Physiological Entomology (1996) **21**, 268–274

Development–activity relationships in nymphal corpora allata of the cockroach, *Diploptera punctata*

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Abstract. In females of *Diploptera punctata* the corpora allata undergo a gradual increase in volume during most of the second nymphal stadium. In the first half of the stadium, steady growth of the glands results from a progressive increase in the size of constituent cells. Late in the stadium, cell size declines but the volume of the glands continues to rise due to an increase in cell number. Changes in cell size during the stadium displayed a distinct pattern in relation to Juvenile Hormone (JH) synthesis. Both cell size and activity increased during the first two-thirds of the stadium, peaked early in the last third of the stadium, and decreased before the moult. The rise in cell numbers late in the stadium corresponded to a wave of cellular mitosis and occurred after a steep decline in the rate of JH biosynthesis. Exposure of late second instars to fenoxycarb, a JH analogue, depressed mitosis significantly, suggesting autocrine regulation of cell proliferation in the corpora allata. Possible mechanisms modulating sequential cycles of growth and atrophy of cells and cell proliferation in these glands are discussed in relation to temporal patterns of JH and ecdysteroid titres in nymphs.

Key words. Cockroach, *Diploptera punctata*, corpora allata, Juvenile Hormone, cell number, cell size.

Introduction

In both immature and adult insects, variations in the volume of the corpora allata have been studied by many authors in an attempt to correlate these changes with fluctuations in the rates of synthesis of Juvenile Hormone (JH) (reviewed by Novak, 1975; Tobe & Stay, 1985; Cassier, 1990). For example, in adult females of the viviparous cockroach, *Diploptera punctata*, a tripling of the volume of the corpora allata during vitellogenesis was reported to correspond with a 10-fold increase in JH biosynthesis, whereas a subsequent decrease in gland volume to the previtellogenic level coincided with declining synthetic activity of the glands preceding ovulation (Szibbo & Tobe, 1981). Similar correlations between gland volume and activity has also been noted in reproductive females of the ovoviviparous cockroaches *Leucophaea maderae* and *Nauphoeta cinerea*, the oviparous cockroach *Blattella germanica*, and the orthopteran *Locusta migratoria* (Scharrer & von Harnack, 1958; Lanzrein *et al.*, 1978; Chiang & Schal, 1994; Johnson & Hill, 1975). Although nymphal corpora allata, like those of adults, display cycles of activity (Szibbo *et al.*, 1982; Kikukawa & Tobe, 1986), changes in the volume of the glands in nymphs have not been reported to coincide with cycles of JH biosynthesis (Szibbo *et al.*, 1982; Johnson & Hill, 1973). Discrepancies between data from nymphs and adults have yet to be resolved.

Cellular mechanisms underlying changes in size of the corpora allata have received considerable attention. In adult cockroaches, either changes in cell number (Scharrer & von Harnack, 1958; Szibbo & Tobe, 1981) or cell size (Engelmann, 1957; Chiang & Schal, 1994) or both (Johnson *et al.*, 1993; Chiang *et al.*, 1996) are largely responsible for alterations in volume. In immature insects the role of these parameters in growth of the glands remains unclear. Results from nymphs of *D.punctata* have suggested that alterations in the volume of the glands are due only to changes in cell number (Szibbo *et al.*, 1982), whereas in nymphs of *L.migratoria* changes in both cell number and size appear to be involved (Johnson & Hill, 1973). Nevertheless, results from both these reports are equivocal, since the conventional stereological techniques employed in these studies for determining cell number and size have recently been criticized as yielding highly biased results (Cruz-Orive & Weibel, 1990; Bertram & Nurcombe, 1992).

The objective of our research has been to elucidate the relationship between changes in cell number and size and fluctuations in volume of the corpora allata and synthetic activity in nymphs of

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D.punctata by direct measurement of all these parameters. In addition, by delineating patterns of mitotic activity within nymphal corpora allata, we have identified the temporal relationship between synthetic activity and development. Our results have led us to investigate JH as a potential modulator of cell proliferation in these glands

Materials and Methods

Animals. The *D.punctata* colony was maintained at 27 ± 0.3°C under a LD 12:12 h photocycle and was supplied with pelleted Purina rat chow (no. 5012) and water *ad libitum*. Neonates were collected within 24 h of parturition, and newly ecdysed second instars were collected from groups of cockroaches that had been monitored since birth. Female nymphs were identified by the presence of a median notch in the ninth abdominal sternite. All cockroaches used in experiments were reared in groups of ten to twenty.

Dissections and measurements of gland volume. Pairs of corpora allata were dissected from insects, cleaned from adjacent tissues, and separated from each other in cockroach saline containing 9.27 g NaCl, 1.314 g KCl, 0.324 g $NaHCO_3$, 0.189 g $NaH_2PO_4.H_2O$, 1.206 g Na_2HPO_4, and 2.7 g glucose per liter (modified from Kurtti & Brooks, 1976). This solution is iso-osmotic with adult *D.punctata* haemolymph (360 mOsM per kg H_2O). The volume of the corpora allata was determined using the formula $V = 4/3\pi abc$, where a, b and c are the radii of the three principal axes as measured with an ocular filar micrometer.

Cell number and cell size. Total cell number per corpus allatum was determined by directly counting all cells in a whole-mount monolayer (modified from Chiang *et al.*, 1989). Individual glands were partially digested with 0.1% collagenase in cockroach saline for 5 min at room temperature, stained with 0.1% safranine in 0.1 M citric acid for 5 min, and then spread beneath a coverslip into a cell monolayer on a gelatin subbed slide. The red-stained nuclei were counted under a microscope with the aid of an ocular grid. Cell size was determined by measuring the maximum diameter of enzymatically dissociated cells as previously described (Chiang *et al.*, 1989).

Metaphase arrest. Cell proliferation was monitored by colchicine block, a technique that arrests proliferating cells in metaphase. Insects were injected through the base of the right metathoracic coxa with isotonic saline containing colchicine (Sigma, St Louis, Mo.). Each insect received a dose of 0.02 µg colchicine per mg fresh body mass. Preliminary tests showed that this dose arrested mitoses at metaphase for at least 6 h without chromosomal disintegration or insect morbidity, whereas injection with 0.2 µg colchicine per mg fresh body mass led to mortality within 24 h. A mitotic index was calculated for each gland by dividing the number of nuclei in metaphase 4 h after colchicine injection by the total number of cells within a gland and converting to a percentage value.

JH biosynthesis. A rapid partition radiochemical assay (Feyereisen & Tobe, 1981) as modified by Holbrook *et al.* (1996) was employed to measure release of JH by corpora allata *in vitro*. Pairs of glands were pre-incubated at 27°C for 90 min in L-15B medium containing 100 µM L-[methyl-^{3}H]methionine (198 mCi/mmol; NEN, Wilmington, Del.) as the sole source of methionine. Glands were then transferred to 100 µl of fresh radiolabelled medium, where they were incubated for 3 h at 27°C. After which they were removed and the medium extracted with 250 µl of isooctane. Release of JH was determined by assaying an aliquot of the isooctane hyperphase in a liquid scintillation spectrometer.

Treatment with JH analogue. To expose insects to a constant low dose of JH analogue, fenoxycarb was administered continuously through tarsal contact with a treated substrate. We avoided injection or topical application of fenoxycarb because these can induce non-specific wound responses in insects (Wyatt *et al.*, 1992). Individual 150 × 25 mm petri dishes were treated with 500 µg fenoxycarb (HLR Sciences) in 500 µl ethanol. After evaporation of the ethanol, day 8 second instars were kept in these treated petri dishes for up to 20 days.

All *t*-tests were unpaired and two tailed. Data are presented as mean ±standard error of the mean (SEM).

Results

General development

Female nymphs took 15.5 ± 0.2 days ($n = 20$) to complete the second stadium. Body mass increased most rapidly in the first half of the stadium and had approximately doubled by the end of the stadium (Fig. 1). The volume of the corpora allata increased progressively and significantly ($P < 0.05$, *t*-test) from day 0 (1.15 ± 0.03 × 10^6 mm^3) to day 12 (2.01 ± 0.10 × 10^6 µm^3), when a maximal size was reached. Although there appeared to be a slight decrease in gland volume between days 12 and 14, preceding the moult to the third stadium, this was not statistically ($P = 0.53$) significant.

Proliferation of cells in the corpora allata

Changes in cell number in the corpora allata (Fig. 2) did not directly correspond with changes in either body mass or gland volume. In the first 8 days of the second stadium, when both body mass and gland volume were increasing cell number in the corpora allata remained constant. Individual glands contained 1578 ± 43 cells on day 0 and 1573 ± 69 cells on day 8. Between days 8 and 14, when body mass increased only slightly, and when gland volume increased and then leveled off, cell number increased at a steady rate. On day 14, individual glands contained 2335 ± 141 cells, significantly more ($P < 0.05$) than on day 8. Thereafter, cell number remained constant through the moult into the third stadium.

A wave of mitosis was responsible for the stage-specific increase in cell number late in the second stadium (Fig. 2). The mitotic index was low during the first half of the stadium and did not increase substantially until day 10, when 0.82 ± 0.32% of nuclei were in metaphase 4 h after colchicine injection. On this day, however, the mitotic index was highly variable among the four pairs of corpora allata examined; there were no mitoses in two of the pairs of glands whereas the remaining two pairs exhibited a high mitotic index (1.6 ± 2.0%). In the next 4 days, mitosis was consistently high in all glands and the mitotic index rose significantly ($P < 0.05$) from day 8 to its peak, 1.59 ± 2.6%, on day 12. After ecdysis into the third stadium, the mitotic index

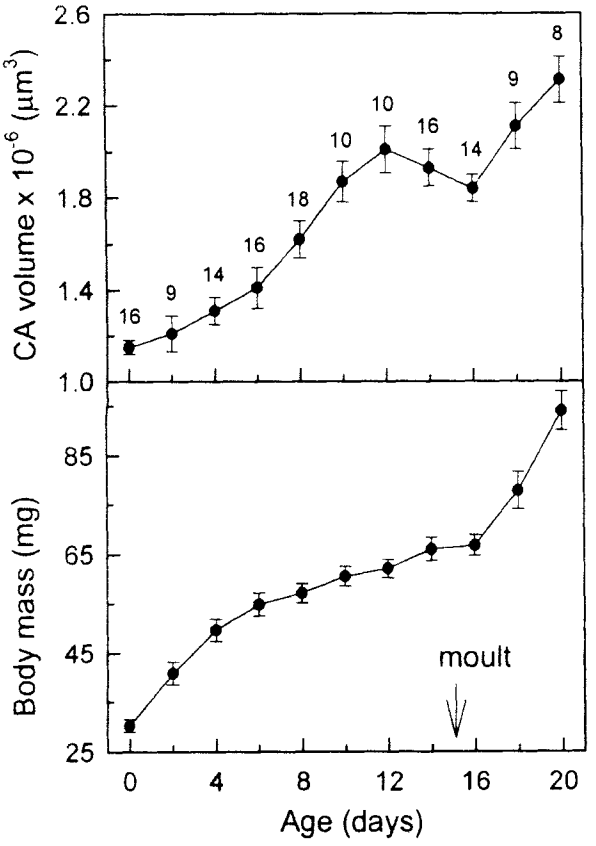

Fig. 1. Changes in volume of the corpora allata and body mass in female nymphs of *Diploptera punctata* during the second and early in the third stadia. Sample size for the measurements of volume of the corpus allatum (CA) is given for each mean. Mean values for fresh body mass were determined by weighing twenty insects at each age. Vertical bars represent the standard error of the mean.

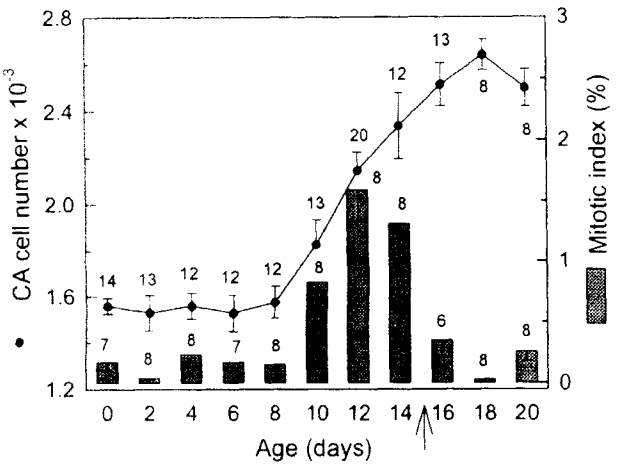

Fig. 2. Changes in total cell number and mitotic activity in single corpus allatum (CA) of *Diploptera punctata*. Mitotic index is the percentage of nuclei in metaphase 4 h after colchicine injection. The arrow indicates the age at which insects moulted into the third stadium. Sample size is given for each mean. Vertical bars represent the standard error of the mean.

decreased rapidly and was near zero on day 18. In both the second and third stadia, mitotic activity was lowest 2 days after the moult. Overall, the pattern of mitosis correlated well with the timing of the increase in cell number.

Changes in cell size in relation to the rate of synthesis of JH

Cells within the corpora allata exhibited a cyclic fluctuation in size during the second stadium (Fig. 3). On each day examined, mean cell size was determined by measuring the maximum diameter of 120 cells in a cell suspension derived from four gland pairs. The largely globular morphology of dissociated cells in nymphs, as in adults (Chiang *et al.*, 1989), ensured that maximum cell diameter was a good measure of cell size. During the second stadium, average cell diameter increased significantly ($P < 0.05$) from 11.6 ± 0.1 μm on day 0 to 15.2 ± 0.2 μm on day 9 and then

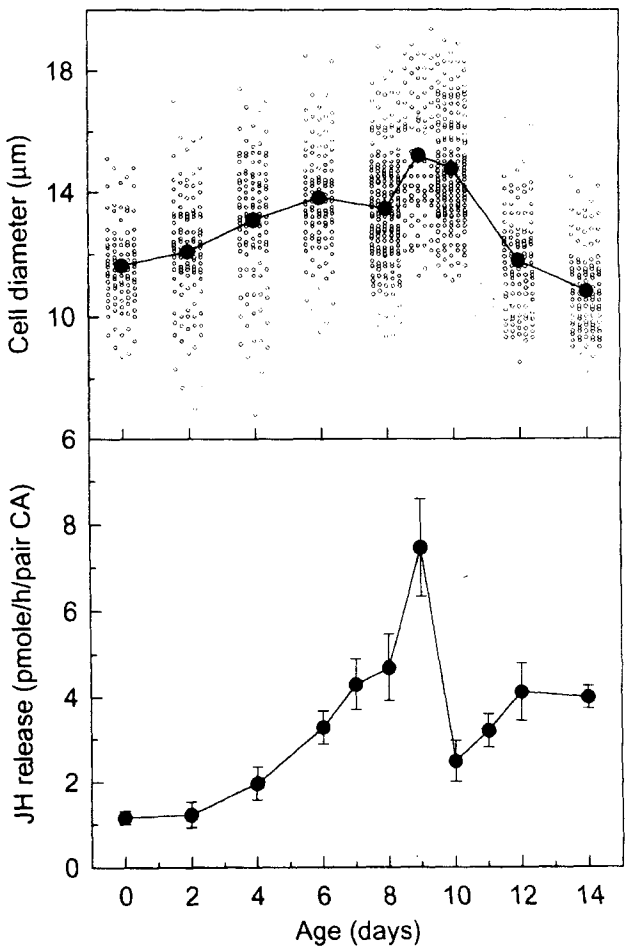

Fig. 3. Changes in the size of the cells in the corpora allata and in rates of release of JH during the second stadium of *Diploptera punctata*. Mean cell size (large circles) was determined by measuring the maximum diameters of 120 cells (small circles) obtained by dissociating glands from four females. Mean rates of release of JH were determined by the radiochemical assay. Vertical bars represent the standard error of the mean; these are obscured in the top figure by the points representing the means.

Table 1. Inhibition of cell proliferation in the corpora allata by JH analogue. All values are means ± SEM (*n*).

Duration of treatment*		Volume × 10^{-6}(μm³)	Cell number/gland	Mitotic index (%)
0 days	Non-treated	1.62 ± 0.08 (18)	1573 ± 69 (12)	0.154 ± 0.050 (8)
4 days	Non-treated	2.01 ± 0.10 (10)	2145 ± 77 (20)	1.591 ± 0.258 (8)
	Treated	1.79 ± 0.06 (10)	1745 ± 135 (12)†	0.001 ± 0.001 (9)†
20 days	Non-treated	3.03 ± 0.23 (8)	5063 ± 602 (8)	0.047 ± 0.009 (5)
	Treated	1.99 ± 0.13 (10)†	2765 ± 212 (9)†	0.001 ± 0.001 (9)†

* Females were continuously exposed to JH analogue since day 8 of the second stadium.

† Means in treated nymphs were significantly lower than corresponding means in non-treated nymphs ($P < 0.05$, two-sample *t*-test).

decreased significantly ($P < 0.05$) to 10.8 ± 0.1 μm on day 14. For each age, the frequency distribution of cell size approximated a normal distribution.

A steady increase in the rate of synthesis of JH until day 9 of the second stadium corresponded with a gradual enlargement of the cells of the corpora allata (Fig. 3). A rapid decline in synthesis after day 9 coincided with a gradual decrease in cell size and was followed by a wave of cell mitosis (Figs 2 and 3).

Hormonal regulation of the development of the corpora allata

Because the rapid increase in cell number occurred only after a decline in synthetic activity of the corpora allata, we speculated that entry of cells into the cell cycle might be inhibited by JH. To test this hypothesis, second instars were treated from day 8 onward with a JH analogue, and volume, cell number and mitotic index were examined in corpora allata 4 and 20 days after onset of treatment. On the fourth day of the experiment, glands of treated insects were smaller, contained significantly fewer cells and exhibited a lower rate of mitosis than those of non-treated insects (Table 1). The inhibitory effect of the JH analogue on cell proliferation and organ growth was even more evident 20 days after initiation of treatment ($P < 0.05$, *t*-test for all three parameters). At that time, glands from treated nymphs were significantly smaller and contained 50% fewer cells than glands from control nymphs. Despite disrupted development of the corpora allata, insects treated with JH analogue displayed no higher mortality than non-treated nymphs, and moulted normally into the third and fourth stadia.

Discussion

Morphogenesis of the corpora allata

For decades, measurements of gland volume have been utilized to identify periods of activity of corpora allata (Novak, 1975). Increase and subsequent decline in volume, for example during reproductive cycles, has often been assumed to indicate a cycle of JH biosynthesis. In second instars of *D.punctata*, the volume of the glands increased progressively as synthetic activity rose, but unexpectedly continued to increase even after synthetic activity had fallen. Two mechanisms appear responsible for growth of the corpora allata in second stadium nymphs. First, during the initial 10 days of the stadium, when rates of JH biosynthesis are rising, a progressive increase in the volume of the glands is due to enlargement of the cells. Second, during the last third of the stadium, when JH biosynthetic rates are declining, increase in cell number is responsible for continued growth of the glands, even as cell size declines. A minor reduction in gland volume preceding the moult has been reported in other cockroaches, such as *N.cinerea* and *L.maderae* (Lanzrein, 1975; Luscher & Engelmann, 1960).

The pattern of change in volume of the corpora allata during the second stadium is clearly different from a previously reported pattern in third instars of *D.punctata* (Szibbo *et al.*, 1982). Szibbo *et al.* (1982) found that volume remained constant for most of the third stadium and rose only late in the stadium, when cell number increased slightly. Although it is possible that differences between the two stadia may be due to stadium-specific events, this seems unlikely because corpora allata from both the second and third stadia display similar patterns of cyclic synthetic activity (see also Szibbo *et al.*, 1982) and increase in cell number (see also Szibbo *et al.*, 1982). It is more probable that differences in techniques employed for measuring gland volume account for different results. Szibbo *et al.* (1982) performed morphometric measurements on histologically fixed tissues, which shrink during fixation (Szibbo & Tobe, 1981), whereas we measured the volume of fresh corpora allata. We have found recently, by examining fresh glands, that the volume of corpora allata does increase early in the third stadium during the rising phase of synthetic activity (unpublished results).

It has been proposed that cycles of JH biosynthesis are, in part, brought about by cyclic changes in cell number. Evidence for this hypothesis was provided in studies on *L.maderae*, an ovoviviparous cockroach, and *D.punctata*, which is viviparous. During the ovarian cycle in adult females of these species, an increase in cell number was reported to coincide with rising synthetic activity, while a subsequent loss of cells was reported to correspond with declining synthetic activity (Scharrer & von Harnack, 1958; Tobe *et al.*, 1984; Johnson *et al.*, 1993). In contrast, in two oviparous cockroaches, *B.germanica* and *Supella longipalpa*, cell number does not change in the corpora allata of adult females, and thus in these species cell proliferation plays no role in modulating JH biosynthesis (Chiang *et al.*, 1991b; Chiang & Schal, 1994). Nevertheless, recently we have reconfirmed the occurrence of cell proliferation during the phase of rising synthetic activity in adult females of *D.punctata* (Chiang

et al., 1996), and this has led us to explore whether a similar increase in cell number occurs in relation to synthetic activity during postembryonic development. Our results, however, show that cell number does not change when JH biosynthesis was increasing early in the second stadium. Rather, cell number increases, in large part, only after JH biosynthesis has begun to decline. Temporal separation of changes in cell number from high rates of synthetic activity was noted previously by Szibbo *et al.* (1982) in third and last stadium nymphs of *D.punctata*.

Although cell number does not change in the first half of the second stadium, this does not preclude the possibility that cells of the corpora allata were undergoing substantial proliferation in early second instars. It was possible that cells are dividing and simply replacing cells undergoing apoptosis and cell death. We addressed this hypothesis by measuring mitotic rates within the glands throughout the second stadium. In doing so, we have, for the first time, clearly elucidated the relationship between JH biosynthesis and cell proliferation in the corpora allata of nymphs. We found that mitosis is rare in nymphal glands when cells are becoming biosynthetically active or are producing JH at high rates (i.e. from day 0 to day 9). In contrast, mitosis is abundant when JH biosynthesis is declining or after it has already declined (i.e. from day 10 to day 14). A similar burst of mitotic activity, late in nymphal stadia, has been reported in *L.maderae* (Luscher & Engelmann, 1960). Our results indicate that cell proliferation play little, if any, role in short-term modulation of synthetic activity of the corpora allata in nymphs.

In many insects, fluctuations in the size of cells of the corpora allata accompany cycles of synthetic activity. For example, in adult females of many cockroaches, including *D.punctata*, cyclic changes in cell size and cellular structures occur during ovarian cycles (Scharrer & von Harnack, 1958; Szibbo & Tobe, 1981; Johnson *et al.*, 1985, 1993; Piulachs *et al.*, 1989; Chiang *et al.*, 1989; Chiang & Schal, 1994; Cheng & Chiang, 1995; Yang & Chiang, 1996). The close relationship between cell size and JH biosynthesis in the corpora allata has led to speculation that factors regulating JH biosynthesis may act by inducing alterations in the amount of cellular machinery available for hormone production. Support for this hypothesis is provided by results from adult females of *B.germanica*, in which both the size and activity of cells in the glands respond concurrently to stimulatory and inhibitory factors from the ovaries (Chiang & Schal, 1991; Chiang *et al.*, 1991a, c). Furthermore, in adult female *D.punctata*, mating induces simultaneous increases in JH biosynthesis and cell size, whereas both these parameters remain unchanged in virgin females (Chiang *et al.*, 1996; unpublished results). Results from our current study suggest that in cockroach nymphs, as in adults, synthetic activity is regulated through modulation of cell size. Changes in cell size were, however, less synchronous in nymphs than in adult females, presumably due to a lack of potent ovarian stimulatory and inhibitory factors that operate in adult females (see also Chiang *et al.*, 1991c).

Regulation of the development of the corpora allata

During the second stadium, increases in body mass and cell number were temporally separated (Figs 1 and 2). Stasis of cell number early in the stadium, when nymphal weight was rapidly increasing, suggests that growth of the corpora allata is not simply a somatic process in which cell number keeps apace with insect size. Rather, the stage-specific increase in cell number late in the second stadium, when body mass is static, indicates that an organ-specific mechanism regulates cell proliferation in the glands. This finding agrees with previous results from Szibbo *et al.* (1982) with *D.punctata* but contrasts with prior studies in Orthoptera showing that mitosis and increases in cell number coincide with general body growth (Mendes, 1948; Johnson & Hill, 1973).

A role for JH in suppressing cell mitosis in *D.punctata* was first speculated by Kikukawa *et al.* (1988), who reported a decline in cell number in the corpora allata of adult virgin females treated with hydroprene, a JH analogue. If anything, however, this report suggested that hydroprene stimulated cell death rather than inhibited cell mitosis, especially since cells proliferate at a very low rate in virgin females (Tobe *et al.*, 1984; Chiang *et al.*, 1996). Two lines of evidence from our study suggest that JH may regulate cell proliferation. First, rates of mitosis in the corpora allata are high only after JH biosynthesis declined late in the second stadium. Second, application of a JH analogue, fenoxycarb, to day 8 nymphs significantly depresses mitosis in the corpora allata for up to 20 days. Further evidence for modulation of the development of corpora allata by JH was provided in studies on two additional insect species. In *Dysdercus intermedius*, fenoxycarb treatment reduced the rate of increase in cell number in the corpora allata of last three nymphal stadia (Hell *et al.*, 1993). Whether the analogue inhibited cell mitosis, however, was not examined. In *L.maderae*, transection of nerves between the brain and the glands in last instars led to both continuous JH production (based on morphometric measurements) and suppression of cell mitosis (Luscher & Engelmann, 1960). All these results suggest that JH acts in an autocrine or paracrine fashion to suppress cell proliferation in the corpora allata during postembryonic development.

Because fenoxycarb did not eliminate cell mitosis or increase cell number, it appears that JH is not the sole factor modulating the development of the corpora allata. Available evidence suggests that ecdysteroids might serve as mitogens, stimulating cell proliferation in the glands. It is known that epidermal cell division in many insect species is most prevalent at or near the peak in ecdysteroid titre (reviewed by Riddiford, 1985). Because the corpora allata are of similar ectodermal origin as the epidermis (Haget, 1977), it is possible that both the epidermis and these glands respond similarly to ecdysteroids. In fact, cell proliferation in the corpora allata in second stadium nymphs of *D.punctata* coincides with a peak in ecdysteroid titre reported previously (Kikukawa & Tobe, 1986). Further evidence suggesting a role for ecdysteroids in regulating the development of the corpora allata comes from *in vitro* studies showing that DNA synthesis in the glands is enhanced by 20-hydroxyecdysone (Chiang *et al.*, 1995; Tsai *et al.*, 1995).

The means by which ecdysteroids may stimulate cell proliferation in the corpora allata is unclear. It is possible, however, that ecdysteroids exert their effect by reorienting cells away from JH biosynthesis towards mitosis. Experimental support for this hypothesis is provided in studies on adult females of *B.germanica*, in which the relative size of the cells of the corpora allata can be influenced by ecdysteroids. Injection of 20-hydroxyecdysone into ovariectomized females induces a reduction in the size of the

cells, which are initially large and biosynthetically active before ecdysteroid treatment (Gadot *et al.*, 1991; Chiang *et al.*, 1991c). In our ongoing research, we are investigating the roles of JH, ecdysteroids and neuropeptides in regulating JH biosynthesis and development in corpora allata both *in vivo* and *in vitro*.

Acknowledgments

This research was supported by a ROC National Science Council grant (NSC85-2311-B007-023) to A.-S. Chiang. At North Carolina State University, research was supported by the Blanton J. Whitmire Endowment, USDA-NRI (9501922) and NSF (IBN-9407372).

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Accepted 16 September 1996