Ecdysteroids govern two phases of eye development during metamorphosis of the moth, *Manduca sexta*

David T. Champlin* and James W. Truman

Department of Zoology, University of Washington, BOX 351800, Seattle, WA 98195-1800, USA

*Author for correspondence (e-mail: champd@zoology.washington.edu)

Accepted 19 March; published on WWW 6 May 1998

INTRODUCTION

Many aspects of development are governed by hormones. Their importance is revealed by the number of developmental disorders that arise when too little or too much of a specific hormone is produced (e.g. Besser and Thorner, 1994). In normal development, the most dramatic examples of hormonal control are found in the metamorphosis of insects and amphibia (Gilbert et al., 1996). During metamorphosis, too little hormone can retard or block development while too much hormone can lead to a dramatic derangement of normal development (Kollros, 1961; Williams, 1968). Recently, the study of several developmentally important hormones, including those that cause metamorphosis, became unified upon discovery that their receptors are members of the highly conserved nuclear receptor gene family (Mangelsdorf et al., 1995).

Insect metamorphosis is caused by steroid hormones, the ecdysteroids, whose action is mediated by a heterodimeric nuclear receptor composed of the ecdysone receptor (EcR) (Koelle et al., 1991) and ultraspiracle (USP) (Yao et al., 1992). Research on the ecdysteroids has been guided for many years by a model for ecdysteroid action proposed by Ashburner and colleagues (1974), in which the ligand-bound receptor complex directly activates transcription of a set of primary response (‘early’) genes. Some of the early genes encode transcription factors that then activate a cascade of downstream gene expression. The cloning of EcR and early genes from several insect species has allowed the key elements of the Ashburner model to be confirmed and refined (Thummel, 1996). The studies that established these principles focused on the role that peaks in blood ecdysteroid titer play in triggering differentiative and maturational responses such as cuticle synthesis by the epidermis, eversion of imaginal discs, chromosome puffing in salivary glands and process elongation by cultured cells (e.g. Riddiford, 1978; Fristrom and Yund, 1976; Ashburner et al., 1974; Cherbas and Cherbas, 1981). Several early genes are indeed induced by the peak in blood ecdysteroid titer that triggers these responses at the end of the larval stage (Thummel, 1996). However, early genes vary in their sensitivity to induction by ecdysteroid (Karim and Thummel, 1992), and other early genes are induced much earlier in development by the low levels of ecdysteroid that precede the metamorphic surge (Andres et al., 1993).

Metamorphosis of the faceted compound eye is similar in the fruit fly, *Drosophila melanogaster* (Wolff and Ready, 1993) and the moth, *Manduca sexta* (Monsma and Booker, 1996), and spans the time period when these different sets of early genes are induced. Initially, the eye primordium is an unpatterned monolayer epithelium. Beginning in the final larval instar, a wave of proliferation and patterning sweeps across the epithelium, leaving in its wake evenly spaced neural cell clusters...
that will eventually form the individual facets (ommatidia) of the adult eye. The anterior edge of this wave is marked by an axial indentation of cells, termed the morphogenetic furrow. Eye development is inherently asynchronous as the furrow is progressing across the primordium because cells behind the furrow are organized sequentially into immature ommatidial clusters while cells anterior to the furrow are not yet committed. After the furrow has traversed the primordium, immature ommatidia throughout the primordium then undergo fairly synchronous maturation of adult eye structures such as rhabdomere formation and synthesis of screening pigments. Here we show that furrow progression and ommatidial maturation are both dependent on ecdysteroids during metamorphosis of *Manduca* but have different steroid requirements. Low to moderate levels of ecdysteroid are required to sustain furrow progression while high levels trigger maturation of the ommatidia. These findings reveal a striking similarity between ecdysteroid control of insect metamorphosis, and thyroid hormone control of amphibian metamorphosis, and are thus likely to have broad implications for the mechanisms by which the ligands of nuclear receptors regulate development.

**MATERIALS AND METHODS**

**Experimental animals**

Larvae of the tobacco hornworm, *Manduca sexta* (L.), were reared in individual containers on an artificial diet at 26°C under long-day conditions (17 hours light:7 hours dark) for continuous development or under short-day conditions (12 hours light:12 hours dark) to induce diapause (Bell and Joachim, 1976). Animals were chilled on ice prior to injection or dissection.

The age of animals was designated with reference to developmental transitions: ec dysis to the final larval instar or pupa (V+0 and P+0, respectively), and onset of wandering behavior (W+0). For example, P+1 day animals had pupated approximately 24 hours earlier.

**Ecdysteroids**

20-hydroxyecdysone (20E; Sigma, St Louis, MO) and ecdysone (Fluka, Buchs, Switzerland) were dissolved in 10% isopropanol and 100% ethanol, respectively, and their concentrations measured spectrophotometrically (E240 =12,670 and E 242 =12,388, respectively). This source of ecdysone is contaminated with less than 1% 20E (R. Lafont, personal communication).

**Culturing of eye primordia**

Pupae were surface-sterilized in 95% ethanol on ice for approximately 3 minutes and rinsed in cold, sterile saline. Dissections were done in cold Levine-modified Weaver’s saline (Levine and Truman, 1985). For stages prior to P+3 days, eye primordia were dissected and cultured with the attached pupal cuticle. Dissected tissues were rinsed twice in Grace’s medium. A few crystals of phenylthiourea were included (Sigma, St Louis, MO). For extended culturing, tissues were transferred to fresh medium every 3 days.

We used Grace’s medium (Grace, 1962), which includes 5% heat-inactivated hemolymph from diapausing pupae. Hemolymph was collected from *Manduca* pupae that had been in diapause approximately 1 month and had been scored for the absence of apolysis of the wings (an indication that development had not resumed). The proboscis was cut and hemolymph collected in a cold microcentrifuge tube containing a few crystals of phenylthiourea. The hemolymph was centrifuged for 10 minutes at 4°C and the supernatant stored at −70°C. Prior to use, frozen samples were heat-inactivated at 60°C for 5 minutes, chilled on ice, and centrifuged for 5 minutes at 4°C. The supernatant was used to prepare medium, which was then sterile-filtered and stored at 4°C. Medium was used within 2 weeks.

**Detection of cells in S phase and M phase**

Cells in S phase were identified by the incorporation of 5-bromodeoxyuridine (BUdR) (Sigma, St Louis, MO) (Gratzner, 1982). Eye primordia were pulsed with BUdR either by injection (250 µg BUdR/g body mass) or by addition to the culture medium (15 µg BUdR/ml). Primordia were fixed overnight at 4°C in 4% parafomaldehyde in phosphate-buffered saline, pH 7.0 (PBS; 130 mM NaCl, 7 mM Na2HPO4, 3 mM NaH2PO4). Fixed eye primordia were processed for BUdR immunocytochemistry as described previously (Hegstrom and Truman, 1996a,b).

Cells in M phase were identified with a 1:4,000 dilution of anti-phosphorylated histone H3 antibody (Hendzel et al., 1997) (Upstate Biotechnology, Lake Placid, NY). Primordia were fixed and processed for immunocytochemistry as above, except that the acid treatment was omitted.

**Analysis of eye maturation**

Precocious maturation of the eye was induced in vivo either by injection of pupae with 10 µg 20E/g body mass or by removal of the source of juvenile hormone (JH) by surgical allacteotomy about 35 hours prior to ec dysis to the final larval stage (Kiguchi and Riddiford, 1978).

Eye primordia to be sectioned were embedded in 8% low-melting point agarose (AT Biochem, Malvern, PA) in HistoPrep molds (Fisher, Pittsburgh, PA). 100 µm horizontal sections were then cut using a Vibratome 1000 (TPI, St Louis, MO) and mounted onto pig skin gelatin-coated coverslips. Sections were stained overnight at 4°C with 5 units/ml BODIPY FL phallacidin (Molecular Probes, Eugene, OR), washed and mounted in 80% glycerol/2% n-propyl gallate.

Since the surface of the mature eye is curved, dissected eye tissues were flattened under a coverslip in order to measure their size. Images of the eyes were collected and analyzed using PhotoShop (Adobe, Mountain View, CA). The surface area was calculated by highlighting the pigmented region using the magic wand tool and scoring the number of pixels in the highlighted area.

**Northern blots**

RNA extraction and northern blot analysis were as described by Palli et al. (1992). Blots were probed with radiolabeled cDNAs of EcR (Fujiiwara et al., 1995) and MHR3 (Palli et al., 1992), and the level of their expression determined using a Molecular Imager Systems, model GS-363 (Bio-Rad, Hercules, CA). The amount of RNA loaded in each lane was normalized by probing the blots with radiolabeled cDNAs of *Drosophila melanogaster* RP49 (O’Connell and Rosbash, 1984) and β1 tubulin (DiBenedetto et al., 1987). The *Drosophila* probes were used at reduced stringency by decreasing formamide in the hybridization buffer to 30% and increasing the washes to 0.5x SSC (75 mM NaCl, 7.5 mM sodium citrate, pH 7.0). Under these conditions, the *Drosophila* probes each identified a single band similar in size to the corresponding *Drosophila* mRNA and which were found to remain constant relative to total RNA under different hormone conditions.

**RESULTS**

**Timing of normal eye development in Manduca**

Fig. 1 shows the timing of developmental events in the adult eye primordium of *Manduca* (Monsma and Booker, 1996, and
Briefly, proliferation in the primordium starts early in the last larval stage as the levels of juvenile hormone decline. The morphogenetic furrow then begins to move anteriorly across the growing primordium as the larva enters the wandering stage and steroid titers rise. Behind the furrow, cells become organized into immature ommatidia. This wave of proliferation and patterning takes about 9 days to traverse the width of the presumptive eye, extending through pupal ecdysis into early adult development. Shortly after furrow progression is completed, maturation of the ommatidia occurs throughout the patterned epithelium. The screening pigments and lens are first...
apparent on P+8 and the photoreceptor rhabdomeres are forming by P+9 days. Each of these components appears fairly synchronously in ommatidia throughout the eye.

Ecdysteroid-dependent furrow progression in culture

To define the role of ecdysteroids in regulating furrow progression, we employed a tissue culture system similar to that used for Manduca epidermis (Riddiford et al., 1979). Eye primordia from P+1 day animals were cultured in medium supplemented with various concentrations of 20E for 30 hours and then the state of furrow progression was assessed. Proliferation of cells flanking the morphogenetic furrow is coordinated with furrow progression (Thomas et al., 1994; Monsma and Booker, 1996) and, thus, markers of cell cycle progression provide an indirect marker for furrow progression. We monitored cell cycle progression by incorporation of the nucleotide analogue, BUdR, into S phase cells followed by immunocytochemistry with an antibody to BUdR (Gratzner, 1982) or by staining of M phase cells with an antibody to phosphorylated histone H3 (Hendzel et al., 1997). As seen in Fig. 2A, a band of S phase cells is evident immediately posterior to the furrow while a more diffuse band is located anterior to the furrow. A similar pattern is also evident for mitotic cells (Fig. 2F).

To sustain furrow progression in culture, we needed to maintain the concentration of 20E between $1.2 \times 10^{-7}$ M and $2 \times 10^{-6}$ M (Fig. 2). Outside of this range, we found little or no BUdR incorporation in cells flanking the furrow, even when the labeling period was extended through 24 hours. In contrast, when eye primordia were cultured with 20E concentrations within the ‘proliferative’ range, a high number of S phase and M phase cells, comparable to that observed in freshly dissected primordia were detected flanking the furrow (Fig. 2). Their frequency appeared to be fairly constant regardless of whether primordia were cultured at the high or low end of the proliferative range (Fig. 2C,H versus D,I). To confirm that the furrow was indeed advancing in culture, primordia were exposed to a second pulse of BUdR 13 hours after the first. The distance between cells labeled by the two pulses of BUdR was similar regardless of whether primordia were cultured in 20E concentrations at the low (Fig. 2L) or high (Fig. 2K) end of the proliferative range, showing that the rate of furrow progression was independent of steroid concentration within this range.

Both ends of the proliferative range show sharp thresholds beyond which the furrow is arrested. The type of arrest, though, is different for the lower and upper ends of the range. The concentration causing 50% arrest (EDS0) for the lower threshold was about $1.2 \times 10^{-7}$ M 20E and was similar for eye primordia isolated either from P+1 or P+4 day animals. Furrow progression stopped in concentrations of 20E below this threshold, but resumed any time the 20E concentration was shifted up into the proliferative range (data not shown). In contrast, the furrow arrest caused by concentrations of 20E above the proliferative range was not reversed when the concentration of 20E was shifted down into the proliferative range even though the furrow had traversed only a portion of the eye primordium (data not shown).

Ecdysone, the precursor for 20E, is widely regarded as an inactive prohormone (reviewed in Smith, 1985; Grieneisen, 1994). However, we noted that the furrow continues advancing early in the pupal stage when ecdysone is essentially the only ecdysteroid detected in the blood (Warren and Gilbert, 1986). Fig. 3 shows that ecdysone was also able to maintain furrow progression in culture at concentrations above a threshold of $6 \times 10^{-7}$ M, a concentration about fivefold higher than the threshold for 20E. However, in contrast with 20E, high concentrations of ecdysone did not cause furrow arrest even at the highest levels that we tested ($2 \times 10^{-5}$ M ecdysone; Fig. 3).

Interruption of furrow progression during diapause

Manduca larvae can be programmed by short-day length to enter a diapause (overwintering) state shortly after they pupate (Bell et al., 1975). Animals destined to diapause are indistinguishable from those programmed by long-day length for continuous development until P+2 days (Bowen et al., 1985), and the morphogenetic furrow continues advancing through this time (Fig. 4A). Prothoracicotropic hormone, the neuropeptide that stimulates ecdysteroid synthesis, fails to be released in short-day animals on P+2 days, the ecdysteroid titer drops to low levels, and diapause ensues (Bowen et al., 1984). By day P+3, furrow progression had ceased in short-day animals at a point about one-third of the way across the primordium (Fig. 4B). The morphogenetic furrow then remains arrested during the months that pupae are in diapause even though the animals may remain at the normal rearing temperature. Diapausing pupae can be induced to resume development at any time by injection of physiological doses of ecdysteroid (Bradfield and Denlinger, 1980). In such animals, incorporation of BUdR in cells flanking the furrow was detected within 12 hours of injection (Fig. 4C).

The concentration of 20E needed to stimulate resumption of furrow progression in eye primordia isolated from pupae in diapause was identical to that needed to maintain furrow progression in primordia isolated from long-day animals (open squares in Fig. 2). In both cases, the EDS0 for stimulating furrow progression was about $1.2 \times 10^{-7}$ M 20E/ml. Consequently, it appears that the furrow arrest seen during diapause is maintained solely by the lack of ecdysteroid.

Precocious eye maturation in culture

Concentrations of 20E above $2 \times 10^{-6}$ M that caused irreversible furrow arrest (Fig. 2) also triggered maturation of ommatidial primordia from P+1 day animals were cultured with various concentrations of ecdysone. After 30 hours in culture, primordia were pulsed with BUdR for 2 hours followed by immunocytochemistry to detect BUdR-labeled nuclei. The graph shows the dependence of BUdR-labeled nuclei flanking the furrow on the ecdysone concentration (closed circles). Each point is the average of ten primordia from independent animals. Scoring as in Fig. 2. For comparison, the data on 20E-dependent BUdR-labeling from Fig. 2 are included as open circles and a dashed line.
components posterior to the furrow. For example, culture of eye primordia above this threshold stimulated production of the screening pigments (Fig. 5B). These first appeared about 45 hours after exposure to 2.4x10^-6 M 20E. Similar kinetics of pigment synthesis were observed at higher concentrations of 20E (2x10^-5 M 20E). In contrast to the tonic requirement of ecdysteroid needed to sustain furrow progression, the maturation response was irreversible and continued even when ecdysteroid was removed. For example, screening pigments appeared at the same time whether the eye primordia were cultured continuously at 2.4x10^-6 M 20E or if 20E was washed out after the initial 10 hours.

Production of other components of the mature eye had ecdysteroid requirements similar to that for the screening pigments. For example, 20E concentrations above about 2x10^-6 M 20E stimulated synthesis of the cuticular lens and production of rhabdomeres by the photoreceptors (Fig. 5D), but these components were not made when primordia were cultured with concentrations of 20E below about 1.2x10^-6 M even after up to 12 days in culture (Fig. 5C).

Eye primordia cultured in very high levels of ecdysone (2x10^-3 M) failed to show furrow arrest (Fig. 3) and also failed to stimulate production of lens, screening pigments or rhabdomeres (not shown).

Precocious eye maturation in vivo

During the prepupal peak (Fig. 1), the ecdysteroid titer rises above levels that cause eye maturation in culture but precocious maturation does not occur. Precocious maturation is prevented by the simultaneous increase in juvenile hormone (JH) (Kiguchi and Riddiford, 1978; reviewed in Riddiford, 1996). Larvae were surgically allatectomized just before ecdysis to the final larval stage so they would go through the larval-pupal transition in the absence of JH. In such animals, the area of the eye primordium posterior to the furrow was already showing the start of ommatidial maturation at the time of pupal ecdysis (8 days earlier than normal), as indicated by the initial synthesis of screening pigments (Fig. 6B).

Following the prepupal ecdysteroid peak, JH disappears and injection of high levels of 20E can now cause furrow arrest and precocious maturation. As shown in Fig. 6, the size of the resulting eye was a function of the time during development at which the maturation program was triggered. The size of such eyes appears to reflect the area that the morphogenetic furrow had covered at the time of high 20E treatment. Cells in the primordium that were presumably ahead of the furrow responded to the high 20E levels by making a scaleless head cuticle. Although the size of the eye was reduced when maturation was triggered prematurely, ommatidia appeared to mature normally (except in the area immediately posterior to the furrow) (Fig. 6E). Irrespective of the age of the animals (P+0 to P+6), screening pigments were first apparent 2 days after injection of high levels of 20E.

A dramatic derangement of metamorphosis is evident in several tissues in addition to the eye following injection of high levels of 20E into early pupae. Precocious retraction of the epidermis occurs followed by premature synthesis of an adult cuticle lacking scales. The ganglia of the CNS retain a pupal organization and fail to fuse. Optic lobe neuroblasts undergo premature apoptosis (Champlin and Truman, 1998). The gut retains its larval organization. The consistency of the fat body becomes more similar to the adult but the cells are not dispersed into the hemolymph. In contrast to the effects observed with 20E, injection of even extremely high levels of ecdysone (up to 70 μg/g body mass) early in the pupal stage failed to disrupt normal development.

Development of ommatidia flanking arrested furrows

Behind the furrow, cells become organized and differentiate into immature ommatidia. We used expression of a neuron-specific marker, ELAV (Robinow and White, 1991), to examine the state of differentiation of ommatidial photoreceptors behind the furrow. In Manduca, ELAV staining is specific for neurons but is cytoplasmic rather than nuclear as in Drosophila. Staining of eye primordia in which the furrow was advancing revealed a graded distribution behind the furrow similar to that observed in Drosophila eye discs (Fig. 7A). This staining pattern is interpreted to reflect a progressive specification and organization of the photoreceptors in successive rows of ommatidia behind the furrow. We found a similar graded distribution of ELAV staining regardless of whether the furrow was advancing or if it had been arrested in low ecdysteroid either in culture or in vivo during diapause (Fig. 7A-C). Propidium iodide staining of nuclei in arrested primordia did not reveal an obvious point in assembly of ommatidial clusters that was ecdysteroid-dependent. Rather, moderate levels of ecdysteroid appear to be required for steps throughout the assembly process.

Development of ommatidia close to the furrow was strikingly different when the furrow was arrested by high levels of 20E. Differentiation of ommatidia behind the furrow continued and high levels of ELAV staining was seen in cells immediately posterior to the furrow (Fig. 7D). In these tissues, ommatidia distant from the furrow contained the proper number of cones cells and photoreceptors (four and nine, respectively, Fig. 7E,F). In contrast, ommatidia in the first several rows behind the furrow contained from zero to seven cone cells and as few as three or as many as 15 photoreceptors (Fig. 7G,H). Therefore, cells immediately behind the furrow were able to mature in response to high levels of 20E even before the entire set of ommatidial cells had been specified, but this often disrupted the proper cell-specification and assembly of ommatidia.

Concentration-dependent induction of early genes in the eye

How can different concentrations of 20E evoke such different responses by cells of the eye primordium? We examined expression of early genes in eye primordia that were cultured with different levels of 20E in the presence of anisomycin, an inhibitor of protein synthesis. Different early genes were induced at concentrations that correspond to the thresholds for furrow progression and for ommatidial maturation, respectively. For example, MHR3 is the Manduca homologue of the Drosophila nuclear receptor DHFR3 (Palli et al., 1992), an early gene that plays an integral role in maturational responses to ecdysteroid (White et al., 1997; Lam et al., 1997). Fig. 8 shows that the induction of MHR3 mRNA requires high concentrations of 20E in the range needed to trigger maturation. In contrast, direct upregulation of the ecdysone receptor mRNA occurs at 20E concentrations in the range that maintains furrow progression (Fig. 8).
DISCUSSION

Development requires coordination between proliferative and maturational programs. For adult eye development in *Manduca sexta*, both programs are controlled by ecdysteroids and the switch from one to the other is regulated extrinsically by changes in the ecdysteroid titer. This dual regulation by ecdysteroids is seen in a number of tissues, including neurogenic cells in the optic lobe (Champlin and Truman, 1998) and myogenic cells in the ventral diaphragm (our unpublished results), but is especially evident in the developing eye.

In terms of its requirements for 20E, the eye primordium can exist in three developmental states. Below $1.2 \times 10^{-7}$ M 20E, development of the primordium is arrested. DNA synthesis, mitosis, progression of the morphogenetic furrow and differentiation of ommatidial precursors all cease under these conditions. At levels of 20E between $1.2 \times 10^{-7}$ M and $2 \times 10^{-6}$ M, the eye primordium shows proliferation, patterning and early differentiation of the epithelium. The response can be stopped by decreasing 20E levels below this range but can be restarted simply by increasing steroid levels back to the appropriate range. Finally, levels of 20E above $1.2 \times 10^{-7}$ M trigger cellular maturation. Unlike the previous response, this is a phasic response and once initiated will continue even if steroid is withdrawn. This response can be evoked early or late in the movement of the morphogenetic furrow. The nature of the response, i.e. production of smooth cuticle or of ommatidial-related products, depends on where the cells are situated relative to the furrow at the time that the high 20E signal appears. The threshold concentrations of 20E that separate these three developmental states remain constant for at least several days during development. Furthermore, the states are discrete. For example, ommatidial maturation does not occur when primordia are exposed to 20E levels at the high end of the proliferative range even when primordia are cultured at this concentration for prolonged periods. Finally, the rate of development does not appear to vary within the range of ecdysteroid concentrations that elicit each state.

The ecdysteroid requirements defined in culture fit the timing of developmental events that occur in vivo (Fig. 1). Primordium formation begins early in the final larval stage as the juvenile hormone titer declines (Monsma and Booker, 1995). Furrow progression then begins as the ecdysteroid titers rise during the wandering stage. Ecdysteroid titers range widely through the subsequent days but stay above the threshold for furrow progression, as defined in culture (Fig. 1). The only time the titer drops below this threshold is in animals that enter diapause.

![Fig. 4. Morphogenetic furrow arrest during diapause. Pupae were pulsed in vivo with BUdR for 4 hours, followed by immunocytochemistry to identify S phase cells flanking the furrow, as in Fig. 1. (A) Short-day reared pupae at P+2 days. (B) Furrow movement and proliferation is stopped 1 day later (P+3) as pupae enter diapause. (C) Eye primordium from pupa that had been in diapause for 1 month and then injected with 2 µg 20E/g body mass. The frequency of S phase cells was assessed 24 hours after injection of 20E and showed the resumption of furrow movement. Green, BUdR-labeled nuclei; red, propidium iodide-stained nuclei. Anterior is to the right. Bars, 100 µm.](Image)

![Fig. 5. Dependence of the eye primordium on 20E for maturation in culture. Eye primordia were isolated from P+4 day animals, cultured for 3 days with various concentrations of 20E and scored for the presence of screening pigments. The graph shows the concentration dependence on 20E for the production of screening pigments (closed circles). For comparison, the data on 20E-dependent BUdR-labeling from Fig. 2 are included as open circles and a dashed line. Examples are shown of eye primordia cultured with $1.2 \times 10^{-6}$ M 20E (A) and $2.4 \times 10^{-6}$ M 20E (B). Arrows in graph point to the 20E concentration used for each panel. A subset of eyes cultured in $1.6 \times 10^{-6}$ M 20E/ml showed faint pigmentation by 7 days. No pigments were observed at lower concentrations even after 12 days in culture. Also shown are horizontal sections through the equator of primordia stained with phallacidin after 12 days in culture with $1.2 \times 10^{-6}$ M 20E (C) or 6 days in culture with $4 \times 10^{-6}$ M 20E (D). White arrowheads identify lens absent in C but present in D. Blue arrowheads identify immature Semper cells in C and Semper cell crystalline cones in D. Red arrowheads identify rhabdomeres absent in C but present in D. Apical is up. Bars, 500 µm (A,B); 25 µm (C,D).](Image)
Furrow progression ceases during diapause and remains arrested until rising ecdysteroid titers terminate diapause months later. During diapause, the furrow remains poised to advance and quickly resumes progressing when the concentration of ecdysteroid rises above the proliferation threshold. In the animal, screening pigments normally appear synchronously across the entire eye beginning late on day P+8, about 2 days after the 20E titer in the blood rises above the level shown to trigger maturation in culture (Fig. 1). Similarly, screening pigments appear 2 days after exposure to high levels of 20E either in culture or when the maturation program is triggered prematurely in vivo.

Different early genes are induced by 20E at the threshold concentrations for furrow progression versus ommatidial maturation. The situation in Manduca appears similar to that seen in Drosophila melanogaster, in which early genes fall roughly into two categories that require either moderate or high concentrations of 20E for their induction (Karim and Thummel, 1992). We propose that early genes induced by moderate concentrations of 20E are involved in proliferation, patterning and other prematurational responses to ecdysteroids in both Manduca and Drosophila, whereas those early genes requiring high concentrations of 20E for induction are involved in maturational responses.

Drosophila eye development may be controlled in a similar manner to that seen in Manduca. For example, 20E is also required for synthesis of screening pigments in cultured eye primordia of Drosophila (Li and Meinertzhagen, 1995). Genetic evidence now suggests that ecdysteroids also play a role in maintaining morphogenetic furrow progression (C. Brennan, M. Ashburner and K. Moses, unpublished). How might ecdysteroids regulate furrow movement in Manduca and Drosophila? In Drosophila, hedgehog can stimulate furrow progression (Heberlein and Moses, 1995). Furrow movement can be arrested with a temperature-sensitive allele of hedgehog, but it fails to resume when animals are returned to the permissive temperature (Ma et al., 1993). The furrow arrest that we see in Manduca is reversible or irreversible, depending on how we shift the 20E concentration. It remains to be determined how different cascades of gene expression evoked by different concentrations of 20E affect the activity of signaling factors such as hedgehog.

**Ecdysteroid control of metamorphic development**

Research on the action of ecdysteroids has emphasized the roles that peaks of blood ecdysteroid titer play in triggering differentiative and maturational responses (see Introduction). Here we have shown that the valleys in the ecdysteroid titers also have an important developmental role. Through the effects of low steroid titers in promoting proliferation and patterning, they determine the size and organization of the structure that will subsequently be formed under direction of the ecdysteroid surge.

The general focus on differentiative and maturational responses to ecdysteroids has also led to the general belief that ecdysone is an inactive prohormone. As expected, we found...
that ecdysone was ineffective in stimulating the comparable response in the developing eye, i.e. ommatidial maturation. However, ecdysone was effective at stimulating furrow progression and did so at levels only about fivefold higher than 20E. Although it is possible that ecdysone is being converted to 20E during the culture period, similar levels of ecdysone have also been shown to be effective in stimulating optic lobe neuroblast proliferation under conditions where conversion does not appear to be occurring (Champlin and Truman, 1998).

The ecdysteroid requirements for ommatidial maturation are similar to those for other maturational responses in Manduca. For example, cuticle synthesis by the epidermis requires a concentration of 20E above about 1 µg/ml (Hiruma et al., 1991), similar to the level that triggers ommatidial maturation but about 17-fold higher than the threshold for furrow progression. In addition, concentrations of 20E that stimulate ommatidial maturation and cuticle synthesis also cause induction of the early gene MHR3 in both the eye primordium and the epidermis (Palli et al., 1992). Finally, a brief exposure to 20E is sufficient to trigger ommatidial maturation or synthesis of a new cuticle by the epidermis (Hiruma et al., 1991) as compared with the tonic requirement to sustain furrow progression.

**Insect and amphibian metamorphosis**

The observation that the type of developmental response made by a tissue is dependent on the concentration of ecdysteroid, reveals a striking similarity between ecdysteroid control of insect metamorphosis and thyroid hormone (TH) control of amphibian metamorphosis. Tonic exposure to moderate levels of TH is required to stimulate early metamorphic development (prometamorphosis) (Kollros, 1961). Development arrests if TH is removed but continues when exposure to TH is resumed. Following prometamorphosis, high levels of TH are required to trigger metamorphic climax. Premature exposure during prometamorphosis to the high levels of TH characteristic of climax triggers precocious and deranged development (Kollros, 1961). It is intriguing to note that premature exposure to high levels of TH in the tadpole (Yaoita and Brown, 1990), and 20E in the Manduca eye primordium (D. T. Champlin, M. Jindra, L. M. Riddiford and J. W. Truman, unpublished), are both associated with the precocious appearance of a second isoform of their respective receptors. The ability to switch the insect eye primordium from one steroid-dependent response to another in vitro should facilitate determination of the roles that receptor isoforms play in the execution of different programs of hormone response.

**Diapause and ecdysteroid action**

It is believed that diapause has evolved independently many times in insects by exploitation of existing mechanisms of hormonal control of development (Denlinger, 1985). Pupal diapause is common throughout the Lepidoptera and, with very rare exceptions, arrest occurs early in the pupal stage rather
than late when the adult is fully formed (Denlinger, 1985). However, diapause early in the pupal stage necessitates interruption of grand developmental events. For example, interruption of morphogenetic furrow progression during diapause in Manduca requires the arrest of diverse processes coordinating cell proliferation, morphogenesis and patterning, commitment to specific fates, and early steps in differentiation. Since all of these events are dependent on the tonic presence of moderate levels of ecdysteroids, they can be coordinately arrested simply by stopping secretion of ecdysteroids during this period. Thus, this tonic requirement for ecdysteroids may have been a useful preadaptation for the evolution of an early pupal diapause. Our results lead us to propose that hormonal requirements may be coordinately interrupted at the appropriate stage simply by reducing the blood ecdysteroid titer below a critical threshold concentration. Months later, development resumes when the ecdysteroid titer rises above this threshold.

We thank Dr Shirley Reiss for excellent technical assistance, Dr Kiyoshi Hiruma for allatectomized animals and Prof. Lynn Riddiford for MHR3 and EcR cDNAs. We also thank Profs DiBenedetto, A. J., Lakich, D. M., Kruger, W. D., Belote, J. M., Baker, B. S. and Wolfner, M. F. (1987). Sequences expressed sex-specifically in wings of Drosophila melanogaster adults. Development 119, 242-251.


Grieneisen, M. L. (1994). Recent advances in our knowledge of ecdysteroid