal, untreated pupa of *G. mellonella*. Each of the pupal epidermal cells (P) produces inverted, concave, cuticles with very thick intercellular junctions, in large contrast to the convex, mushroom-like cuticles of the larval cells (see figure 12). Scale bar = 5 μ m. Figure 14. The ultrastructure of an integument on the mesothoracic tergite of a larval pupal intermediate in *G. mellonella* (see figure 2). The cuticles of this heterochronic specimen show a mosaic distribution of true larval cells (L) with the convex mushroom-like cuticles (see the corresponding pattern in figure 12), intercalated individually or in groups among true pupal cells (P) with the inverted concave pupal cuticle (see the corresponding pattern in figure 13). Scale bar = 20 μ m. Figure 15. Enlarged detail from a similar SEM preparation to that shown in figure 14, showing that individual larval cells with the mushroom-like cuticles (L) have been sharply separated from the heterogenic, quite dissimilar, pupal cells with the inverted cuticles (P); this micrograph provides evidence that epidermal cells that entered the larval option (+JH), as well as the cells with the pupal option (–JH), can independently develop side-by-side, producing in the end profoundly dissimilar cuticles. Scale bar = 5 μ m. Figure 16. A similar preparation to that illustrated in figure 15 which documents, conversely, that an isolated island of pupal cells (P) can develop via the pupal metamorphosis programme and fully retain its pupal identity even in the ocean of larval cells (L). There are no ambivalent intermediate cells in between. Scale bar = 5 μ m. Figure 17. Ultrastructure of the integumental surface on the third abdominal tergite of a larval pupal intermediate produced by exogenous application of JH activity in *M. sexta* (see figure 3 for macroscopic appearance). The image shows mosaic displacement of true pupal cells producing smooth pupal cuticle (P) among the true larval cells (L) (with dense cuticular evaginations and poorly discernible cell boundaries). Scale bar = 20 μ m.

Figure 18. Ultrastructure of the integumental surface from a similar preparation to that shown in figure 17, which shows a few islands of true larval cells (L) among true pupal cells (P) with a smooth cuticle and poorly visible cell boundaries. There are no intermediate cuticular structures between the sharply delimited, extreme, types. Scale bar = 10 μ m.

remains a biologically important, though very often neglected, factor, namely the critical timing of the developmental events. We have already indicated that individual cells become virtually insensitive to large dosages of JH after traversing the short sensitive period (see Sláma *et al.* (1974) for more information). By analogy, we can exemplify the role of developmental timing in JH action by saying that an electrical switch cannot function without electricity.

The virtual absence of gradual responses of single cells to JH, which we have observed in all the investigated species, indicates that a cell cannot proceed simultaneously through the qualitatively different +JH (somatic growth) and -JH (metamorphosis) pathways. Such a strict choice seems to be well secured by a feedback mechanism associated with termination of the JH-sensitive period. Some earlier authors believed that a single, intermediate or 'composite' cell could develop when metamorphosis had been blocked by JH at a point between the two developmental stages (Sehnal 1981, 1984). Apparently, this view has been derived from the common mixture of heterochronic characters that are spread over the whole bodies of intermediates (see figures 1–3). We have already explained, however, that according to our experimental data a single immature insect epidermal cell has only two qualitatively different developmental options at the given ontogenetic stage, i.e. +JH and -JH. It can never be stopped at some imaginary intermediary stage or be directed back towards the outlived structures because the +JH and -JH pathways have a common origin, but different destinations, and no common intermediate point. We are conscious that the above interpretations of JH action may conflict with the current, concentration-dependent, high-intermediate-low JH theory of Piepho (Piepho 1951; Gilbert & Schneiderman 1961; Sehnal 1984; Riddiford 1972, 1985), which has been used by insect endocrinologists for almost half a century (cf. Gilbert & Schneiderman 1961; Willis 1969; Williams & Kafatos 1971; Riddiford 1972). Some authors refrained from using the theory after some time

(Wigglesworth 1970; Riddiford 1985, 1993, 1996), mainly because of the critiques directed against the attributed 'reversal of metamorphosis' (Bodenstein 1953; Pflugfelder 1958; Hinton 1963; Novák 1975, 1991). Arguing against the theory was always extremely difficult. The experiments of Piepho have never successfully been repeated or confirmed, and the actual values for what would be the high, intermediate, or low concentrations of JH have never been exactly determined. On the other hand, there exists some experimental evidence, obtained by means of artificially induced heterochronic developmental deviations, which has seriously invalidated the quantitative JH theory (Sláma 1975, 1985, 1995).

The basic idioms of the high-intermediate-low JH theory have been applied to individual epidermal cells by several authors (Lawrence 1966, 1969; Willis *et al.* 1982; Sehnal 1981, 1984). They concluded that, just as in the whole body, a single epidermal cell could also produce a gradual mixture of larval, pupal, or adult structural patterns, depending on the concentration of JH circulating in the haemolymph. Willis *et al.* (1982) described a common presence of these ambivalent 'composite' cells in several insect species. We have a feeling, however, that there might be some misunderstandings related to the use of non-specific stage characters, like the hairs or bristles of Hemiptera, or the black melanin pigment of Lepidoptera (Lawrence 1969; Willis 1969; Willis *et al.* 1982; Sehnal 1981, 1984). We have found that larval and adult bristles of *Pyrrhocoris* are quite similar (see bristles in figures 4, 6, 8, 9 and 11). Moreover, the black melanin pigments are deposited into the overlying cuticle, outside the epidermal cells. These cuticular pigments can diffuse over the territory of a single cell giving, under the light microscope, a false impression of a half-pigmented cell.

The most intriguing features of our SEM study are fundamental differences between the cuticles produced by the larval and the pupal cells in *Galleria* (cf. figures 12–16). This is perhaps the reason why Willis *et al.* (1982) were unable to find any intermediate larval-pupal cells in this species, although Piepho's JH theory was, quite ironically, founded just on studies of the epidermal cells of *Galleria* (Piepho 1951), and it has been always propagated by authors working with this species. According to Sehnal (1981, 1984), for example, high amounts of JH caused reversal of metamorphosis in epidermal cells back to a previous stage, or the cells which divided in the presence of JH could dictate their programme to the neighbouring cells. It was further hypothesized that gradual responses of individual cells, the so-called composite cells, could develop when metamorphosis was blocked by JH at a point between the two developmental stages (Sehnal 1981, 1984). Our results do not support this interpretation of JH action, which is based on the unrealistic assumption that larval-pupal transformation of a single cell is a continuous process that can be arrested at different stages by high-to-intermediate-to-low concentrations of JH.

Finally, we need to come back to the most essential point in JH action, which is represented by the critical developmental moment at which a single immature

epidermal cell becomes suddenly reversibly insensitive to JH (cf. Sláma 1985, 1995). From this point of view, the actual decision (i.e. the 'yes' or 'no' response) of a cell to JH would depend, primarily, on whether the cell in question occurs before or after the critical moment of its sensitivity to JH. Practical assays performed earlier with hundreds of synthetic JH analogues (Sláma *et al.* 1974) revealed clearly that, after traversing the JH-sensitive period, the epidermal cells of a number of insect species refrained from responding to JH activity, even if JH was applied in concentrations 500 million-fold in excess of the usual effective dose. It is a pity that the quantitative high-intermediate-low JH theory seriously underestimated the factor of alternating tissue sensitivity and insensitivity to JH. According to our results, the critical switchover between the sensitivity and insensitivity of epidermal cells to JH takes place at the very beginning of developmental stimulation by the complex of hormones, long before endogenous peaks in ecdysteroid concentration. In conclusion, we are convinced that the described switchover in cell responses to JH might somehow be associated with somatic recombinations in the distribution of developmental instructions on the genome during mitosis or endomitosis of the somatic cells. This post-zygotic, qualitative, 'yes-or-no' distribution of hormonal developmental messages provides a strong analogy to the Mendelian distribution of genetic information during meiosis of the gonial cells.

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