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Figure 1. Participants in the XV Ecdysone Workshop

Abstracts are listed in alphabetical order by the last name of the senior author.

Ftz-f1 is required for gonadal guidance and germline differentiation in *Caenorhabditis elegans*

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FTZ-F1 is one of the ancient nuclear receptors and its ortholog is termed NHR-25 in *Caenorhabditis elegans*. The important roles of this molecule were identified genetically in mice (differentiation of primary steroidogenic glands and synthesis of steroid hormones) and in *Drosophila melanogaster* (embryonic segmentation, larval emolting, and metamorphosis). In insects FTZ-F1 acts in the ecdysone cascade. *ftz-f1/nhr-25* mRNA expression was detected by *in situ* hybridization throughout embryonic development. Zygotic expression was seen particularly in the gut and epidermal precursor cells. Epidermal expression persisted until L2 stage, when the developing gonadal cells took over the expression. Adult gonads were loaded with the *nhr-25* transcript (Asahina *et al*. 2000. Genes to Cells 5:711-723). Activities of transgenic *nhr-25::gfp* constructs mirrored the embryonic and the larval epidermal mRNA pattern. We isolated a *nhr-25* deletion mutant (*jm2389*) which is recessive embryonic lethal at the 1.5-fold stage (at the time of cuticle synthesis and elongation of the embryo), consistent with the expression pattern mentioned above. To address a possible FTZ-F1/NHR-25 role in postembryonic stages, we performed RNA interference by injection. RNAi confirmed the embryonic lethality and additionally showed molting defects at L1-L2 (Asahina *et al*. 2000. Genes to Cells 5:711-723; Gissendanner and Sluder, 2000. Developmental Biology 221 :259-272) and gonadal defects from L3 to adult stage. The gonad was typically tumorous, misshapen and undifferentiated. These worms were vulvaless and sterile as adults. A polyclonal antibody was raised against NHR-25. The carboxyl-terminal end of the hinge region and part of the ligand binding domain were used as an antigen to avoid cross-reaction with other nuclear receptors. The antibody recognized a single band on immunoblots. Immunocytochemistry was performed on adult gonads. Strikingly, NHR-25 was highly expressed in the nuclei of somatic gonadal cells, namely the distal tip cells (DTC) and the sheath cells. DTC and sheath cells are known to be important for the gonadal guidance and the differentiation of the germline. The molting defects observed in RNAi treated worms suggested that NHR-25 and its insect orthologs FTZ-F1 share an evolutionarily conserved role. Further, somatic gonadal expression of NHR-25 perfectly correlates with the RNAi phenotype, strongly suggesting that NHR-25 is involved as a transcription factor in the signaling pathway directing the gonadal development.

Molecular mechanisms of ecdysone action in *Drosophila melanogaster*

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Our laboratory is using *Drosophila melanogaster* as a model system for understanding the molecular mechanisms of steroid hormone action during development. Pulses of the steroid hormone ecdysone direct the major developmental transitions during the *D. melanogaster* life cycle. The best characterized of these pulses occurs at the end of the third larval instar, triggering puparium formation and signaling the onset of prepupal development. The prepupal stage lasts ~12 hours and is terminated by another ecdysone pulse that marks the prepupal-pupal transition. During metamorphosis, most larval tissues are destroyed by programmed cell death and replaced by adult tissues and structures that develop from small clusters of progenitor cells. The net effect of these changes is the transformation of a crawling larva into a highly mobile and reproductively active adult fly. Like vertebrate hormones, ecdysone exerts its effects through a heterodimer of two members of the nuclear receptor superfamily, EcR and USP. This hormone-receptor complex directly induces the expression of primary-response genes including early genes that encode transcription factors. Three early genes have been characterized in detail: the *Broad-Complex* (*BR-C*), *E74*, and *E75*. The *BR-C* encodes a family of zinc finger transcription factors. *E74* encodes two isoforms of an ETS domain transcription factor, designated E74A and E74B, and *E75* encodes orphan members of the nuclear receptor superfamily. The early transcription factors directly regulate large sets of secondary-response late genes, defining ecdysone-triggered regulatory hierarchies. These cascades of gene expression direct the appropriate biological responses to each pulse of ecdysone during development. Our current studies are focused on defining the regulatory cascades that control the two major biological responses to ecdysone during metamorphosis – the growth of adult tissues and the massive programmed cell death of larval tissues. We have defined roles for *BR-C* and *E74* in both of these pathways and are identifying other key regulators through genetic screens. Our lab is also studying the roles of the EcR/USP heterodimer and orphan nuclear receptors in transducing ecdysone pulses during development. These studies utilize a number of approaches including loss-of-function genetics as well as antibody stains of the giant larval salivary gland polytene chromosomes in order to identify potential regulatory targets. We use transgenic animals that carry the GAL4 DNA binding domain fused to the ligand binding domains of nuclear receptors as a means of determining when and where hormones or critical co-factors are present in the animal. Taken together, these studies are clarifying our understanding of nuclear receptor function in insect development as well as providing insights into how their vertebrate homologs might function.

Rhythm of ecdysteroids level in the moths testes and its effect on sperm release in the cotton leafworm, *Spodoptera littoralis*

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In the cotton leafworm, as in other moths, sperm cell development begins in the preadult stage so that the testes of late pupae contain mature eupyrene (nucleated) and apyrene (anucleated) spermatozoa. The first sperm release takes place a few days before eclosion and is preceded by the maturation of the biological clock, which controls many events including sperm transfer through multicellular layer that separates the lumina of the testicular follicles from the lumen of the upper vas deferens (UVD). Earlier studies showed that in adult males the exterior sheaths surrounding testicular compartments are the main source of 20-hydroxyecdysone (20-HE), a hormone crucial to insect metabolism, development and reproduction. We examined the daily pattern of 20-HE release from testes of pupae and adult cotton leafworms, *Spodoptera littoralis*. Since sperm transfer, glycoprotein production by internal epithelium of the UVD and V-ATPase activity in this tissue are circadially regulated by autonomous clock (functionally independent from the brain), we investigate the variability of 20-HE in the testes and hemolymph along with its effect on sperm release. Furthermore we asked the question whether the biological clock could utilise a hormonal intermediary to regulate this rhythm. A distinct temporal pattern of 20-HE attendance was revealed in testicular tissues of the adult males and a robust rhythm of its release into the culture medium during the *in vitro* study. This rhythm persisted under light:dark (LD) conditions as well as constant darkness (DD) for two days but was almost completely abolished by continuous light (LL). Moreover, based on our preliminary results that showed a crucial time of pupal development when the initiation of clock function occurs seen as a first sperm release, we described a daily pattern in 20-HE variance that implies its potential role in this process. It was observed that the decline in hemolymph 20-HE levels seems essential for the initiation of the rhythmic release of sperm from the testes. Finally, we described that the infusion of 20-HE into pupae inhibits the process of sperm release in a dose-dependent and age-dependent fashion. This study was supported in part by State Committee for Scientific Research (KBN) grant No. 6PO4CO1916.

Identification of Ca^{2+} influx stimulating signalling pathways in the ecdysteroid secreting prothoracic glands of the silkworm, *Bombyx mori*

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Measurements of Ca^{2+} influx in Fura-2/AM loaded prothoracic glands (PGs) of the silkworm, *Bombyx mori*, have identified distinct Ca^{2+} mobilizing pathways that participate in the ecdysteroid secretory activity of these glands. The first signalling pathway, which can be triggered by recombinant PTTH (prothoracicotropic hormone), mediates Ca^{2+}-influx through amiloride-sensitive and dihydropyridine-insensitive plasma membrane channels and includes

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release of Ca^{2+} from IP_3-dependent intracellular Ca^{2+} stores. Activation of this pathway results in increased level of cAMP and stimulation of ecdysteroid secretion. The second pathway is initiated by increase of cAMP and mediates Ca^{2+} influx, perhaps via a cAMP–dependent protein kinase A, through dihydripyridine–sensitive plasma membrane channels. This mechanism can be mimicked by the agonist of L–channels, Bay K 8644, and is inhibited by the prothoracicostatic peptide, Mas-MIP/ Bom-PTSP that also inhibits ecdysteroid secretion. This second pathway seems to be part of a positive feed-back, triggered by the initial increase of cAMP through the PTTH-dependent Ca^{2+} influx, which maintains elevated intracellular Ca^{2+} levels necessary for increased ecdysteroid secretion. The antagonistic hormone, Mas-MIP/ Bom-PTSP, can interfere in this feedback by blocking the dihydropyridine–sensitive channels and thus inhibiting ecdysteroid secretion.

Isolation of genes involved in ecdysteroids biosynthesis from *Achyranthes japonica*

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In order to isolate genes involved in ecdysteroids biosynthesis in plants, total RNA was isolated from *Achyranthes japonica* (Nakai) and RT-PCR was performed using degenerate primers designed based on the results of multi-alignment of four cytochrome P450 genes from plants and a putative ecdysone 20-hydroxylase gene from an insect. Fourteen partial cDNA clones showing unique base sequences were obtained, out of which six showed homologies at the levels of nucleotide and amino acid sequences to the other cytochrome P450 genes known to be involved in the ecdysteroid biosynthesis. Of the six clones, four showed relatively high homologies to a putative ecdysone 20-hydroxyase gene isolated from an insect.

The RNA expression patterns of EcR and USP during the process of gene amplification and DNA puffs activity in the salivary gland of *Bradysia hygida* (Diptera, Sciaridae)

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In *Bradysia hygida*, in two of the three morphologically distinct salivary gland regions (S1 and S3) about eight chromosomal sites undergo gene amplification at the end of the 4th larval instar. This process is triggered by 20-OH ecdysone (20-HE) and results in the formation of DNA puffs, that expand in two different groups. We have shown, in vitro and in vivo, that the activity of the first group of amplified genes demands high titer of 20-HE, while the hormone presence is inhibitory for the activity of the second group of amplified genes. We are interested into understand the role of 20-HE in the control of the gene amplification process and in the control of amplified genes activity. Some time ago, by RT-PCR, a 0.6 kb DNA fragment was isolated containing part of the DBD and LBD domains of *BhEcR (BhEcR 0.6)*. S1, S2 and S3 regions from larvae, at different ages during the last 3 days of 4th instar, were separated, total RNA was isolated and analyzed by Northern blots, using the 0.6 kb fragment as a probe. Two main transcripts with homology to *BhEcR* 0.6 were detected, 5.8 kb and 0.9 kb in size. The patterns of the 5.8 kb RNA expression were very similar in the three regions, with a peak at age E3 (when the process of gene amplification starts), 18 h later. At age E5 (when the first group of DNA puffs begins to expand) there was a decrease in the amount of this RNA. From E7 (about 2h after E5) to E7+12h there was very little 5.8 kb RNA. At E7+16h the RNA attained a new peak and decreased towards the pupal molt. In S2 this second peak was attained later, at E7+22h. Curiously, the small transcript starts to be detected at E7 and shows high expression levels only in the S1 and S3 regions when the first group of DNA puffs begins its regression. The maximum amount of this RNA was present at E7+12h, time for the second group of DNA puffs start to expand. At E7+16 h it was almost undetectable. We have also found two main transcripts, 1.7 kb and 1.3 kb, with homology to a *Dmusp* probe. The 1.7 kb transcript seems to be expressed very weakly and only in S3, not accompanying the expression pattern of *EcR*. On the other hand, the 1.3 kb RNA is highly expressed, but only in the S2 region. At the beginning of the fourth instar it is already detected, its maximum amount is attained at E3 (when the gene amplification process is starting in the other two regions), at E7 its amount decreased to the level of E1, at E7+14h it was a very faint band. From this information plus other results previously obtained in *B. hygida*, a tentative model is proposed to explain the role of 20-HE and its receptor in the control of gene amplification and in the control of the activity of DNA puffs. We thank FAPESP, CAPES, FAEPA for financial support.

Tissue-specific properties of the ecdysone receptor

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When a dominant negative mutant EcR (EcR-DN) is expressed in targeted tissues of *Drosophila melanogaster*, individual tissues become defective in the ecdysone response, while the rest of the animal is unaffected. We have used this technique to inactivate ecdysone receptor function at the onset of metamorphosis in a variety of tissues, including eye discs, larval epidermis, fat body, salivary glands, and the border cells of the egg chamber. In each case a characteristic developmental defect ensues. Targeted expression of EcR-DN was combined with targeted over-expression of putative components of the ecdysone response, or with mutation in the genes encoding such proteins. In this way, we could assess the function of these proteins in the ecdysone response of each targeted tissue. We will present data describing tissue-specific isoform requirements and co-activator functions. We will also describe a molting checkpoint, which was discovered as a by-product of these studies.

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Disruption of development in any epidermal region, whether by EcR-DN or by other means, usually prevents further development in the entire animal. This global inhibition of development appears to result from an inhibition of ecdysone secretion, reminiscent of the injury effect described many years ago by Wigglesworth, Williams, and others.

A new chromatin-associated, set domain protein, trithorax-related (TRR), is a putative coregulator of EcR and βFTZ-F1 nuclear hormone receptors

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The *Drosophila melanogaster* trithorax (trxG) and polycomb (PcG) group genes encode chromatin-associated proteins that maintain the proper expression of homeotic genes, among others, during development. The Trithorax-related (TRR) gene, cloned by our group, shares high sequence similarity to Trithorax (TRX). TRR encodes an essential, 260 kDa protein, which contains several protein motifs which are highly conserved among trxG and PcG proteins, including a SET domain known for its histone methyltransferase activity. Similar to other trxG and PcG proteins, TRR is also a component of several large chromatin-associated protein complexes. Our data suggest that despite these similarities, TRR is not involved in regulation of homeotic genes, and that its binding sites do not overlap with those of other trxG proteins on polytene chromosomes. It is therefore likely that TRR regulates a set of target genes different from that of the classic trxG proteins. TRR contains three LxxLL nuclear receptor interacting motifs, a signature motif of nuclear hormone receptor coactivators. We demonstrated that TRR exhibits an almost complete colocalization on polytene chromosomes with ecdysone receptor (EcR), in particular its β1 isoform, and very extensive colocalization with the orphan receptor βFTZ-F1. EcR and βFTZ-F1 also co-immunoprecipitate with TRR in embryonic nuclear extracts. Further, after several chromatographic steps, βFTZ-F1 co-migrates with TRR during size-fractionation as a large (≥2Mda) complex. This suggests the novel possibility that nuclear hormone receptors can be stably-associated components of large chromatin/coregulator complexes during development. TRR is necessary for eye development. In particular, TRR is necessary for maintaining proper *hedgehog* transcript levels which is crucial for advancing the morphogenetic furrow in the developing eye. Interestingly, temperature sensitive *ecdysone* mutants show similar defects in furrow progression and reduction in *hedgehog* transcription during eye development. TRR is also required for wing, leg and bristle development, while mutant clones exhibit various ectopic tissue growth. Many of these developmental processes also involve ecdysone- as well as βFTZ-F1-induced genes. The biochemical and genetic data, thus, provide strong evidence that 1) TRR may modulate some of the same ecdysone, and βFTZ-F1, induced target gene expressions through its putative role as a nuclear hormone receptor coregulator; 2) nuclear hormone receptors may potentially form stable associations with TRR-containing coregulator complexes in addition to forming transient receptor-coregulator interactions; 3) TRR may further represent a novel class of nuclear hormone receptor coregulators involved in maintenance of gene expression. Although the exact mechanisms for TRR maintenance of gene expression remain unclear, it is likely that it shares with trxG and PcG proteins similar biochemical modes of gene regulation based on their structural homology.

Characterisation of two FTZ-F1 nuclear receptors in the German cockroach, *Blattella germanica*

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Transcriptional competence is a crucial mechanism for refining ubiquitous hormonal signals into different tissue- and stage-responses. However, the molecular basis of transcriptional competence remains poorly understood, especially in heterometabolous insects such as our model insect, the German cockroach *Blattella germanica* (L.) (Dictyoptera, Blattellidae). In *Drosophila melanogaster*, the nuclear receptor βFTZ-F1 has been shown to be responsible for stage-specific response to ecdysteroids during metamorphosis. In the present work we report the cloning of two cDNAs of FTZ-F1 of *B. germanica*. We also describe the detailed analysis of the developmental expression of both BgFTZ-F1nuclear receptors during the pre-adult (last larval instar) as well as in different tissues of the adult female of *B. germanica* during the first reproductive cycle. Our results suggest that both BgFTZ-F1 may be involved in regulating competence in different tissues in this heterometabolous species.

The steroidogenic function of *Drosophila* ring gland: facts and hypotheses

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Post-embryonic development of insects is regulated by molting hormones, the ecdysteroids. In *Drosophila melanogster* larvae, ecdysteroids are produced by the large lateral cells of the so-called ring gland, a composite organ which includes the equivalent of both the prothoracic gland, the corpora allata and the corpora cardiaca evidenced in other insects. The steroidogenic cells of the ring gland present a complex function: (1) “ecdysone” synthesis results from a multi-step and still uncompletely elucidated metabolic pathway. For proper ecdysteroid production, the coordinated expression of many different genes is needed, involving steroidogenic enzymes as well as proteins necessary for cholesterol trafficking in the cell; (2) the steroidogenic capacities fluctuate over a wide range during post-embryonic development. These variations imply that a multifactorial and tight regulation of hormone production takes place, which involves both transcriptional and post-transcriptional levels. The

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recent biochemical, molecular and genetical studies, which lighten ecdysteroid biosynthetic pathway as well as control mechanisms of ring gland activity will be reviewed in order to present our current understanding of the steroidogenic function of *D. melanogaster* ring gland.

Use of double-stranded RNA to analyze gene function in a *Bombyx mori*-derived cell line

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Double-stranded RNA (dsRNA) has recently been found to specifically interfere with the expression of the homologous mRNA in *Caenorhabditis elegans* and *Drosophila melanogaster* by a phenomenon that is known as RNA interference (RNAi). To test the feasibility of dsRNA to mediate RNAi in Lepidopteran species, we introduced dsRNA to a *Bombyx mori*-derived tissue culture cell line (Bm5 cells) and monitored its effects on the activity of several nuclear receptors implicated in the ecdysone response as well as the transcription factor BmGATAβ. DsRNA was prepared from cloned sequences encoding nuclear receptors such as the ecdysone receptor (BmEcR), the *B. mori* homologue of the Ultraspiracle protein USP (BmCF1), the ecdysone-regulated orphan receptors BmHR3A and BmE75C as well as the transcription factor BmGATAβ and marker proteins such as green fluorescent protein (GFP) or chloramphenicol acetyl transferase (CAT). Co-transfection of Bm5 cells with each of the respective dsRNA(s) and appropriate expression plasmids resulted in strong, specific and dose-dependent inhibition of mRNA and protein expression as well as transactivation function, when judged by Northern blot analysis, Western blot analysis and the ability of each factor to regulate the transcription of appropriate target constructs. For example, dsRNA for BmEcR dose-dependently inhibited activation of an ecdysone responsive reporter cassette by the ecdysone receptor complex and dsRNA for BmE75C could reverse the inhibition of BmHR3A following overexpression of BmE75. Our dsRNA experiments also indicate a possible differential role for BmCF1 in the primary ecdysone response, mediated by 20-hydroxyecdysone versus tebufenozide. Our results show that RNAi occurs in Lepidopteran cells and that it can be used as a valuable tool in clarifying signal transduction pathways such as ecdysone-regulated gene expression. Finally, we are also exploring the potential of dsRNA to interfere with specific gene expression in the growing ovarioles of *Bombyx* in organ culture using a transgenic silkworm race that constitutively expresses GFP.

Ecdysteroid receptor (EcR/USP) agonist pharmacophore identification through ecdysteroid/diacylhydrazine superimposition and multidimensional QSAR

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Ecdysteroid receptors, in common with other classes of nuclear receptors, interact with ligands of diverse chemical classes as agonists or antagonists. Elucidation of these interactions will provide useful information for i) understanding ecdysteroid-regulated gene expression in arthropods, ii) the development of further target-specific pest control agents, iii) improvement of gene-switching mechanisms based on the ecdysteroid system and iv) provide baseline data for the assessment of potential endocrine disruptors in invertebrate systems. We are characterising ligand interaction with the *Drosophila melanogaster* ecdysteroid receptor complex by quantitative structure-activity relationship (QSAR) studies and molecular modelling (Comparative Molecular Field Analysis [CoMFA] and 4D-QSAR). Training sets of over 70 ecdysteroids and over 100 diacylhydrazines were examined in the ecdysteroid-responsive *D. melanogaster* B_{II} cell line as a measure of DmEcR affinity (EC_{50} values) to generate independent models for each chemical class and also models for the combined data. The quality of the models was assessed with appropriate test sets consisting of analogues for which the biological potencies had not been previously determined. The class-specific models generate specific, and testable, predictions concerning how the two agonist classes interact with the ligand-binding domain of the receptor. For example, 4D-QSAR modeling of the ecdysteroid data predict that C-2 and C-22 hydroxyls are H-bond acceptors and that there is a sterically favored/hydrophobic region extending out from C-11/C-12. The unified data from both chemotypes were used to construct multidimensional QSAR models using the CoMFA and 4D-QSAR methods applied to a number of steroid/diacylhydrazine superimpositions. Relative model robustness allows inferences pertaining to ligand overlap and a consensus pharmacophore. These conclusions are examined with respect to a DmEcR homology model.

Structure-activity studies on natural and non-natural ligands for ecdysteroid receptors

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The *Drosophila melanogaster* B_{II} bioassay provides a convenient assay for the relative potencies of ecdysteroid agonists and antagonists. Over recent years, we have used the assay to identify plant natural products acting as agonists (maocrystal E) and antagonists (cucurbitacins, withanolides, limonoids, stilbenoids, phenylalkanoids). Additionally, the assay has proved very useful for the preliminary screening of environmental chemicals for the identification of potential endocrine disrupting chemicals active on arthropods. It is apparent from all these studies that a wide range of ligands with very diverse chemistries can interact with the ligand-binding domain of the ecdysteroid receptor complex. In this presentation, we shall review the structure-activity and molecular

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modelling studies performed on these various classes of compounds and consider their implications for ligand-receptor interactions. Major studies have concerned ecdysteroids (ca. 125 analogues), bisacylhydrazines (ca. 160 analogues) and cucurbitacins (ca. 30 analogues). This approach is being broadened to encompass intracellular ecdysteroid receptors from other species and taste receptors of phytophagous insects. This research has implications for i) understanding of ecdysteroid-regulated gene expression, ii) the design of novel, receptor-active molecules, iii) protection of crop plants by modification of phytoecdysteroid profiles and iv) identification of endocrine disrupting chemicals detrimental to arthropods.This research was supported by: BBSRC, EU-INTAS, Rohm & Haas Co. and AstraZeneca Ltd.

Juvenile hormone regulates expression of the E75a nuclear hormone receptor both in S2 cultured cells and during oogenesis in *Drosophila melanogaster*

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By employing the differential display technique, previously we identified genes that show elevated transcript accumulation in *Drosophila melanogster* cultured cells treated with a synthetic juvenile hormone (JH) analog, methoprene. Because our initial screen focused exclusively on RNA species whose abundance increased continuously during the period of hormone treatment, we would have overlooked RNA species that were only transiently abundant. So we went back to the original DD-gels searching for RNAs that became abundant only transiently during the first two hours of methoprene treatment. Several candidates were identified in a preliminary screen, but there was one of particular interest. This RNA was transiently expressed during the first 1-3 hours of methoprene treatment, but its abundance continued to increase over a 6-hour period when cycloheximide was present in methoprene containing medium. The sequence analysis identified this transcript as the *E75A* RNA encoded by the *E75* early gene involved in the ecdysone regulatory cascade. Because *E75* is known exclusively as an ecdysone inducible gene, we characterized in details its response to JH. A time course analysis of *E75A* transcript accumulation confirmed the transient nature of its JH-dependent synthesis. Elevated transcript levels were evident at hormone concentrations as low as 1 nM, and peak induction was seen at 10-50 nM, well within the physiological range of JH levels in larvae and adults. Induction was specific to JHIII and methoprene, and failed to occur when other chemically related compounds were used. We also compared the response of S2 cells to ecdysone and methoprene. In the presence of methoprene we observed the accumulation of JhI transcripts described previously and *E75A* RNA, but not *E75B* or *E75C* transcripts. In the presence of ecdysone there was no evidence of *JhI* gene expression, while the abundance of both *E75A* and *E75B* RNAs rose rapidly to very high levels. We re-examined *E75A* expression during development. As expected, high levels of *E75A* RNA were found during metamorphosis. In embryos, *E75A* mRNA appeared in two peaks. One was very brief and occurred at the beginning of embryogenesis (6-8 h after egg laying) and the other was broader, occurring from 16 to 22 h. The latter peak extended into larval development, when *E75A* showed a steady level of transcript accumulation in the first and second instars. Following female eclosion, *E75A* transcripts are synthesized in ovaries. With *in situ* hybridization, we found a very dynamic pattern of *E75A* expression during oogenesis. *E75A* transcripts were first found in posterior follicle cells during stages 6-9. At late stage 9-10, there was a switch, after which *E75A* transcripts began to accumulate in nurse cells, where they persisted until late stage 12. We did not detect any hybridization in the nascent or mature oocyte. Ectopic accumulation of *E75A* transcripts in ovaries can be induced by topical methoprene application. In ap^4 mutant adults defective in JH secretion, *E75A* RNA levels are severely reduced, but normal abundance is rescued to a high degree by topical methoprene treatment. The JH-dependent expression of *E75A* raises a possibility that E75A protein is a JH receptor or a transcriptional co-activator required for the expression of JH response genes.

The expression of the *LET-7* small regulatory RNA is controlled by ecdysone during metamorphosis in *Drosophila melanogaster*

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In *Caenorhabditis elegans*, the heterochronic pathway controls the timing of developmental events during the larval stages. A component of this pathway, the *let-7* small regulatory RNA, is expressed at the late stages of development and promotes the transition from larval to adult (L/A) stages. The stage-specificity of *let-7* expression, which is crucial for the proper timing of the worm L/A transition, is conserved in *Drosophila melanogaster* and other invertebrates. In *D. melanogaster*, pulses of the steroid hormone 20-hydroxyecdysone (ecdysone) control the timing of the transition from larval to pupal to adult stages. To test whether *let-7* expression is regulated by ecdysone in *D. melanogaster*, we examined the effect of altered ecdysone levels on *let-7* expression in mutant animals, organ cultures, and S2 cultured cells by Northern analysis. Similar experiments were conducted to test the role of *Broad-Complex* (*BR-C*), an essential component in the ecdysone pathway, upon *let-7* expression. We show that ecdysone and *BR-C* are required for *let-7* expression, indicating that the ecdysone pathway regulates the temporal expression of *let-7*. These results demonstrate an interaction between steroid hormone signaling and the heterochronic pathway in insects.

Dual roles for 20-hydroxyecdysone in programmed cell death

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Steroid hormone exerts its effects through the nuclear receptor with its partner protein. There are two other modes of action of steroid hormone, one through PI(3)K and the other through a membrane receptor. Though we know three different pathways by which steroid hormone exerts its function, there is no instance that the same hormone shares two or three different modes of action, except for estrogen. Although estrogen exerts its effects through nuclear receptor or IP(3)K, two signal transfers are not known to occur in a same tissue to trigger the same phenomenon. Ecdysteroids are important regulators of insect growth and development and programmed cell death of larval specific tissues. The silk gland of the silkworm, *Bombyx mori* is a larval specific tissue that undergoes programmed cell death (PCD) shortly after pupation. The gland PCD is reproduced during *in vitro* culture with 20-hydroxyecdysone (20HE) and completed through five distinctive morphological changes that end after 144-160 h of culture. The 20HE stimulus is required for the first 42 h of the *in vitro* death process, which is divided into three phases with respect to the hormone requirement. Phase one occurs when 20HE activates early genes that must be mediated by EcR and its partner proteins. The second phase is characterized by an abrupt progression of PCD that is triggered by protein synthesis inhibitors, although the molecular mechanism of 20HE action in this phase is obscure. Completion of the PCD requires the presence of 20HE by the end of third phase. This includes nuclear condensation, DNA fragmentation and nuclear fragmentation. Withdrawal of 20HE in this phase suppresses the progression of PCD, and dbcAMP can take the place of 20HE, a strong indication that 20HE may exert its effects through a membrane-bound receptor but not a nuclear receptor. The receptor must be associated with adenylyl cyclase activation and elevation of cytoplasmic cAMP level. This is the first case showing that a steroid hormone exerts its effects on the same developmental event through two different modes of action. (References: Tsuzuki, S. et al. Insect Biochem Molec Biol 2001; 31: 321; Terashima, J. et al. Dev Genes Evol 2000; 210: 545).

In vivo interaction of ecdysteroids and juvenile hormone in nymphal and adult cockroaches

Full text available in the Journal of Insect Science 2:20.

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In insects, ecdysteroids (Eds) and juvenile hormones (JHs) are key players in directing molting, metamorphosis, and vitellogenesis. The presence of both Ecd and JH promotes the continued juvenile development, Ecd in the absence of JH allows metamorphosis to occur, whereas presence of JH in the absence of Ecd appears to be essential for the facilitation of vitellogenin (Vg) synthesis in the adult fat bodies of most insect species. In cockroaches, fat bodies of penultimate instars do not respond to JH. Competency of the fat bodies to respond to JH and synthesize Vg develops during the last nymphal instar in the absence of JH. A progressive increase in responsiveness to the JHA methoprene, expressed as rate of synthesis of Vg, is observable during the early days of the last instar. It is followed by a dramatic reduction in response in the second half of this instar when the prothoracic glands (Pgs) are highly active and liberate Ecd. A few days before the metamorphic molt the Pgs become physiologically inactive and are programmed to atrophy following emergence. However, active mid-instar Pgs implanted into adult females two weeks after emergence remain active for many months, as shown by the induction of a molt of these adult animals. During the same period, these implanted active Pgs inhibit Vg synthesis in the host in two ways: 1.) via the direct inactivation of the corpora allata (no JH), and 2.) the inhibition of Vg production by the fat bodies. Adult fat bodies that had been exposed to Ecd for some time and no longer produced Vg are still capable to synthesize Vg in the absence of Ecd. This observation suggests that the metamorphosed fat body had not reverted to the conditions of the penultimate instar. The mode of action of Ecd on the fat bodies in nymphs or adults and the control of Vg synthesis under these conditions will be discussed.

Interaction between the BR-C Z1 transcription factor and cytoskeletal components for the implementation of ecdysone-triggered salivary gland histolysis in *Drosophila melanogaster*

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During metamorphosis of *Drosophila melanogaster*, larval tissues, such as the salivary glands, are histolysed whereas imaginal tissues differentiate into adult structures forming at eclosion a fly-shaped adult. The disintegration of the larval salivary glands is triggered by the steroid hormone ecdysone and occurs 12-13 h after pupariation (AP), although vacuolation of the cytoplasm takes already place 4-5h AP, at a much earlier time point than that of the activation of the death genes (10-12h AP). Previous work showed that apoptosis of the salivary glands requires the function of p127, a cytoskeletal protein encoded by the *l(2)gl* gene, and that the timing of salivary gland histolysis displays a *l(2)gl*-dose response. Reduced p127 expression delays disintegration whereas over-expression accelerates this process without affecting the duration of third larval instar and pupal development. Since histolysis of larval salivary glands fails to occur in *rbp* alleles of the ecdysone-inducible *Broad Complex* (*BR-C*) locus, we examined the distribution of the BR-C Z1 transcription factor in salivary glands deficient for *l(2)gl*. In wild type late 3rd instar larvae, BR-C Z1 was detected in the nucleus of salivary gland cells, whereas, in aged *l(2)gl* larvae, it accumulated in the cytoplasm and nuclear lamina. This data provide strong evidence that $p127^{l(2)gl}$ acts at an early stage of programmed cell death by controlling the access of BR-C Z1 to the nucleus and chromatin. Moreover, since $p127^{l(2)gl}$ acts as a negative regulator of the contractility of myosin II we investigated whether expression of *zipper* (*zip*) would affect salivary gland disintegration. Ectopic expression of myosin II during late third instar larvae retarded considerably salivary gland histolysis and caused a marked retention of BR C Z1 in the cytoplasm

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and nuclear lamina. Furthermore combination between the neomorphic *zip$^{E(br)}$* mutation and *l(2)gl* resulted in developmental arrest of the transheterozygous animals at the larval pupal transition phase and produced the formatiopn of tumours in the brain similar to those seen in *l(2)gl*. In salivary glands, BR C Z1 was detected in the cytoplasm and nuclear lamina whereas the Ecdysone Receptor (EcR) and Ultraspiracle (Usp) proteins were located inside the nucleus. These results show that p127$^{l(2)gl}$ and myosin II regulate the nuclear uptake and chromatin accessibility of BR C Z1 in salivary gland cells and in this way may control the implementation of ecdysone triggered cell death in this tissue.

Molecular characterization of *ecdysoneless* in *Drosophila melanogaster*

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Ecdysteroids are important regulators of insect growth and development. The *ecdysoneless* (*ecd*) locus of *Drosophila melanogaster* was mutagenized both chemically (EMS) and with gamma-ray irradiation. The several resulting alleles exhibited phenotypes ranging from a thermosensitive lethal or female sterile (*ecd^1*) to non-conditional larval lethal (*ecd^2* and all other alleles). *ecd* mutants are characterized by low ecdysone titer, which seems to be the cause of the defects, at least in *ecd^1*. Cell autonomous defects in sensory bristle formation suggested that *ecd* is not simply participating in ecdysone synthesis but may play a more general role in development. Although *ecd* mutants have been used for studies of ecdysone response for decades, the molecular identity and thus the mechanism of *ecd* action remained unknown. In the present study we set out to clone *ecdysoneless*. Using complementation tests between *ecd^2* and deficiencies affecting the 62D region, we localized *ecd* into a 60-kb sequence containing 7 to 8 predicted genes. Genomic fragments including these genes were used to prepare rescue constructs in the CaSpeR P-element vector. Flies carrying these constructs were generated by germline transformation. The molecular identity of *ecd* determined by the ability of the transgenic lines to rescue *ecd^2* and its expression profile will be presented.

Whitefly ecdysteroids

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Although whiteflies [particularly *Trialeurodes vaporariorum*, the greenhouse whitefly (GHWF), and members of the *Bemisia* complex which includes *Bemisia argentifolii*, the silverleaf whitefly (SLWF), and *B. tabaci*, the sweet potato whitefly] are serious pests of more than 500 different species of plants and cause hundreds of millions of dollars in damage each year, little is known about the endocrinology of this homopteran group. Since the development of insect control strategies based on the disruption of endocrinological events is dependent upon the availability of information concerning the hormonal and neurohormonal regulation of insect life processes, we undertook to examine the timing and regulation of molting in the greenhouse and silverleaf whitefly. Whiteflies are hemimetablous insects that undergo three larval molts and a fourth metamorphic molt to the adult. Precise staging systems for tracking development during the third and fourth instars and during pharate adult development have been described by our laboratory. Just prior to, or at the initiation of, the adult molt, ecdysteroid titers peak at approximately 120 fg/ug protein and 400 fg/ug protein, respectively, in the GHWF and SLWF. These values are approximately 10-1000 x lower than those reported for other insect orders. Histological examination of 4th instar whiteflies revealed that adult eye and wing development are initiated at a later stage in the SLWF (Stage 6) than in the GHWF (Stage 4). However, diffusion of the pigment present in the larval eye is first observed in Stage 6 of both whiteflies. Thus, adult eye and wing development precede pigment diffusion in GHWFs and accompany diffusion in SLWFs. In the GHWF, ecdysteroid titers are at peak levels between Stages 3 and 6, while in the SLWF, the peak is narrower, i.e., between Stages 4 and early 6. Predominant ecdysteroids present in the SLWF premolt adult peak are ecdysone and 20-hydroxyecdysone. The critical feeding period, time at which approximately 50% of whitefly 4th instars can be removed from the leaf and yet complete development and emerge as adults is at Stage 2 and 4, respectively, for the SLWF and GHWF. Thus, the GHWF requires a food source up to the onset of adult development while the SLWF may complete adult development even if deprived of food well before the adult molt.

Influence of the dimerization partner ultraspiracle on the dynamics of ligand binding to the ecdysteroid receptor

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Intermolecular signaling between different proteins is a posttranslational mechanism for modulation of protein function. Modification of ligand binding properties by dimerization partners is a well known mechanism observed in steroid hormone receptors. Ligand binding to Ecdysteroid receptor (EcR) promotes dimerization with Ultraspiracle (USP) and dimerization enhances ecdysteroid binding to EcR. Analysis of mutants created by site directed mutagenesis revealed that this is presumably due to stabilization of the same 3D structure of the ligand binding pocket of EcR either by ligand or by USP although with different efficiency (Grebe et al. (submitted) Mol. Endocrinol). According to competition experiments (EMSA) with ligand binding parts (C-terminus of D- and E-domain) of EcR and USP the affinity constant for heterodimerization is about tenfold higher than homodimerization. USP increases the velocity for association of ligand with EcR and decreases dissociation, which results in a lower affinity for ligand binding to EcR compared to EcR/USP. In the presence of ecdysteroids association of EcR and USP is enhanced considerably compared to heterodimerization in

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the absence of hormone. The pathway for ligand entry and exit are different (Kosztin et al. 1999. Biophys. J. 78, 188-197). Analyses of mutants created by site directed mutagenesis allow to identify the amino acids involved in association and dissociation of ligand. Different functional roles of helix 12 in EcR and USP are proposed and compared with the 3D structure of USP (Clayton et al. 2000. Proc. Nat. Acad. Sci. 98, 1549-1554; Billas et al 2000. J. Biol. Chem. 276, 7465 -74). The significance of the results for the development of insecticides interfering with the hormonal regulation of insect development is discussed.

Ecdysteroid levels and oogenesis in *Drosophila virilis* under heat stress

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Ecdysone (E) and 20-hydroxyecdysone (20HE) levels, expression of the Yp3 gene and oogenesis were studied in a wild type, stress responsive, strain (101) and in a mutant, stress nonresponsive, strain (147) of *Drosophila virilis* under normal and stress conditions. Sexual dimorphism of ecdysteroid levels in the wild type flies was found: 20HE content was higher and E content lower in females than in males. The levels of E and 20HE in females of the 147 strain were significantly higher than those in 101 females. Different dynamics of the response of E and 20HE to heat stress was demonstrated in females of 101 and 147 strains: 101 females responded to 60-min heat stress (38 °C) by an increase of levels of both E and 20HE, while in the 147 strain females ecdysteroid levels did not to change upon such exposure; 3h stress exposure resulted in a striking increase in 20HE and decrease in E levels in 147 females and an increase of both 20HE and E in 101 females. Under normal conditions, *in situ* hybridization revealed that the Yp3 gene was expressed in 96,6% of stage 9-11 eggchambers of wild type flies, while its expression was found in only 35,5% of eggchambers of these stages in mutant 147 strain flies. Following 1h heat stress, Yp3 gene expression sharply declined in wild type females and it remained unchanged in mutant ones. Following 4h heat stress wild type 101 females underwent apoptosis at stages 9 and 10 of oocyte development, a delay in passing through stage 10, and had twice as many mature oocytes (stage 14) compared to normal conditions. The 147 strain females showed an obvious delay of passing through stage 10 and some apoptosis at stages 8–10 under normal conditions. Following 4h heat stress they revealed more frequent apoptosis compared to normal conditions (although not so abundant as in wild type flies under stress) at stages 8 and 9, and apoptosis was also observed at later stages, mainly 10-12. In contrast to wild type, the 147 strain females did not display the delay in oocytes passing through stage 10 or any increase in the number of stage 14 eggs under stress. A role for ecdysteroids in the control of reproduction of *D. virilis* under stress will be presented.

Cloning and comparative characterisation of ecdysone receptors from five insect species spanning three orders

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The ecdysone gene regulatory system plays a central role in controlling and coordinating the expression of genes during insect development. Our laboratories are studying ecdysone receptors as: 1) targets for the development of environmentally friendly insecticides, and 2) elements for the construction of "ecdysone switches" for the control of transgenes in heterologous systems. We have cloned and characterised full-length cDNAs encoding both EcR and USP subunits for ecdysone receptors from the cotton bollworm *Helicoverpa armigera* (Lepidoptera), the Australian sheep blowfly *Lucilia cuprina* (Diptera) and three members of the Hemiptera, the peach aphid *Myzus persicae*, the silverleaf whitefly *Bemesia tabaci* and the green vegetable bug *Nezara viridula*. Phylogenetic relationships based on sequence data will be presented. The cDNAs have been placed into expression vectors and transfected in to CHO and CV1 mammalian cells along with reporter genes functionally linked to ecdysteroid responsive promoters. The ability of the receptors to activate reporter genes in response to insect ecdysteroids, phytoecdysteroids and a commercial synthetic compound has been studied. In some cases it has been necessary to create chimeric proteins in order to achieve function in mammalian cells. The responses of our cloned ecdysone receptors to different ligands will be discussed. Full-length receptor encoding cDNAs have been expressed in an *in vitro* transcription and translation system to examine the protein products of their open reading frames. Regions from within both EcR and USP subunits of our cloned receptors have also been subcloned for expression in a baculovirus system. Studies of the properties of the expressed protein segments, including competition ligand binding *in vitro*, are in progress.

Functional dissection of the hexamerin receptor and its ligand arylphorin in the blowfly *Calliphora vicina*

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Endocytosis mediated by cell-surface receptors is an essential process in all eukaryotes and is required for the uptake of nutrients and the recycling of membranes and membrane proteins. In insects, the best known examples are the uptake of low-density lipoprotein (LDL) and the uptake of yolk by growing oocytes. In both cases the uptake process follows a common scheme in vertebrates and in invertebrates. The receptors involved belong to the LDL receptor supergene family characterized by a highly conserved arrangement

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of cystein-rich repeats which form the ligand-binding domain. By contrast, the process of receptor-mediated uptake of hexamerin storage proteins from insect haemolymph by fat body cells is a unique feature of the class Insecta involving a receptor which does not belong to the LDL receptor family. In the blowfly, *Calliphora vicina*, the cleavage of precursor into the active hexamerin receptor is controlled by ecdysteroid hormone at a posttranslational level. We identified the binding domains of the receptor and the hexamerin ligand arylphorin in the blowfly, by means of the yeast-two-hybrid-system. The receptor-binding domain of arylphorin is located within an epitope of 49 amino acids in the domain 3 of the arylphorin monomer. The ligand-binding domain of the hexamerin receptor was mapped to the first 24 amino acids of the N-terminus of the receptor. The binding domains identified exhibit no similarity to any functional domains known to date and, therefore, represent a new species of interaction-domains. We identified two previously unknown protein-interactors of the hexamerin-receptor, (i) the anterior fat body protein (AFP), and (ii) a delta-adaptin subunit (d-AP3). The results of this study provide further insight in the mechanism of the receptor-mediated endocytosis of storage proteins in insects.

Preparation of dimeric and side chain modified ecdysteroid analogues by photochemical transformation and dehydration of phytoecdysteroids

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Preparation of various ecdysteroid analogues is stimulated by the need to extend current structure-activity relationship studies (Ravi M. et al. 2001. J. Chem. Inf. Comput. Sci. 41: 1587-1604) and to synthesise putative intermediates for biosynthetic experiments. One way to prepare such derivatives is photochemical transformation. This was first applied to 20E almost 20 years ago and afforded a series of modified analogues (Canonica L. et al. 1987. Helvetica Chim. Acta 70: 701-716). Our photochemical experiments confirmed production of identical and other similar compounds, but yielded also a new unusual dimeric analogue of 20E as main product (Harmatha J, Budesinsky M, Vokac K. 2002. Steroids 67: 127-135). The dimeric structure corresponds to 7,7´-*bis*-14-deoxy-iso-20E. This compound has unexpected HPLC characteristics and could have been easily overlooked in any previously analysed natural or synthetic mixtures. It also possessed unexpected and interesting biological activity in the *Drosophila melanogaster* B_{II} bioassay (Harmatha J., Dinan L., Lafont R. 2002. Insect Biochem. Molec. Biol. 32: 181-185). In order to obtain more information to explain the activity of dimeric ecdysteroids, photochemical transformation of ponasterone A and ajugasterone C was performed, and the results are now reported. In order to get more information on the activity of so far rare ecdysteroid analogues with a side chain lactone or cyclic ether moiety, a targeted search in plant sources (Vokac K., Budesinsky M., Harmatha J. 2002. Collect. Czech. Chem. Commun. 67: 124-139), and also in chemically transformed ecdysteroid products was performed. Production, isolation and identification of further side chain modified analogues is here also presented. Interaction of dimeric ecdysteroid analogues and side-chain modified ecdysteroid derivatives with the ecdysteroid receptor in B_{II} bioassay is reported separately (Harmatha J., Dinan L., Abstracts of this Workshop). Supported by GACR, grant No. 203/01/0183 and partly by the research project Z4 055 905.

Interaction of dimeric and side-chain modified ecdysteroids with the ecdysteroid receptor in the B_{ii}-bioassay

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Over recent years we have prepared a series of natural and chemically modified ecdysteroids for structure-activity relationship studies of ligands effective on the ecdysteroid receptor (Harmatha J., Dinan L. 1997. Arch. Insect Biochem. Physiol. 35: 219-225; Harmatha J., Dinan L., Lafont R. 2002. Insect Biochem. Molec. Biol. 32: 181-185). They were tested in the B_{II} bioassay, in which the potency reflects the affinity of binding to the ligand-binding site of the *Drosophila melanogaster* ecdysteroid receptor. The natural ecdysteroids were isolated from higher plants or from fungi. The modified structural analogues were prepared by chemical transformations or by phototransformations (Harmatha J., Budesinsky M., Vokac K. 2002. Steroids 67: 127-135) of 20-hydroxyecdysone (20E). The resulting activity data contributed to the expression of the quantitative structure-activity relationship, for the first time in a complex form of a pharmacophore hypothesis (Dinan L., Hormann R.E., Fujimoto T. (1999), J. Computer Aided Molec. Design 13: 185-207). These results led us to design further targeted structural modifications and prepare new analogues in order to explore more this phenomenon and to find suitable compounds which would be potent and useful in the regulation of insect growth and development. We compare here the activities of the relatively large ecdysteroid dimers prepared from 20E, ponasterone A and ajugasterone C, and also side-chain modified, natural and chemically transformed ecdysteroid analogues, with the parent or related monomers and with the parent or assumed side-chain precursors. The dimers all show a surprisingly high level of activity. Amongst the side-chain modified ecdysteroids, we identify the first ecdysteroid analogue to demonstrate antagonistic activity in the B_{II} bioassay. The presented results enlarge our understanding of ecdysteroid QSAR (Ravi M., Hopfinger A.J., Hormann R.E., Dinan L. 2001. J. Chem. Inf. Comput. Sci. 41: 1587-1604) and correct some of the former general presumptions obtained from earlier bioassays and implicate a new aspect to the mode of ligand binding. Supported by GACR, grant No. 203/01/0183 and partly by the research project Z4 055 905.

Characterization of ecdysteroid phosphotransferases in *Spodoptera littoralis*

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Regulation of insect development and reproduction by ecdysteroids requires major changes in the hormone titre, with the mandatory occurrence of peaks at specific stages in development. Regulation of the hormone titre occurs at the level of synthesis, sequestration, inactivation and excretion. Ecdysteroid inactivation may occur via several routes, depending upon the insect species, tissue and developmental stage. These include: (i) conversion into various phosphate conjugates (at C-2, C-3, C-22, or in some cases, C-26), or fatty acyl ester conjugates, (ii) conversion via 26-hydroxy-ecdysteroid into the 26-oic acid, or (iii) transformation via 3-dehydro-derivative into 3-epi (3α)-ecdysteroid. Ecdysteroid phosphate conjugates are unique steroid phosphates and the first ones characterized from eggs were the 22-phosphates, which represent inactive maternal storage forms of ovarian hormone. These are passed into the eggs for use, following enzymatic hydrolysis, in the early developing embryos before differentiation of the prothoracic glands. Subsequently, not only have 22-phosphates been characterized as storage forms in eggs of a range of species, but such conjugates as well as ecdysteroid 2- and 3-phosphates have been identified during hormone inactivation in immature stages of many species. Consistent with the functions of ecdysteroid phosphates in immature and adult stages of development, the ATP/Mg^{2+}-dependent ecdysteroid phosphotransferases apparently undergo marked changes in activity during development. Characterization of ecdysteroid phosphotransferases (kinases), primarily from the midgut of the cotton leafworm, *Spodoptera littoralis* will be reported.

cDNA cloning and characterization of *Bombyx mori* orphan receptor, BmHR78

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We have identified a novel member of the nuclear receptor superfamily from the silkworm *Bombyx mor*i, and named it as BmHR78, the *B. mori* orphan receptor. The DNA binding domain of BmHR78 shows high similarities to those of *Tenebrio molitor* hormone receptor 78 (TmHR78), *Drosophila melanogaster* hormone receptor 78 (DHR78), and mammalian testicular receptor 2 (TR2), whereas the ligand binding domain is not well conserved. Northern blot analysis showed that BmHR78 gene was most abundantly expressed in the testis. From the fourth to fifth instar, BmHR78 gene was constantly expressed in the testis. In the anterior silk gland, the level of BmHR78 gene expression was developmentally changed. In the late fifth instar, another BmHR78 transcript with the smaller size appeared. Ultraspiracle (USP) isoform also appeared at the same stages in this tissue. BmHR78 forms not only a homodimer, but also a heterodimer with USP in a yeast two hybrid assay. The direct interaction between BmHR78 and USP was confirmed by pull down assay. Deletion mutant analysis showed that BmHR78 interacts with USP via the ninth heptad repeat in helix ten of the E region. This repeat is well conserved in RXR and its heterodimer partners, and shown to be an interface for their dimerization. In insect, only the ecdysone receptor and hormone receptor 38 (DHR38) are known thus far to dimerize with USP. Thus, BmHR78 is a third dimerization partner for USP and may modulate the molecular action of USP, including the ecdysone signal cascades.

Roles of EcR-A and USP in the regulation of MR3 expression by 20-hydroxyecdysone

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MHR3 is an ecdysone-inducible transcription factor whose expression in both *Manduca sexta* epidermis and the *M. sexta* GV1 cell line is induced by 20-hydroxyecdysone (20E) *in vitro.* Previous studies have shown that there are four putative ecdysone response elements (EcRE) in the 2.6 kb flanking region of the MHR3 promoter and that the most proximal EcRE1 is necessary for activation of the promoter by 20E in the GV1 cells (Lan *et al*. 1999: Mol. Cell. Biol. 19, 4897-4906). High expression of the ecdysone receptor (EcR) isoforms, EcR-B1 and USP-1, under the control of the *Autographa californica* baculovirus promoter, $pIE1^{hr}$, increased 20E activation of the 2.6 kb promoter, whereas the additional high expression of USP-2 prevented this activation. The EcR-B1/USP-1 heterodimer was shown to bind to EcRE-1, and this binding was prevented by the addition of excess USP-2. The upstream putative EcREs, EcRE2 and EcRE3, were essential for activation but bound neither EcR-B1 nor either of the USPs. Thus, the 20E activation of MHR3 is thought to be mediated by EcR-B1 and USP-1, the predominant isoforms in the epidermis during the intermolt period. In the epidermis, both EcR-A and USP-2 levels are high at the time that MHR3 mRNA begins its decline. When we expressed EcR-A alone or in combination with either or both isoforms of USP, the promoter containing EcREs 1-3 was not activated by 20E. High expression of EcR-A also had no effect on the activation of this promoter by 20E in the presence of high levels of EcR-B1/USP-1. When the most distal EcRE4 was present, the activation by 20E-EcR-B1/USP-1 was increased twofold. This increased activation was suppressed by the addition of high levels of EcR-A. Similarly, in the presence of all 4 putative EcREs, the expression of high levels of EcR-A and either or both USPs in the absence of excess EcR-B1 allowed only about 50% of the normal induction. These findings suggest that EcR-A with either isoform of USP may bind to EcRE4 in the presence of 20E to inhibit the expression of MHR3. Gel mobility shift assays are underway to test this hypothesis. Supported by the USDA.

Ecdysteroid receptors in crustaceans: DNA and ligand binding properties

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Ecdysteroid signaling is critical to crustacean growth. In the fiddler crab, *Uca pugilator*, and other crustaceans, several major ecdysteroids circulate in the hemolymph. The level of ecdysteroids in the hemolymph increases at the same time that major molt-related events such as proliferation of epidermal cells, secretion of new cuticle, and construction of new exoskeleton are occurring. A specialized form of growth that can accompany the normal adult growth and reproduction cycle in crustaceans is limb regeneration. During the late stages of limb regeneration, increases in rates of protein synthesis are responsive to ecdysteroids, but the role(s) of hormones in the specification of a new limb blastema immediately following limb loss are not understood at this time. To aid in an examination of the roles of ecdysteroids in these processes, we constructed cDNA libraries from late regenerating limb bud tissue and have isolated *U. pugilator* cDNA clones that are structural homologs of the ecdysteroid (*UpEcR*) and retinoid-X/USP (*UpRXR*) classes of nuclear receptor (NR). We have generated in vitro synthesized proteins and produced polyclonal antibodies to both common and A/B domains of both receptors. Using Western blots, immunocytochemistry, and ribonuclease protection assays, we have shown that both mRNA and the mature receptor proteins are present in limb buds throughout all stages of regeneration. The mRNA of UpEcR and UpRXR are present in blastemas at the onset of regeneration as well as in final stages of limb regeneration, but the pattern of expression of mRNA's does not correlate with patterns of circulating ecdysteroids. The cloning of these receptors has allowed us to begin characterizations of their physical properties. GST-pulldown experiments indicate that the UpEcR and UpRXR proteins are capable of forming a hetero-complex, and EMSA analysis indicates that both proteins are required for binding to canonical ecdysteroid HREs. EMSAs are now being used to determine DNA binding affinities to various HREs and the role of ligand on DNA binding relative to HRE sequence. Ligand binding studies are also in progress to determine the binding affinities of UpEcR and UpRXR for a variety of ligands. UpRXR appears to bind t9-cis-retinoic acid as a homodimer and a second putative ligand for UpRXR has been isolated from early blastemal tissue.

Multidimensional quantitative structure-activity relationships of diacylhydrazine toxicity in *Spodoptera exigua* and *Leptinotarsa decemlineata*

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A training set of 109 *N-t*-butyl-dibenzoylhydrazines with modification at two benzoyl moieties was examined for toxicity in larvae of *Spodoptera exigua* (beet armyworm) and *Leptinotarsa. decemlineata* (Colorado potato beetle) by topical application. The resulting LD_{50} values were used to construct CoMFA (comparative molecular field analysis) QSAR models. The best CoMFA models are compared to previously calculated classical (Hansch & Fujita) QSAR equations and to preliminary 4D-QSAR models for identification of the pharmacophore significant to toxicity and selectivity of the diacylhydrazine class of insect growth regulators.

Antifeedant activity of 20-hydroxyecdysone relative to larvae and imago of *Leptinotarsa decemlineata*

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Although the ecdysteroids are well known as a deferent to insect species, the antifeedant properties of phytoecdysteroids in relative to *Leptinotarsa decemlineata (Say)* have not been investigated. We have found that water solution of 20-hydroxyecdysone (20E) shows the singificant antifeedant activity on larvae and imago of *L. decemlineata*. The effective and threshold concentration of 20E solvents as also the continuation of effect after treating of *Solanum tuberosum L*. leaves.

Ecdysone receptors link pattern and process in butterfly eyespot development

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Butterfly wing patterns provide important models for the study of both development and evolution. Among these patterns, the eyespots of *Bicyclus anynana* show a striking seasonal polyphenism in both size and colour. In this species, wet season morphs have large eyespots of several colours whereas dry season morphs have much reduced eyespots of fewer colours. These two morphs can be mimicked by artificial selection for eyespot size in the laboratory, resulting in HIGH- and LOW-selected lines which represent wet and dry season equivalents, respectively. Eyespot development is initiated by an ordered succession of homeotic gene transcription and concludes with the maturation and pigmentation of the differently coloured scale cells in each of the concentric colour rings. However, the processes that link homeotic gene dependent pattern with final colour pigmentation have remained obscure. Here we show that the pattern of expression of nuclear hormone receptors for ecdysteroids (EcRs) correlates with the final colour of the scale cells themselves. Moreover, these expression patterns differ between HIGH and LOW-selected lines, differences that can be rescued either by rearing butterflies at high temperature or by injection of ecdysone itself. Our results show that EcR expression forms a critical, and ligand self-regulated, link between pattern formation and

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colouration. These data explain how complex changes in wing pattern can be effected by simple changes in environmental parameters such as temperature, resulting in butterfly morphs with presumptive selective advantages for their different environments.

Ecdysteroid controls pupal commitment and cell cycle in the wing discs of the silkworm, *Bombyx mori*

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Cell number of a wing disc (WD) of lepidopteran larvae increases at a relatively constant rate until the final larval molt after which the cell proliferation rate abruptly increases. When a pair of corpora allata is removed from penultimate instar larvae of *Bombx mori*, the WD cells are pupally committed within24 h, and the cell number increases rapidly. Similarly, in the fifth instar, WDs are pupally committed shortly after the final larval molt, and the WD cells respond to 20-hydroxyecdysone (20E) with an acceleration of cell proliferation. These suggest that 20E accelerates cell proliferation in the pupally committed discs. We cultured WDs of fourth instar larvae under various hormonal conditions to examine the relation of the cell phases to the change in commitment. S-phase cells were detected by BrdU incorporation followed by immunostaining using anti-BrdU monoclonal antibody, and M-phase cells using anti-phospho histone H3 antibody. The WDs obtained at 66 h contained both S- and M-phase cells. When these WDs were cultured with 20E for 24 h, pupal commitment was not induced, and both S- and M-phase cells disappeared irrespective of the presence of 20E. By contrast, in the WDs at 12 h prior to the final larval molt (M-12h), the number of S-phase cells remained at the initial level after the culture in a hormone-free medium, but M-phase cells disappeared. Exposure of M-12h WDs to 20E increased the number of cells in both S- and M-phase, and induced pupal commitment in those discs as well. Ecdysteroid thus appears to have a critical role in the control of G2 checkpoint in the pupally committed cells. Taken together, we suggested that there are likely two different hormonal controls of cell cycle in the WD. One is the cell cycle that is independent of 20E, while the other is 20E-induced cell cycle that occurs at M-12h and thereafter. The present results indicate that the acquirement of competence of the cells to respond 20E in respect of cell phase dynamics is tightly associated with the initiation of the change in commitment.

Development of high through-put screening systems for insect growth regulating substances using transformed insect cell lines

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The isolation and cloning of the ecdysone receptor combined with the identification of ecdysone-responsive elements (EREs) in target genes that are bound by the activated ecdysone receptor complex has opened new possibilities for the development of cell-line based screening systems for compounds with ecdysone agonist or antagonist activity. An ecdysone-inducible reporter cassette was constructed in which 7 repeats of the *Drosophila melanogaster hsp27*-derived ERE were cloned upstream of a basal promoter-driven reporter gene. Transient expression experiments using silkmoth-derived (Bm5) cells that constitutively express the ecdysone receptor heterodimer show that approximately 2,000-fold activation of the reporter construct is achieved following addition of submicromolar quantities of 20-hydroxyecdysone (20E) or the ecdysone agonist tebufenozide. To develop a cell-based screening system for ecdysone mimetic compounds, Bm5 cells were permanently transformed for an ecdysone-responsive GFP reporter construct and five homogeneous cell lines were obtained after selection by fluorescence-activated cell sorting (FACS) that show a very strong response to 20E. The ecdysone-responsive cell lines are currently being used to screen for the presence of ecdysone agonist and antagonist activity in plant extracts. Ecdysone mimetic compounds were identified in extracts from spinach and *Chenopodium album* of which one was purified and characterized by mass spectrometry as 20E. A variety of antagonistic substances were shown to be present in extracts from several *Citrus* species and their purification by HPLC is in progress. A similar rationale is currently applied to develop cell-line based screening systems for juvenile hormone (JH) agonistic and antagonistic compounds. The system for screening of JH-like compounds is based on the observation that JH causes multimerization of the Ultraspiracle (USP) nuclear receptor in yeast two-hybrid assays. We are currently constructing a similar two-hybrid system for the silkmoth homolog of USP, the nuclear receptor BmCF1, in Bm5 tissue culture cells to test whether JH can cause multimerization of BmCF1 in this system.

Practical uses for ecdysteroids in mammals and humans: An update

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The pioneering "heterophylic" studies of Burdette in the early sixties concerning ecdysteroid actions on mammals revealed effects on cell proliferation and protein synthesis. Subsequent experiments extended investigations to many different areas, e. g. hyperglycaemia, hypercholesterolaemia and immune reactions; together these results led to the development of a wide array of ecdysteroid-containing preparations, mainly used for their anabolic and/or "adaptogenic" properties on humans. In the same way, many patents have been filed describing various beneficial effects of ecdysteroids in for medical or cosmetic treatments, which make ecdysteroids very attractive candidates for practical use. The interest in ecdysteroid receptor systems in 'gene switching' strategies adds to the importance of understanding the pharmacological effects of

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ecdysteroids on mammals. We consider several aspects of the topic: Firstly, the major proposed uses of ecdysteroids and the corresponding commercially available preparations will be reviewed. Secondly, some selected experiments on which these uses are based will be critically analyzed with regard to the protocols used and the statistical relevance of the results. Third, current hypotheses on the possible modes of action of ecdysteroids on vertebrates will be described in the light of what is presently known for neurosteroids and oxysterols, especially as concerns membrane effects of steroids. Finally, some recent data on the metabolic fate of ecdysteroids in mammals and humans will be reported.

Ecdybase [http://ecdybase.org] - The Ecdysone Handbook, 3rd edition

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The aim of Ecdybase is to provide general data on all natural ecdysteroids described so far. Each data file contains (whenever available) biological, chemical, structural, spectroscopic (UV, IR, MS, NMR), chromatographic and bio-activity data together with selected relevant references. This database is a 3rd Edition (now on web) of The Ecdysone Handbook, originally created by René Lafont and Ian D. Wilson, published in 1992 (1st Edition by the Chromatographic Society, Nottingham, U.K.). As the ecdysteroid family continued to increase (from ca. 170 compounds in the 1st Edition), a 2nd enlarged Edition was published in 1996 (with 262 compounds). The number of compounds is still growing (312 compounds, described at the onset of 2000), and thus requiring new updated Editions. Owing to the limited number of people interested by such Handbooks, a printed version is no longer justified. Moreover, the problem of updating content was a major one, too. This led to the idea of developing a new concept, making a proper use of modern communication tools. A decision was taken to transform the Handbook in a Database made freely available on the web to anyone interested in ecdysteroids. Between that decision and the realisation, more than 2 years have elapsed, and the project has involved several additional key contributors. First, Frédéric Marion-Poll collaborated with several students from the INA-PG to design the general organisation of the database, defining the different fields and their links. Then came the enormous work of converting the original MacIntosh data (Pagemaker® files) to the present "Ecdybase". Juraj Harmatha and his son Tomas have mainly done this conversion. The last part of the work was the design of the web interface, done by Tomas Harmatha (Cybersales, a.s.), to create data files rather similar to the data sheets of the Handbook. Owing to compatibility problems all figures had to be redrawn (by J. H.), but in counterpart this provided an opportunity to correct several mistakes from the previous paper editions. The Ecdybase will also include data on the biological activity of ecdysteroids (when available), added by Laurence Dinan. The Ecdybase, as presently available, is still considered by the authors as a developing source, which can be continuously improved and extended with the help of all other ecdysonists. Forms will be available for the electronic submission of additional data for new ecdysteroids or for providing more information on already known ones. In the future, functions will be improved and allow e.g. a search of all compounds present in a given animal or plant species. More references will be added, although we do not plan to include physiological data (the literature is too vast). However, we included a catalogue of commercial products containing ecdysteroids, with direct links to the homepages of the relevant producers. Supported partly by GACR, grant No. 203/01/0183 and by the research project Z4 055 905.

Dimerization interface (DIF-) model for nuclear receptor interaction

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At previous Ecdysone Workshops we reported about our yeast two-hybrid studies on heterodimerization between the ligand binding domains (LBDs) of EcR and USP (see abstract 18, 1998, and abstracts 1.9 and 1.10, 2000), and the effect of ecdysteroids (Ec) on this interaction. We also reported that site-directed mutations were made, some of them decreased but, surprisingly, others enhanced heterodimerization. We were especially puzzled by two mutations in the ligand binding pocket (LBP) of USP which potentiated the effect of Ec on heterodimerization. Since USP is generally not regarded the receptor protein for Ec we tried to explain that phenomenon by an allosteric effect of the USP LBP on the EcR LBP, in analogy to a similar mutation described in mouse RXRα (F318A) (Vivat et al., EMBO J. 16, 5697, 1997). I recently developed a model which can explain this and similar phenomena by two simple but valid assumptions: (a) subdomains in LBD are structurally and functionally coupled through a three-dimensional network of interhelical links and (b) there exist conformational states of the EcR and USP dimerization interface (DIF) which are more compatible than others. The DIF of the mentioned LBP mutations (I323A and L322G) in USP fit the DIF of the Ec-induced EcR (holo-EcR) better than that of the non-liganded EcR (apo-EcR). With superinducible EcR mutants (like T619A), ligand increases the affinity to the DIF of USP I323A more than it does with wild-type EcR. On the other hand, there are constitutive EcR mutants (e. g. K497A) which hardly react to ligand and which therefore prefer the DIF of wild-type USP over the DIFs of the USP mutants I323A and L322G which are adapted to interact preferably with the DIF of holo-EcRs. The presented DIF model may help to understand not only the interaction between various EcR and USP mutants but also between alternative dimerization partners such as SVP or DHR38, and the regulation of these interactions by different ligands. Moreover, it is applicable to any dimerizing nuclear receptor types and may often replace other more complicated models used for

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explaining apparent transmolecular cross talk or ligand mimicking effects.

Female specific wing degeneration of a tussock moth, *Orgyia recens*, is induced by ecdysteroid

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Adult females of a tussock moth, *Orgyia recens*, have vestigial and nonfunctional wings. Their wings shrink during the pupal stage, although they develop normally until pupation. We studied the developmental processes of this event in minute and found that more than 80% of female wing cells disappear at 1.5days after pupal ecdysis. The female specific cell degeneration completed until Day 2. In male wings, only peripheral region (distal to bordering lacuna) was lost until Day 2, like in other Lepidoptera. During cell degeneration, huge numbers of phagocyte-like cells exist in female wings but rare in male wings. The timing of the female wing degeneration during pupa seems to be concurrent with the timing when ecdysteoroid titer in hemolymph is going up. To test the effects of ecdysteroid on this developmental event directly, we cultured the wings from females and males at Day 0 just after pupal ecdysis with and without 20-hydroxyecdysone (20E). With various concentrations of 20E from 0.01 to 10 μg/ml, the female wing degeneration could be induced, but no morphological changes were observed without 20E. Higher dose of 20E could induce the cell degeneration rapidly. The ecdysone (E), less effective than 20E, could also the same events. After 2 days of exposure to 1 μg/ml of 20E, only female wings dramatically underwent degeneration, while male wings did not show such morphological change. In male wings, only the region just outside bordering lacuna degenerated. DAPI staining clearly showed that cells not only outside but also inside the bordering lacuna disappeared in female wings. This observation suggests that ecdysteroid itself triggers the excessive cell death specifically in female wings during pupal development. To test whether cell death in female wings are caused by apoptosis, we performed TUNEL analysis on cultured pupal wings. In male wings, many signals were only observed in the region distal to the bordering lacuna. In female wings, however, a dozen of signals were observed both inside and outside of bordering lacuna. This results support the above observation. However, we need more evidences to understand the mechanisms of female wing degeneration precisely. To understand the difference of ecdysteroid action between male and female, we further tried to study the EcR isoforms expression by *in situ* hybridization. The preliminary results showed the differential expression of EcR isoforms between the inside and outside regions of bordering lacuna in region specific manners.

Molecular cloning, expression analysis and functional confirmation of ecdysone receptor (EcR) and ultraspiracle (USP) from the rice stem borer, *Chilo suppressalis*

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In our previous studies, we investigated the substituent effects of non-steroidal ecdysone agonists, N-*tert*-butyl-N,N'-dibenzoylhydrazines, on the larvicidal and molting hormonal activities against the lepidopteran rice stem borer, *Chilo suppressalis* Walker. We found that the larvicidal activity was linearly correlated with the molting hormonal activity measured in a cultured integument system. Even though various physiological and pharmacological data for these ecdysone agonists have been published, their biochemical and molecular mechanisms are still unknown. The use of structure-activity relationship studies of the binding activity of ecdysone agonists with EcR and USP proteins could explain the detail for the ligand-receptor interaction profile. Here, we report the cDNA cloning and functional expression of EcR and USP from *C. suppressalis* as well as a binding assay using expressed EcR and USP proteins. We have determined the cDNA sequences for the EcR-A, EcR-B1 and USP-2 isoforms from *C. suppressalis*. Their deduced amino acid sequences showed high identities to other insects' counterparts. In gel mobility shift assays, *in vitro* translated *C. suppressalis* EcR (CsEcR) and USP (CsUSP) proteins bound to Pal1 or *Drosophila melanogaster* hsp27 probe as a heterodimer. [^{3}H]Ponasterone A bound to the EcR-USP complex constructed from *in vitro* translated EcR and USP, and the binding was competitive with ecdysteroids or non-steroidal ecdysone agonists.

Calcium plays crucial role in the activation of prothoracic glands in locusts

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Larval prothoracic glands (pg) of the desert locust (*Locusta migratoria*) secrete ecdysteroids when cultured *in vitro*. Their co-incubation with brains taken from certain developmental stages of locusts enhances the secretion. A similar increase of ecdysteroid production is achieved by the addition of tris (hydroxymethyl aminomethane) to the incubation medium to a 50 mm concentration. The stimulatory effect of tris is maximal with the prothoracic glands of larvae of days 2 to 4 of the penultimate or the last larval instars. These prothoracic glands exhibit very high spontaneous ecdysteroid secretion, apparently in response to their previous stimulation by the brain *in situ*, and the exposure to tris further elevates the secretion up to 16-fold. By contrast, the prothoracic glands from the start and the end of these instars naturally produce much less ecdysteroids and the addition of tris to the culture medium enhances the secretion only 2-4 times. The aim of our study is to elucidate the stimulatory effect of tris. In the experiments performed so far we found that the

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stimulation was negligible in a calcium-free medium. The stimulatory effect of tris in the normal medium is suppressed in the presence of 1 mM cadmium that is known as general blocker of calcium channels. These observations suggest that extracellular calcium is involved in the tris-stimulated cascade. To explore possible role of the intracellular calcium, the culture medium was supplemented with 1mM TMB-8 that specifically inhibits mobilisation of the intracellular calcium. The stimulatory effect of tris was totally eliminated, indicating that the opening of calcium channels in the endoplasmic reticulum plays pivotal role in the stimulatory action of tris.

Trimethylsililation of 14 αA-hydroxy group of ecdysteroids.

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Trimethylsililation of ecdysteroid hydroxy group is essential for target transformation and for gas chromatography analysis. Meanwhile the sililation of 14 αα-hydroxy group involving mainly using N,O-bis(trimethylsilil)acetamide and N-(trimethylsilil)imidazole runs in rigid conditions (up 100 °C) and complicated by undesirable secondary processes. We are found that 14 αα-hydroxy group of poststerone and 20-hydroxyecdysone in mild conditions (THF, 0 °C, 2 min) transforms into trimethylsililoxy group by treating of equivalent amounts of (trifluoromethyl)trimethylsilane under the $Bu_4N^+F^-$ as a catalyst.

Ecdysteroids derived from 20-hydroxyecdysone and their biological activities.

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Phytoecdysteroids are spread very unevenly in nature. Most of them are available in only minor amounts from plants and hardly available in adequate amounts for research and practical applications. A more preferable strategy is the synthesis of rare natural ecdysteroids by chemical transformation of common, wide-spread phytoecdysteroids, such as 20-hydroxyecdysone (20E). A regio- and stereoselective route to the natural phytoecdysteroid shidasterone (stachysterone D) synthesis based on one-step transformation of 20E by treating with trifluoroacetyl anhydride in chloroform has been achieved. Along with shidasterone, 20,22-*O*-(1*RS*-hydroxy-2,2,2-trifluoroethylidene)-20-hydroxyecdysone was isolated by column chromatography. Stachysterone B and ponasterone A have been synthesized by dehydration of the hydroxyl groups at C-14 and C-25, respectively, of 20E diacetonide. 20-Hydroxyecdysone 6-oxime has also been prepared. The structures of all compounds were confirmed by IR, UV, ^1H and ^{13}C NMR and mass spectrometry. Ecdysteroid activities in the *Drosophila melanogaster* B_{II} bioassay has been determined for all the synthesised compounds. The implications of these activities for ecdysteroid structure-activity studies will be discussed.

Ecdysteroid induced cell death and cell proliferation in pupal wings of Lepidoptera.

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The wings of insects that show striking morphological changes from larval to adult transformation are good systems in which study ecdysteroid responses in tissue differentiation. The remarkable developmental event in Lepidopteran wings during pupa is scale formation and programmed cell death of epithelia at the periphery. It is known that the outline of adult wings of moths and butterflies emerges as a result of the disappearance of peripheral region. This wing margin of adult wings is defined by the position of a special structure, " bordering lacuna (BL)". To determine the effect of 20-hydroxyecdysone (20E) on these events, we cultured the silkworm pupal wings with or without 20E and analyzed regional specificity for cell death by the TUNEL method and cell proliferation by 5-bromodeocyuridine labeling. Programmed cell death was induced by 20E after 5 days of culture and observed only in the region distal to BL. Cell proliferation after 1day of culture were also inducible by 20E and detected in the region proximal to BL. These results suggest that two types of two types of pupal wing cells, which are divided by the position of BL. Two cellular responses are induced by ecdysteroid in different manners. Higher concentrations more than 1000 ng/ml 20E repressed the scale formation, while the peripheral cell death could not be repressed even with 5000 ng/ml. The ecdysteroid may work both as a trigger to make the wing margin and scale formation and as a developmental timer to arrange. Interestingly, the wingless (wg) mRNA seemed to be expressed specifically in the region distal to BL from larval to pre-pupal stages in Lepidoptera. This observation suggests the relationship among the wingless driven cascade, ecdysteroid action and programmed cell death.

Characterisation of 3-dehydroecdysone 3α-reductases

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The ecdysteroid titre exhibits obligatory, distinct peaks at specific stages in development. In immature stages, these arise by increased

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ecdysteroid synthesis in the prothoracic glands, whereas decreases in titre result from enhanced ecdysteroid inactivation reactions together with elevated excretion. A number of pathways may contribute to the inactivation of ecdysteroids depending on the species of insect, stage of development and tissues. These include: (i) conversion into 26-oic acids via 26-hydroxyecdysteroids; (ii) transformation into phosphate ester or fatty acyl conjugates; and (iii) formation of 3-epi (3α-hydroxy)ecdysteroids, which are regarded as hormonally inactive. Production of 3-epiecdysteroids occurs in many insect orders, but is apparently prominent in Lepidopteran midgut cytosol and occurs by ecdysone oxidase-catalysed formation of 3-dehydroecdysteroid, followed by NAD(P)H-dependent irreversible reduction by 3-dehydroecdysteroid 3α-reductase to 3-epiecdysteroid. The 3-dehydroecdysteroid may also undergo NAD(P)H-dependent 3-dehydroecdysteroid 3β-reductase-catalysed reduction back to 3β-hydroxyecdysteroid; the significance of this is uncertain. Similar reactions occur with ecdysone and 20-hydroxyecdysone. We previously demonstrated the occurrence of two forms of 3-dehydroecdysone (3DE) 3α-reductase in midgut with attached Malpighian tubules of the cotton leafworm, *Spodoptera littoralis*, a 26 kDa form, which may exist as a trimer with apparent molecular mass of 76 kDa, and a monomer with approximate molecular mass of 51 kDa. The 26 kDa form was cloned and the transcripts shown to be expressed in Malpighian tubules. The enzyme belongs to the short-chain dehydrogenases/reductases (SDR) superfamily. We now report characterisation of the 51 kDa form of 3DE 3α-reductase.

Ecdysone receptor-based gene switch for simultaneous regulation of multiple genes

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Some of the applications in proteomics, functional genomics, gene therapy, cell-based high throughput screening assays and large-scale protein production require controlled expression of multiple genes in the same cells. At present, two different gene switches such as ecdysone receptor (EcR)-based system and tetracycline-resistant operon-based system are used for parallel control of expression of two genes. We developed an EcR-based gene switch that can be used for regulation of two genes simultaneously. A *Choristoneura fumiferana* EcR (CfEcR) that responds to non-steroids such as GS™-E but not to steroids such as ponasterone (PonA) or Muristerone A was developed as a non-steroid receptor. Some mutant versions of CfEcR as well as wild-type EcRs such as *Drosophila melanogaster* EcR (DmEcR) that respond better to steroids than to non-steroids were developed as steroid receptors. The CfEcR(DEF) fused to LexA DNA binding domain, DmEcR(DEF) fused to GAL4 DNA binding domain, HsRXRβ-LmRXR(EF) chimera fused to VP16 activation domain and two reporter constructs p5XGALRESEAP (secreted alkaline phosphatase reporter controlled by a promoter containing 5X GAL4 response elements and TATAA) and p80PLUC (luciferase reporter controlled by a promoter containing eight LexA operators and TATAA) were transfected into NIH3T3 cells. The transfected cells were exposed to either non-steroid, GS™-E, or to steroid, PonA, or to both. Addition of GS™-E induced SEAP (1200-fold above background levels) but not luciferase. On the other hand, addition of PonA induced luciferase (400-fold) but not SEAP. Addition of GS™-E and PonA induced the activity of both SEAP and luciferase reporters. These results showed that the two EcRs and the two ligands tested are orthogonal and are useful for simultaneous regulation of expression of two genes within the same cell.

Variations of ecdysteroid biosynthesis and expression of steroidogenic enzymes are correlated in *Drosophila melanogaster* ring gland

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During *Drosophila melanogaster* larval development, ecdysteroid titers present large temporal fluctuations, which imply that synthesis and/or degradation of ecdysteroids are tightly regulated. Even if the biosynthetic pathway of ecdysteroids is still incompletely understood, several genes coding for putative steroidogenic enzymes have been recently identified from *D. melanogaster* Halloween group mutants (Chavez, M. *et al.* 2000, *Development* 127, 4115-4126). In order to understand to which extent the steroidogenic activity of the ring gland depends on the expression of steroidogenic enzymes, a series of experiments was conducted on third instar synchronized larvae. Expression of Halloween genes in the larval ring glands was studied by *in situ* hybridization. Variations of expression were further precised by quantitative RT-PCR. In parallel, the steroidogenic capacity of the ring gland was measured by enzymatic immunoassay of ecdysteroids produced *in vitro*. It appears that genes expression and ecdysteroid synthesis peak at the same moment in the third instar. The implication of the variations of enzyme expression in the control of ecdysteroid biosynthesis will be discussed.

The halloween group genes spook, phantom, and shade regulate ecdysone biosynthesis in *Drosophila melanogaster*

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The steroid hormone ecdysone (E) controls larval molting, metamorphosis and reproduction in insects. The biosynthesis of ecdysone involves a cascade of hydroxylation steps, which are catalyzed by both the mitochondrial and the microsomal P450 enzymes. Little is known about the genes that encode enzymes involved in the steroidogenic pathway of ecdysteroid production.

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We have previously reported that *disembodied* (*dib)* codes for a new member of the cytochrome P450 family designated CYP302a1 that is involved in ecdysone biosynthesis. Since *dib* mutants exhibit defects in embryonic cuticle formation, we searched for other mutants characterized by abnormal cuticular patterning. We found that the phenotypes of mutants in the Halloween class, including *phantom (phm)*, *shade (shd)*, and *spook (spo)* are strikingly similar to the phenotype of *dib* mutants. All of these genes code for P450-type products. We have identified at least one mutant allele for each gene. All mutants show reduced expression of the ecdysone-inducible gene IMP-E1 and low ecdysone levels. The characterization of the enzymatic activities of these P450-type products is pending. The expression patterns of *spo*, *phm*, and *shd* suggest that ecdysteroid production may occur in tissues other than the classical endocrine organs such as ring glands and ovaries. While all of the genes are expressed in the follicle cells of the ovaries, and *phm* is expressed in the ring glands, *spo* and *shd* do not show any staining of the ring glands. *phm* and *shd* show a segmental pattern of expression in the epidermal cells, suggesting perhaps the involvement of these cells in the regulation of ecdysone synthesis. There are also high levels of *spo* expression in the yolk nuclei and amnioserosa, which are not normally thought of as endocrine organs. The yolk nuclei have been implicated in the storage of ecdysteroid conjugates and subsequent timed release of free ecdysteroids during embryogenesis, as well as endoderm migration and midgut formation. The role of amnioserosa in embryonic development is also poorly understood. In order to understand the role of tissues other than the ovaries and the ring glands in ecdysteroid production, we have designed rescue constructs that will allow for specific expression of various biosynthetic enzymes in individual tissues such as amnioserosa, epidermis, central nervous system and others. The ability of tissue-specific expression of the different enzymes to rescue the mutant phenotypes is under study.

Interaction of the Gal4-DNA binding domain fused to the ligand binding pocket of ultraspiracle with dna and influence on ligand binding to the heterodimerization partner, the ecdysone receptor

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The Gal4-DNA binding domain (Gal4-DBD), widely used for two hybrid studies, is generally considered as an inert partner of the fused protein and changes in reporter gene activity are interpreted as consequences of the interaction with additional proteins fused to the activating domain (Gal4-AD). In contrast to the autonomous hormone binding properties of the ecdysteroid receptor ligand binding domain (EcR-LBD), which is not influenced by additional proteins like comodulators and heat shock proteins and other domains of the EcR molecule, a profound influence of the protein moiety fused to the Gal4-DBD is observed. We demonstrate the influence of helix 12 (USP) and the importance of the antagonistic position of helix 12 for DNA binding of the fusion protein. By mutational analysis we investigated the dimerization properties of USP and the role of amino acids located in the ligand binding domain of USP, which mediate the interaction with a non specifically bound fatty acid. The consequences for the interpretation of two hybrid data are discussed.

Inducible double-stranded RNA produced from transgenes can direct gene product inactivation with cell-, stage-, and isoform-specificity

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The functionnal ecdysteroid receptor complex in *Drosophila melanogaster* consists of a nuclear receptor heterodimer of the ecdysteroid receptor (EcR) and ultraspiracle (USP) proteins. The EcR gene encodes three ecdysone receptor isoforms (EcR-A, EcR-B1, and EcR-B2) that have common DNA and hormone binding domains but a distinct N-terminal region. EcR isoforms are differentially expressed in tissues with distinct developmental fates. EcR-A is the predominant isoform expressed in imaginal discs while EcR-B is the predominant isoform expressed in most larval tissues. Expression of a UAS/GAL4 driven transgene with portions of a cDNA cloned in an inverted repeat orientation results in the synthesis of double-stranded RNA and subsequent inactivation of the corresponding gene. We have tested this approach to inactivate a set of genes including the *EcR*, *usp* and *batman* genes. Our results indicate that ubiquitous expression of a specific dsRNA throughout development phenocopies the effect of null or hypomorphic mutations of the corresponding gene. We demonstrate that EcR isoforms can be specifically inactivated. In addition, tissue- or cell-specific GAL4 drivers can direct inactivation of the ECR-B1 protein in the fat body and Batman protein in the posterior compartment of imaginal discs. Finally, we used a heat inducible GAL4 driver to trigger the expression of batman dsRNA at mid third instar and determined the kinetic of the disappearence of Batman protein. We found by western analysis that Batman protein disappears completely 8 hours after heat shock. Our results provide strong support to the use of double-stranded RNA produced from a transgene as a powerful tool to inactivate gene products in a tissue-, stage- and isoform-specific manner.

Prothoracicotropic hormone stimulates extracellular signal-activated receptor kinase (ERK) activity through Ca^{2+}- and cAMP-dependent mechanisms

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The synthesis of ecdysteroids by the prothoracic gland of Lepidoptera is acutely regulated by the brain neuropeptide hormone prothoracicotropic hormone (PTTH). PTTH is believed to bind to receptors on the plasma membrane of prothoracic glands. PTTH binding initiates a series of events within the cells that proceeds from Ca^{2+} influx through Ca^{2+}-dependent cAMP generation, activation of protein kinase A and S6 kinase, phosphorylation of the S6 ribosomal protein, and culminates in protein synthesis and protein synthesis-dependent ecdysteroidogenesis. Recent work has shown that the signal transduction cascade activated by PTTH in prothoracic glands of the tobacco hornworm *Manduca sexta* involves at least one additional phosphorylation pathway (Rybczynski *et al.*, Molec. Cell. Endocrinol. 184: 1-11, 2001). Specifically, PTTH rapidly stimulates the activity of an extracellular signal-activated receptor kinase (ERK) in the *Manduca* prothoracic gland. ERKs are members of the mitogen-activated protein kinase (MAPK) family and regulate a wide variety of intracellular processes from translation and microtubule stability to gene expression. The use of ERK pathway inhibitors at low micromolar concentrations resulted in the inhibition of both PTTH-stimulated and basal ecdysteroid synthesis in prothoracic glands studied *in vitro*. The path by which PTTH stimulates ERK phosphorylation has been investigated using prothoracic glands challenged *in vitro* with a Ca^{2+} ionophore and a cell-permeable cAMP analog at concentrations that stimulate ecdysteroid synthesis to maximal *in vitro* levels. The results indicate that Ca^{2+} influx-dependent processes, other than cAMP generation, probably play the major role in eliciting ERK phosphorylation and activity. At 50 to 100 µM the Ca^{2+} ionophore A23187 stimulates ERK phosphorylation 20- to 40-fold, levels that are also elicited by physiological doses of *Manduca* PTTH. Dibutyryl cAMP treatment (5 to 10 mM) results in a much lower stimulation of ERK phosphorylation (≈2-fold increase). However, simultaneous treatment of prothoracic glands with dibutyryl cAMP and a lower dose of A23187 (25 µM) results in ERK phosphorylation much higher than when these two reagents are administered singly (i.e. the observed increase in ERK phosphorylation indicates that cAMP and Ca^{2+}-dependent processes may act synergistically to activate the ERK pathway). The treatment of several organs other than prothoracic glands with A23187 does not result in large increases in ERK phosphorylation, suggesting that Ca^{2+} influx-dependent processes in the prothoracic gland are unique to this endocrine tissue. Incubation of prothoracic glands with GTP and GDP analogs indicates that PTTH-stimulated ERK phosphorylation likely involves the action of one or more GTP-binding proteins. Candidate GTP-binding proteins include a G-alpha protein, likely associated with the PTTH receptor, and a small G-protein such as *ras*.

Docking studies of putative ligands in the ligand binding pocket of the ultraspiracle protein

Full text available in the Journal of Insect Science 2:25.

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Ultraspiracle (USP) belongs to the superfamily of nuclear receptors which are ligand-inducible transcription regulators. USP is the insect homologue of the vertebrate retinoid X receptor (RXR). Like RXR, USP heterodimerizes with other receptors to form active receptor complexes. In particular, it is the key heterodimerization partner of the ecdysone receptor (EcR) implicated in development, reproduction, molting and metamorphosis of insects. The heterodimerization of EcR with USP is necessary for high affinity binding of ecdysteroids to EcR and transcriptional activity. In addition to EcR, USP also heterodimerizes with DHR38, another insect nuclear receptor. A fundamental question arises whether the activity of USP is mediated through ligand binding or not. Indeed, USP is an orphan receptor for which no endogenous ligand has been established yet unambiguously. For RXR, agonistic ligands like the 9-cis retinoic acid are known to bind to RXR and modulate the activity of the RXR dimeric partner. For USP, juvenile hormones (JHs) have been proposed as endogenous ligands which would directly modulate the activity of the EcR/USP complex. In fact, JHs, which belong to the family of terpenes just like the retinoic acid, have been known for a long time to prevent metamorphosis by modulating the ecdysteroid action at the outset of the ecdysteroid rise for the molt. Recently, the crystallographic structures of the ligand binding domain of USPs from the lepidopteran *Heliothis virescens* (hvUSP) and the dipteran *Drosophila melanogaster* have been solved. The stuctures show the canonical fold of nuclear receptor ligand binding domains. However, a major structural feature is observed in the USP structures for the first time. The segment connecting helices 1 and 3 adopts a position which precludes the canonical agonist conformation. The highly conserved sequence of this stretch suggests the functional relevance of this conformation unique to USPs. Moreover, the structures reveal the presence of a fortuitous copurified ligand which was identified as a phospholipid. Although this molecule is not the physiological ligand, it suggests that USP can bind ligands and therefore that it is most probably ligand-regulated. Furthermore, the ligand binding pocket determined experimentally allows the docking of molecules and the design of high affinity ligands. We present here a systematic study of the docking of JHs and JH analogs in the ligand binding pocket of USP. For the sake of comparison, we use as docking templates the crystal structure of hvUSP, as well as one model based on the crystal structure of hsRXR. The ligand binding cavities of USP and RXR have one part in common, close to helices 3 and 5, but the cavity is wider for USP. In fact, we show that some JHs and JH analogs can fit well in a USP-like cavity, whereas they sterically interfere with residues in a RXR-like cavity. The comparison of the docking results in both types of pockets leads us to propose an hybrid model which includes characteristics of both crystal structures. In particular, the pocket includes all important features of the pocket determined experimentally for USP, except that the region occupied by the phospholipid is shrunk to the size observed in the RXR structure. From the shape of the pocket and its chemical nature, interactions between ligand functional groups and residues belonging to the interior surface of the pocket are examined. Furthermore, the hybrid model is used as a template for modelizing the ligand binding domain of USPs from different types of insects.

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Identification and characterisation of ecdysteroid-regulated proteins in crustaceans using a proteomic-based approach

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It has been demonstrated that ecdysteroids are involved in regulation of yolk protein synthesis and reinitiation of meiosis in prophase I-arrested oocytes during ovarian development in crustaceans. However, despite these reports very little is known of the molecular effects of ecdysteroids on gene and protein expression in crustacean tissues. In order to characterise the effects of ecdysteroids on regulation of gene and protein expression in crustacean ovaries, we have undertaken a proteomic-based approach to identifying ecdysteroid-regulated proteins. For this, analysis of the proteome of ovary tissue was carried out using a combination of two-dimensional gel electrophoresis (2DE), image analysis of the 2DE maps to identify differentially regulated proteins and mass spectrometric analysis to generate *de novo* sequence information for the proteins of interest. For the experiments, a baseline map of the global changes in protein expression and ecdysteroid titre throughout ovarian development was produced using 2DE and radioimmunoassay, respectively. This map provides a reference for comparison any effects of *in vitro* incubation of ovary tissue, with physiologically relevant concentrations and ratios of ecdysteroids. This allows identification of those proteins whose expression changes following natural variation of ecdysteroids *in vivo*. Modulated proteins have been further characterised by *de novo* peptide sequencing, using tandem mass spectrometry.

Structural diversity of prothoracicotropins and bombyxins in Lepidoptera

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Amino acid sequences of prothoracicotropins (PTTH) and bombyxins have been elucidated in just a few species representing a couple of lepidopteran families. Related antigens have been detected immunohistochemically in various insect orders, indicating that the hapten regions are preserved. They probably correspond to conserved amino acid sequences that can be identified by comparing known homologues of the hormones. The conserved parts of the PTTH are located around the cysteine residues. An QxIxDPPCxC sequence is close to the N-terminus, NCxxxQxxCxxPYxCKE in the central region, and SVxCTRDY close to the C-terminus. Surprisingly, a long stretch of conserved sequence DYxNMxxNDVxLLDNSxETRTRKRG, which includes the cleavage site (underlined) and initial glycine of the PTTH, occurs at the end of the intercalated peptide that is assumed to have no function. The conserved regions of bombyxins are also found around the cysteines. The core of A-chain (total length 25-28 residues) has the sequence GxxxECCxxxCxxxxLxxYC and that of B-chain (total length 20-23 residues) YCGRxLxxxxxxxC. The C-chain, which is discarded during prohormone processing, is of different length but always includes a tetrapeptide WxWL (x = P, R or K) in its central part and a cleavage and arginine-amidating sequence RGKR at its carboxy-terminus. It must be emphasized that "x" does not indicate a random variation of amino acid residues. Residues alternating in most of the positions are of similar properties. For example, K may be replaced with R, V with I, S with T, D with E, etc. Another conserved feature is the distance between individual cysteine residues. This research has been supported by grant ME425 of the Czech Ministry of Education, Youth, and Sports.

A partially characterized small (0.9 kb) *EcR* transcript from the salivary gland of *Bradysia hygida* bears a small part of the LBD that is upstream of the two zinc fingers

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In *Bradysia hygida* (Diptera, Sciaridae), as in other Sciaridae, the process of gene amplification and DNA puff formation, in the salivary gland, is triggered by the molt hormone, 20-OH ecdysone (20-HE). Eight chromosomal sites develop the larger DNA puffs: C7, C5 and C4 are the first to expand and are called the first group of DNA puffs. When these three puffs had already receded, the second group of DNA puffs (A14, B3d, C6 and X4) starts its expansion. DNA puff B10 is an intermediary puff, being unique between the periods of activity of first and second groups. The salivary glands of *B. hygida* present three physiologically and morphologically different regions, S1, S2 and S3. Interestingly, the large DNA puffs develop only in S1 and S3 regions. In S2 the first group of DNA puffs is absent and the puffs of second group are very small. We have shown, in a recent paper (Carvalho et al., Insect Biochem. and Mol. Biol. 30, 541, 2000), that in vitro and in vivo, 20-HE, exerts opposite control on the activity of the two groups of amplified genes. Its presence is necessary for the first group activity and is strongly inhibitory for the second group. So, these characteristics: 20-HE is able to trigger the process of gene amplification in S1 and S3 regions, but not in S2; 20-HE stimulates the activity of a group of amplified genes and inhibits the activity of the other, make the salivary gland of *B. hygida* a very provocative model for the study of 20-HE and its receptors functions. In the accompanying Abstract (Carvalho et al., this meeting), we show that, in the salivary gland of *B. hygida*, two main transcripts (5.8 and 0.9 kb) were detected by a small (0.6 kb) cDNA probe from EcR gene (*BhEcR*). Two other main transcripts (1.7 and 1.3 kb) were detected by a (0.5 kb) probe from *Drosophila melanogaster usp* gene (*Dmusp*). Each of the four transcripts presents a specific pattern of expression in each of the salivary gland regions during the period of gene amplification and first and second group of DNA

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puffs activity. These facts, associated to other information from our laboratory and from the group of Dr. M.L. Paçó-Larson, were used to propose a model to explain the participation of 20-HE in the control of those events. Among the four transcripts detected, by no doubt, the *EcR* 0.9 kb is the more intriguing subject. It starts to be expressed during the time the first group of DNA puffs is in activity, its amount increases concomitantly with the regression of these puffs and attains its maximum when those puffs have already receded. At this time the DNA puffs of the second group are beginning their expansion. To the presumed small protein product of this transcript we attributed two main functions: 1) to contribute in the switching off of the first group of amplified genes; 2) to participate in the activation of the second group of amplified genes. Our prediction was that such small protein should be produced in large amounts and contain the DBD and/or part of the LBD, in order to, in some way, compete with the normal EcR. In this work we describe the partial characterization of the 0.9 kb transcript. Several independent experiments were performed and about 563 bp were sequenced. The sequence of amino acids was deduced and compared with a fragment, already characterized, from the cDNA of the 5.8 kb RNA. Unexpected features were found: a) in the DBDs the second zinc fingers are identical. In the 5.8 kb isoform, the first finger is identical to the one of *DmEcR*, in the 0.9 kb isoform it is completely different, presenting two repeats of the P-box motif; b) only part of the D domain is present in the 0.9 kb isoform and, interestingly, it contains the nuclear localization signal and a polyadenylation site; c) a very small part of the LBD is also present and carries the complete τi region. Surprisingly the LBD segment is not in its usual position, it precedes the DBD. These two domains are separated by a sequence formed by two repeats of 15 amino acids that are not present in other EcRs. The possible origin and functions of this isoform will be discussed. We thank FAPESP, CAPES and FAEPA for financial support.

Interaction of natural and synthetic agonists and antagonists with the ecdysteroid receptor in *Spodoptera* (Lepidoptera)

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The larvicidal toxicity of a series of *N-t*-butyl-dibenzoylhydrazines with various substituents in the two benzoyl moieties was measured against the beet armyworm, *Spodoptera exigua* (Hübner) and the cotton leafworm, *Spodoptera littoralis* (Boisduval). The substituent effects on pLD_{50}s were analyzed using the classical quantitative structure-activity relationship (QSAR) procedure. In another series of experiments, we measured the biological activity in cultured imaginal discs and in a continuous cell line of *Spodoptera*. In addition, the receptor-binding affinity was determined in competition with ^{3}H-ponasterone A. Several ecdysteroids (20-hydroxyecdysone, ponasterone A, makisterone A, inokosterone, cyasterone and ecdysone), the dibenzoylhydrazines (tebufenozide, methoxyfenozide, halofenozide and the unsubstituted RH-5849) and two brassinosteroids (24-epibrassinolide and 24-epicastasterone) were tested. In these *in vitro* bioassays, a good correlation was found between pEC_{50}s and pIC_{50}s. A structure-activity relationship model for the physiological action of dibenzoylhydrazines to mimic the insect molting hormone in Lepidoptera is discussed. We thank Dr. G.R. Carlson (PA, USA), Profs. J. Coll (Barcelona, Spain) and G. Adam (Halle, Germany), and the Fund for Scientific Research (Belgium) and the Japanese Ministry of Education, Science, and Culture.

Binding of ecdysteroids and insect growth regulators in the Colorado potato beetle

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The biological activity of several ecdysteroids (20E, PonA, MakA, Ino, Cya, E) and dibenzoylhydrazines (halofenozide, methoxyfenozide, tebufenozide and RH-5849) was tested on a continuous cell line of the Colorado potato beetle *Leptinotarsa decemlineata* (Say). For PonA, 50% of cell growth inhibition was measured at pEC_{50} (M) of 9.76. Among nonsteroids, halofenozide was the most potent against *L. decemlineata*. However, this compound was previously shown to be only weakly active against *Spodoptera* caterpillars and cells in vitro. In this study and for the first time in literature, a quantitative structure-activity relationship (QSAR) analysis was calculated for a coleopteran species in vitro using pEC_{50}s and pIC_{50}s for the hormone action of several ecdysteroids and ecdysteroid agonists. It was of interest to compare this with a QSAR equation from complete beetle larvae that we estimated before, and remarked differences with Lepidoptera are discussed. Molecular differences at the receptor site are hypothesized to explain differences in physiological activity of ecdysteroid-mimicking insect growth regulators (IGRs) in beetles as compared to caterpillars. These new insights should help in better understanding the toxicity of such new group of IGRs in controlling pest insects. The authors express thanks to Dr. G.R. Carlson (PA, USA), Dr. A. McIntosh (MI, USA), Prof. J. Coll (Barcelona, Spain), the Fund for Scientific Research (Belgium) and the Japanese Ministry of Education, Science, and Culture.

Invasion and development of *Heterodera avenae* in wheat as affected by a moulting hormone and plant extracts

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The effects of the moulting hormone, 20-hydroxyecdysone, and plant extracts on the invasion and development of *Heterodera avenae* in wheat (cv Egret) was tested. Infective stage juveniles (J2) of *H. avenae* were treated with 20-hydroxyecdysone, and leaf and root extracts of spinach and oats before inoculating onto wheat. In another

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test, *H. avenae* J2 were subjected to varying concentrations of 20-hydroxyecdysone and inoculated onto wheat. After 30 days from inoculation, the treatments had no effect on invasion except for the leaf extracts, which reduced invasion. However, development of the nematode was affected with only 2-7% of the treated J2 developed to J3 and J4 and none to adults. More than 50% of the controls (10% methanol and water) developed to J3, J4 and some to adults. J2 treated with spinach leaf extract did not invade the roots and only 2% of the J2 treated with oat leaves invaded. Before inoculation, J2 from these treatments were immobile. Incomplete and abnormal apolysis was observed in 20-hydroxyecdysone treated J2 in the soil and roots. This was observed in J2 in the soil as early as 7 days after inoculation. Root invasion and development of J2 treated with less than $2x10^{-4}$ mg/ml of 20-hydroxyecdysone did not differ from the controls. No abnormal moulting in J2 was observed at these concentrations. Therefore, apolysis or even ecdysis could be induced by 20-hydroxyecdysone in *H. avenae* at a concentration higher than $2x10^{-4}$. Plant extracts hindered penetration and development of the nematode although abnormal moulting was not observed even with spinach extract, which was confirmed to contain 20-hydroxyecdysone.

Application of fluorescent proteins for the study of nuclear receptor function: Nuclear transport of USP

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The transport of nuclear receptors (transcription factors) into the nucleus is mediated by a conserved nuclear localization signal present C-terminal to the second zinc finger of vertebrate nuclear receptors. In the vitamin D receptor an additional motif between the two zinc fingers has been described. Both regions are characterized by a prevalence of basic amino acids. In EcR and USP motifs with a high percentage of basic amino acids are present at comparable sites. Green (GFP) and blue (BFP) fluorescent proteins were coupled at the N-terminal end of DmUSP and the fusion product transfected into vertebrate cells. Fusion proteins with USP were found selectively in the nucleus. In contrast controls with GFP alone were equally distributed in cytosol and nucleus. Intracellular localization was studied with USP wild type and in vivo, missense mutations presumably involved in nuclear transport. The results were compared with in vivo effects of the same mutant. The role of nuclear transport for the biological action of USP during development and metamorphosis in *Drosophila melanogaster* is discusssed.

Expression and affinity purification of ecdysteroid receptor and ultraspiracle

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In vitro biochemical inv estigations of *Chironomus tentans* ecdysteroid receptor (cEcR) and ultraspiracle (cUSP) require substantial amount of functional, highly purified full length receptor proteins. Recombinant expression as fusion protein with glutathione-sulfotransferase (GST) or with polyhistidine-tag (his-tag) provide a feasible strategy for production and gentle purification (Arbeitman, M. N. 2000. Cell 101, 67-77; Grebe M. *et al.* (in press) Insect Mol. Cell. Biol.). Now, we aimed to optimize expression and purification to get enhanced yield for the considerably large and hydrophobic cEcR and cUSP proteins. GST-tagged receptor proteins were expressed in *Escherichia coli* strain BL21 and several culturing conditions, including composition of medium, temperature, oxygenization, as well as concentration of promoter IPTG, and time period of expression were compared. As alternative strategy, his-tagged receptor proteins were expressed in SF9 cells. Purity of the receptor preparates were analyzed by silver stained denaturing gel electrophoresis. Hormone- and DNA-binding functions were tested in radioligand binding and gel mobility-shift assays. Different approaches for affinity purification were examined and the results concerning yield and functionality were compared.

Cloning of ecdysone oxidase and 3DE 3β-reductase from *Drosophila melanogaster*

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In insects, moulting, development and aspects of reproduction are regulated by moulting hormones (ecdysteroids). The ecdysteroid titre exhibits distinct peaks at specific times in development. These changes in titre are regulated by the rate of ecdysteroid synthesis, inactivation and excretion. In midgut cytosol of Lepidoptera, ecdysteroids can be inactivated by ecdysone oxidase-catalysed conversion into 3-dehydroecdysteroids, which undergo 3-dehydroecdysteroid 3α-reductase-catalysed reduction to 3-epi-ecdysteroids. In most Lepidopteran species studied, the major ecdysteroid product of the prothoracic glands is 3-dehydroecdysone (3DE). 3DE 3β-reductase-catalysed reduction of 3DE to ecdysone is viewed as an important activation step. Previously, we have isolated the genes encoding ecdysone oxidase and 3DE 3β-reductase from the cotton leafworm, *Spodoptera littoralis*. We now wish to further investigate the significance of these enzymes in the ecdysteroid hormonal system in *Drosophila melanogaster*. The homologous genes encoding these enzymes in *D. melanogaster* were searched for in the genomic sequence based on the similarity of the amino acid sequences. 12 candidate sequences were discovered for ecdysone oxidase and 6 for 3DE 3β-reductase, but none of them had significantly high similarity to ecdysone oxidase or 3DE 3β-reductase. In order to find the homologous genes, we are screening these candidates using an *in vitro* expression cloning approach.

Corazonin affects the development by modulating the hemolymph ecdysteriod level during the spinning stage in the silkworm, *Bombyx mori*.

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Corazonin was first isolated from corpora-cardiacum (CC) of an American cockroach *Periplaneta americana* as the most potent cardiostimulatory peptide. Recently, it has been identified that the factor inducing dark color present in the brain and CC of the locust, *Locusta migratoria*, was [His^7]-corazonin by using an albino strain of *L. migratoria* as a novel bioassay. Furthermore, some factors identical or similar to corazonin are found to be present in the brain-retrocerebral neuroendocrine system and ventral nerve cord of 11 insect orders except coleopteran insects by implantation experiment and immunohistochemistry. These results suggest that corazonin has some physiological functions in many insects but the physiological functions of corazonin-like compounds in these insects other than locust and cockroach remain to be known. In the silkworm, *Bombyx mori*, [Arg^7]-corazonin has been identified from the larval brain and injection of corazonin into the final-instar larvae prolonged the spinning period but reduced the weight of cocoon layers. Even a 1 pmol of corazonin caused the significant prolongation of spinning period when it was injected 2 days before the initiation of spinning. However, injection of corazonin did not affect the duration of feeding period. In the larvae injected with corazonin, the haemolymph ecdysteroid increased slowly and reached to the peak level 1 day later than in the control larvae then decreased slowly during the spinning period. These results suggest that corazonin affects the development by modulating the ecdysteroid level during the spinning stage.

Prothoracicotropic hormone (PTTH) and prothoracicostatic peptide (PTSP) activities in the brain of the 5th instar larvae of eri-silkworm *Samia cynthia ricini*

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The prothoracic gland (PG) is the defined site of ecdysteroid biosynthesis in insect and its activity is regulated by intrinsic and extrinsic factors. PTTH activates the PG to synthesize and release ecdysone. Recently a peptide inhibiting ecdysteroid biosynthesis in the PG was isolated from the larval brains of silkworm, *Bomyx mori* (Hua et al., 1999). The present study aims at the isolation of the PTTH and PTSP factors from the larval brains of eri-silkworm, *S. cynthia ricini*. Brain exrtracts of 5th instar day 2 larvae were purified with Sephadex G-75 chromatography or Sep-Pak C18 fractionation and C 18 reverse-phase HPLC. The biological activity is monitored with in vitro bioassay-ecdysteroid RIA. Two separated fractions with PTTH and PTSP activities acting in a dosage-dependent manner respectively were recovered. Further purification is being undertaken.

A 2-D gel analysis of the effects of ecdysteroids on *Brugia malayi*

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Filarial parasites are responsible for several serious human diseases with symptoms such as lymphoedema, elephantiasis, and blindness. Over 130 million people are affected worldwide with over 500 million living in high risk areas. These nematode parasites require both an insect and a mammalian host for their development. An understanding of how they cross developmental checkpoints may suggest potential targets for intervention. Previous work by Huw Rees and colleagues (Barker, G., et. al., Parasitol. Res., **77**, pg 65, 1991) has demonstrated an increase in microfilarial production when *in vitro* cultured adult female *Brugia* were treated with ecdysone. We have extended these experiments by 2-D gel and 2-D western blot analysis. Adult female and male *B. malayi* worms were cultured in the presence of ^{35}S-methionine and treated with various ecdysteroids and analogs. We observe distinct differences in the 2-D pattern of proteins between treated and untreated females. These differences are reproducible and different for different compounds. No differences were observed when females were treated with β-estradiol or males treated with 20-hydroxy-ecdysone. Western blot analysis reveals isoelectric point shifts for some *B. malayi* proteins such as Bmnhr3 (a homologue of insect EcR), but not for other proteins such as cyclophilin.We are continuing these analyses with the goal of identifying more ecdysone-affected proteins.

Addressing the role of FTZ-F1 via induced ectopic expression and RNAi in transgenic *Bombyx mori*

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Genetic evidence for the developmental roles of ecdysone response genes thus far derived exclusively from studies on *Drosophila melanogaster*. The newly pioneered germline transformation using the transposon *piggyBac* and the discovery of RNA interference (RNAi) now open ways for causal tests of gene functions also in non-drosophilid insects. One can interfere with the normal function of a gene by overexpressing its wild-type or dominant-negative version or silence the gene via RNAi by expressing its double-stranded (ds) RNA. The two prerequisites for this approach are the ability (1) to transform the insect, and (2) to drive the ectopic expression of the transgene. We achieved stable germline transformation of the silkmoth *Bombyx mori* with the *piggyBac* transposon vector 3xP3-EGFP [Horn C. et al. 2000. Dev Genes Evol 210: 623-629] and showed that the *D. melanogaster hsp70* promoter can be used for heat-inducible transgenic expression in live silkworms. We chose to overexpress the *Bombyx* nuclear receptor Ftz-F1 (BmFtz-F1). *ftz-f1* is an ecdysone response gene required for metamorphosis in *D. melanogaster* (Broadus J. et al. 1999.

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Molecular Cell 3: 143-149; Yamada M. et al. 2000. Development 127: 5083-5092) and for molting in both *D. melanogaster* and the nematode *Caenorhabditis elegans* (Asahina M. et al. 2000. Genes to Cells 5: 711-723). BmFtz-F1 is induced by the decline of ecdysteroid titer during larval and metamorphic molts (Sun GC. et al. (994. Developmental Biology 162: 426-437) and presumably is also important for these processes in *B. mori*. The BmFtz-F1 coding sequence was cloned between the *hsp70* promoter and the terminator in the *piggyBac* vector, and transgenic larvae carrying this construct were tested for heat-inducible BmFtz-F1 expression. The transgenic BmFtz-F1 mRNA was induced by 60-90 minute exposures of second, fourth and fifth instar larvae to 42 °C. Although basal expression at 25 °C was detectable by RT-PCR, Northern blot hybridization showed only the heat dependent expression. Low levels of the mRNA were still present 6 hours after the heat treatment. Immunostaining of epidermis using anti-BmFtz-F1 antibody showed an increase of nuclear signal 90 minutes after a heat shock. We will present our current analysis of ectopic gene expression and phenotypic changes in the heat shock-BmFtz-F1 animals. The above results suggested that the *hsp70* promoter may be sufficient for RNAi and encouraged us to transform *B. mori* with a *piggyBac* vector for heat-inducible expression of the BmFtz-F1 dsRNA (Lam G, Thummel CS 2000. Currrent Biology 10: 957-963). In this construct, 820-bp portions of the BmFtz-F1 cDNA are cloned in two copies, oriented head-to-head and separated by an artificial 360-bp intron. Results obtained with the RNAi approach will also be presented.

Time of intermolt, survival and hemolymph parameters of juveniles of *Carcinus aestuarii* under exposure to β-ecdysone in laboratory conditions

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It has been shown that moulting of juvenile crabs and probably even of larvae are under the control of β-ecdysone. In this work, we have tested the hypothesis of a sex-specific pheromonal action of the moulting hormone as a synchroniser of moulting for female crabs addressed by our preliminary findings. In fact, after Kittredge et al. (1971) report on crab behavioural displays, we have measured sex-specific respiratory responses after bath treatment with *pM* and *nM* concentrations of β-ecdysone, suggesting a possible role as a sex-pheromone. The chemical structure of the female pheromone of *Carcinus aestuarii* has not been described but it plausibly could be some conjugated form of the moulting hormone, by analogy with sexual chemical communication in fishes. Sex-specific responses to B-ecdysone treatment by either bath or by injections indicate that it is not an endogenous action of this hormone after drinking the moulting hormone itself, but a more specific pheromonal action on external receptors. This effect occurs in the antennules, binding specific receptors with astonishing affinity (till 10^{-13} m). Samples of hemolymph were extracted from the arthrodial membrane of the second leg and hemolymph osmolarity and lactate concentration were measured.

Molecular and biochemical characterization of two p450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*

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As many as seven different enzymatic activities, catalyzed by both microsomal and mitochondrial cytochrome P450 monooxygenases, have been hypothesized to be implicated in the biosynthesis of ecdysone, although none have been characterized completely, either at the biochemical or molecular levels. The present data show that the wild type genes mutated in two members of the Halloween Family of cuticular embryonic lethals, *disembodied* (*dib)* and *shadow* (*sad)*, code for P_{450} enzymes that catalyze the last two hydroxylation reactions in the ecdysteroidogenic pathway in *Drosophila melanogaster*, namely the C_{22}- and C_2-hydroxylases, respectively. *Sad* and *dib* expression is limited to the prothoracic gland component of the embryonic and larval ring glands and the follicle cells of adult ovaries, sites of endogenous ecdysteroid production. When transfected into *D. melanogaster* S2 cells, *sad* (CYP315A1) metabolizes 2-deoxyecdysone (2dE) to ecdysone (E), while *dib* (CYP302A1) converts the ^{3}H-ketotriol (2, 22-dideoxyecdysone) to ^{3}H-2dE. These data were confirmed when cells transfected with both *dib* and *sad* were shown to transform the ^{3}H-ketotriol to ^{3}H-E in high yield. Consistent with prior partial biochemical characterizations, these enzymes appear to be targeted to the mitochondria, based on their charged and hydrophilic N-terminal residues as well as their sequence similarities to other known mitochondrial cytochromes in both insects and humans. We are presently investigating the involvement in *D. melanogaster* ecdysteroidogenesis of three other P_{450} enzymes shown to be the targets of mutations in the related Halloween mutants, *spook*, *phantom* and *shade*.

Misexpression of the broad-complex provides new insights into the control of pupal development and juvenile hormone action

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The Broad-Complex (BR-C) set of transcription factors are induced by ecdysone in *Drosophila melanogaster* at the time of the mid-third instar transition and persist through the pupal development period during which time they are required for normal development. They then disappear as adult development begins. Similarly, in *Manduca sexta* epidermis, they first appear at the time of pupal commitment, persist through pupal cuticle formation, then disappear with the onset of adult development. When treated with the juvenile

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hormone (JH) mimic pyriproxifen before the onset of adult development, *M. sexta* pupae formed a second pupal cuticle and *D. melanogaster* pupae formed a second pupal abdomen but the head and thorax were adult. In both species BR-C was re-induced in the tissues that repeated pupal development. Heat-shock misexpression of the BR-C Z1 isoform during adult development in *D. melanogaster* (45-60 hr after puparium formation) mimicked the application of JH in that it induced the formation of a second pupal cuticle. This included the reinduction of the pupal cuticle genes *Edg78E* and *pcp* and the suppression of the adult cuticle gene *Acp65A*. Misexpression of BR-C Z2 caused re-expression of the two pupal cuticle genes but no suppression of the adult cuticle gene, whereas misexpression of BR-C Z3 had its primary effect in the suppression of the adult cuticle gene. In both cases the resultant cuticle was adult in morphology. Misexpression of BR-C Z4 had relatively little effect. By contrast, misexpression of each isoform earlier during the onset of bristle outgrowth caused severe truncation of the bristles. Thus, BR-C is sufficient to prevent adult-specific differentiation and also to redirect the pupal program in epidermis that has already begun adult differentiation. Similarly, when BR-C Z1 was misexpressed during the second instar larval molt, the pupal cuticle gene *Edg78E* was prematurely expressed and the animals could not ecdyse. None of the other isoforms caused expression of *Edg78E* but all prevented ecdysis to the third larval stage. Thus, BR-C is both necessary and sufficient to specify pupal development, but the different isoforms play different roles. Therefore, the ability of JH to regulate the transitions both into and out of this stage appear to be mediated through its actions on the expression of this gene complex. Supported by NIH.

Ecdysteroid distribution during development of perennial species *Silene frivaldszkyana*

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Silene frivaldszkyana is characterised by high ecdysteroid level and rich composition. These plants good acclimatize in siberian climate conditions. During the growth and development of S.frivaldszkyana phytoecdysteroids are dynamically cycled between different organs and in various leaves pairs too. The quantity of 20-hydroxyecdysone (mg per plant) increase in first, second,third and fourth generative leaves pairs during the first 42 days (the beginning of vegetation), but in fifth and sixth leaves until 66 days. The levels of 20-hydroxyecdysone decrease to 77 days in the flowering phase in all leaves pairs. Maximum quantity of 20-hydroxyecdysone was observed in the third leaves. The 20-hydroxyecdysone content (%, dry weigh) and quantity (mg per plant) in vegetative leaves decrease until the flowering period and quickly increase to the end of vegetation because ecdysteroids make for the winter leaves. We shall discuss ecdysteroid distribution in all organs *S. frivaldszkyana* at comparison with annual Silene species.

Chemotaxonomic value of phytoecdysteroid profiles in the genus *Silene* (Caryophyllaceae)

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The genus *Silene* consists of ca. 700 species. It is the plant genus which has been most extensively studied for the presence of ecdysteroids and many phytoecdysteroids were first isolated and identified from *Silene* spp. Previous tlc analyses and ecdysteroid agonist bioassay data for species in this genus had shown that many species belonging to the sections sclerocalycinae and otites accumulate ecdysteroids. The availability of many indigenous and naturalised species belonging to this genus prompted us to undertake a more detailed study in order to evaluate the possible chemotaxonomical value of ecdysteroid patterns in the genus *Silene*. An extensive study of ecdysteroid profiles was undertaken on *Silene frivaldszkyana, S. Chlorifolia, S. Radicosa, S. Oligantha, S. Tatarica* (Sect. Sclerocalycinae) and *S. Roemeri, S. sendtneri, S. Otites, S. Pseudotites* (Sect. Otites), which included isolation and spectroscopic characterization of as many compounds as possible in all these species. These analyses showed that the same major components (20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone, ecdysone, 2-deoxyecdysone, and polypodine B) are present in the majority of these species. On the other hand, minor components, such as various conjugates (acetates, benzoates and glycosides), have a more restricted distribution and can be to some extent correlated with the systematic position of species within the *silene* genus. We shall discuss the chemotaxonomic significance of phytoecdysteroid both in relation to the specific example of the genus *silene* and more generally in the plant world. This research was supported by EU-INTAS.

The organization of silk fibroin in pyralid moths

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The excellent mechanical properties of insect silk depend on the molecular arrangement of heavy-chain fibroin. Regular iterations of short amino acid repeats in the fibroin molecule allow formation of β-sheets and their stacking into crystallites reinforcing the silk fiber. The fibroins of *Bombyx mori* silkworms contain GAGAGS repeats and those of *Antheraea* silkmoths contain stretches of 5-15 alanine residues. Very different fibroin organization was found in the moths *Galleria mellonella* and *Anagasta kuehniella* of the family Pyralidae. The core of their fibroins consists of three long and highly

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ordered repeats. In *G. mellonella*, the A type repeat (63 amino acid residues) alternates with B_1 (43 residues) and B_2 (18 residues) repeats in about 12 assemblies $AB_1AB_1AB_1AB_2(AB_2)AB_2$ that make up most of the 500 kDa fibroin. The core of *A. kuehniella* fibroin consists of homologous and similarly alternating repeats *A*, B_1 and B_2. The *A* and B_2 repeats are of similar length as the A and B_2 repeats of *G. mellonella*. The B_1 repeat is considerably longer (75 residues) than B_1 but sequence homologies between the two are obvious. Sequence similarities among all repeats suggest that they have evolved from a common ancestral sequence. Fibroin amino acid composition is dominated in both species by G, A, and S and residues with long hydrophobic side chain (L, I and V). The fibroin of *A. kuehniella* is enriched in P, N, and Y that occur in some of the positions occupied by G, A, and L in *G. mellonella*. Pleated β sheets and crystallites might be formed by alanine arrays such as SSAASAAAA that are present in both species or by the G-rich motifs such as GLGGLG present in *G. mellonella*. The fibroin of *A. kuehniella* is characterized by relatively high frequency of the triplets GPY, GPN and GLN. Conservation of the motif PVIVIED in the repeats A, *A*, and B_1 indicates the importance of hydrophobicity and electrostatic charge in H-fibroin cross-linking. The research was supported by grant 204/00/0019 of the Grant Agency of the Czech Republic.

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