Juvenile hormone synthesis in relation to corpus allatum development in embryos of the viviparous cockroach *Diploptera punctata*

GLENN L. HOLBROOK[1], ANN-SHYN CHIANG[2], YUAN-JU LEE[2], CHIH-YUNG LIN[2] and COBY SCHAL[1*]

[1]*Department of Entomology, North Carolina State University, Raleigh, NC 27695-7613, USA*
Tel. +1 (919) 515-1821; Fax +1 (919) 515-7746; e-mail: coby_schal@ncsu.edu
[2]*Institute of Life Science, National Tsing-Hua University, Hsinchu 30043, Taiwan*
Fax: +886 (35) 571-5934; e-mail: lscas@life.nthu.edu.tw

Summary

Few studies have addressed endocrinology of the corpora allata (CA) in insect embryos. We now report on development and biosynthetic activity of CA in embryos of a viviparous cockroach, *Diploptera punctata*. When newly-eclosed adult females of *D. punctata* were mated, they oviposited and gave birth, respectively, about 8 and 73 days later; thus, gestation and corresponding embryogenesis lasted approximately 65 days. Dorsal closure, which coincides with differentiation of the CA, was concluded when embryos were about 13 days old and had completed 20% of embryogenesis. Reverse phase-high performance liquid chromatography revealed that embryonic CA released predominantly juvenile hormone III (JH) *in vitro*. Furthermore, an *in vitro* radiochemical assay showed that between day 28 of embryogenesis (43% of embryonic development completed) and hatch rates of JH synthesis rose, plateaued and then fell. When CA activity was increasing or was high, from day 28 to 54 (83% development), mitosis occurred at low and constant rates within embryonic CA, and corpus allatum cell number increased gradually. Between days 56 (86% development) and 60 (92% development), CA activity fell to a low level, rates of mitosis peaked, and corpus allatum cell number rose rapidly. Throughout embryogenesis, CA volume increased in parallel with CA cell number, suggesting that glandular growth was due largely to cell proliferation. Although CA activity and volume changed considerably in embryos, the diameter of corpus allatum cells, as measured from enzymatically dissociated CA, remained surprisingly constant at 11–12 μm on days 32, 46 and 60 (49, 71 and 92% development). Ultrastructural observations confirmed the large size of cells in low-activity CA of 60-day-old embryos and also showed that these cells, like those in highly active CA of 46-day-old embryos, contained abundant cytoplasm, ribosomes, microtubules and mitochondria.

Key words: Cockroach, viviparity, embryo, corpora allata, juvenile hormone, cell proliferation, ultrastructure

*Corresponding author.

Introduction

The corpora allata (CA) are insect endocrine organs that synthesize juvenile hormone (JH), a regulator of morphogenesis and reproduction (Tobe and Stay, 1985). Over the past 60 years, a vast amount of research spanning dozens of insect species has been directed toward understanding the development, function, and regulation of CA in larval and adult insects. During this time, however, comparatively few investigations have dealt with CA endocrinology during embryogenesis, despite the early discovery by Novak in 1951 of JH activity in heads of milkweed bug embryos.

In recent studies, high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry have been employed to indisputably show the presence of JHs in embryos of ametabolous, hemimetabolous and holometabolous insects (Bergot et al., 1981; Baker et al., 1984; Temin et al., 1986; Short and Edwards, 1992). Meanwhile, the role of embryonic CA in producing these JHs has been examined only in cockroaches. In the lobster cockroach, *Nauphoeta cinerea*, the CA at mid-embryogenesis were shown to synthesize large amounts of both juvenile hormone III (JH III) and its unepoxidized precursor, methyl farnesoate (MF) (Lanzrein et al., 1984; Bürgin and Lanzrein, 1988). In contrast, JH III was reported to be the predominant product of CA from embryos of the beetle cockroach, *Diploptera punctata* (Cusson et al., 1991). Nevertheless, since Cusson et al. examined CA products only during late embryogenesis, when CA of *N. cinerea* synthesize little MF (Bürgin and Lanzrein, 1988), production of MF by embryonic CA of *D. punctata* cannot be excluded. Thus, the significance of MF as a biosynthetic product of CA from embryos other than *N. cinerea* remains unknown.

Although it is now well accepted that embryonic CA are biosynthetically active, there is presently a dearth of information on dynamics of CA activity in embryos. Two recent studies, with differing results, addressed patterns of JH synthesis in cockroach embryos. In *N. cinerea* CA activity was shown to peak just after dorsal closure and to decline thereafter very gradually through mid-embryogenesis to hatch (Bürgin and Lanzrein, 1988). In contrast, in *D. punctata* rates of JH synthesis were reported to increase substantially and then fall sharply during mid-embryogenesis (Kikukawa and Tobe, 1987). Meaningful comparison of CA activity between the two species is nevertheless hampered by the limited nature of the report on *D. punctata*, which covered less than a third of embryonic development.

While little is known about CA activity in embryos, even less can be said of CA development. Although mitosis has been reported in early allatal rudiments of *Oncopeltus fasciatus* (Dorn, 1972) and in late embryonic CA of *Locusta migratoria* (Aboulafia-Baginsky et al., 1984), a detailed pattern of CA cell proliferation has yet to be established for embryos of any insect species. Therefore, the relationship between CA development and activity, which has received considerable attention in larvae and adults (Engelmann, 1957; Scharrer and von Harnack, 1958; Johnson and Hill, 1973; Szibbo and Tobe, 1981; Tobe et al., 1984; Chiang et al., 1989; Chiang et al., 1996a, 1996b), is unknown in embryos.

The viviparous cockroach, *D. punctata*, has served as a model in investigations of CA development, function, and regulation. To date, few studies (Kikukawa and Tobe, 1987; Cusson et al., 1991; Holbrook et al., 1996) have dealt with embryonic CA in this species. In our current report we have examined both development and synthetic activity of CA throughout the latter half of embryogenesis in *D. punctata*. Moreover, we have described in detail the relationship between fluctuations in embryonic CA activity and changes in CA cell number, size, and ultrastructure.

Materials and Methods

Insect rearing and embryo staging

The *D. punctata* colony was reared at 27±0.3°C under a 12 h light:12 h dark photoperiod and was provided rat chow and water *ad libitum*. Adult females were mated immediately after imaginal ecdysis and were maintained thereafter in groups of 10–20 under conditions similar to the colony. Embryo broods were obtained from pregnant females by applying gentle posterior-directed pressure to females' abdomens. Broods were partitioned with blunt forceps into individual embryos under cockroach saline (Kurtti and Brooks, 1976) modified to 360 mOsm/l. Embryo weight was measured after embryos were blotted to surface-dryness with filter paper.

Because adult females were mated on day 0, embryo age was calculated as adult female age minus mean oviposition time. The total length of embryogenesis was calculated as mean parturition time minus mean oviposition time, and the percentage of embryonic development completed was determined by dividing embryo age by the total length of embryogenesis. All calculated values are expressed to the nearest unit day or percent.

Corpus allatum cell number, mitotic rates, cell size and gland volume

By dissecting embryos on successive days after definitive dorsal closure, which occurred when embryos were about 13 days old (20% of embryonic development completed), we found that CA first became evident, dorsal to the esophagus, in 28-day-old embryos (43% development). Before day 28, CA were undergoing dorsal migration and were difficult to locate under a dissecting microscope. We therefore examined CA only in embryos which had completed at least 43% of embryogenesis. To obtain CA, embryos were decapitated and severed heads were transferred into cockroach saline, where CA were carefully isolated from remaining tissues with fine forceps.

Corpus allatum cell number was determined with a monolayer technique (Chiang et al., 1989). Individual glands were partially digested in 0.1% collagenase in cockroach saline for 5 min at room temperature and were then drawn into pipet tips containing 5 μl of a nuclear stain solution composed of 0.1% safranin and 0.1 M citric acid in distilled water. Glands were subsequently ejected into 5 μl drops of nuclear stain solution on the surface of gelatin-subbed slides. After 5 min, glands were spread beneath coverslips into mono-layers, and cell number per corpus allatum was determined by counting all red-stained nuclei with the aid of a grid in the ocular of a compound microscope.

Mitotic activity within embryonic CA was examined by arresting nuclei in metaphase with colchicine (Tsai et al., 1995). Gland pairs were incubated for 4 h in 0.01% colchicine in L-15B medium (Munderloh and Kurtti, 1989), after which glands were prepared into monolayers, as described above. The total number of metaphase nuclei per gland pair was counted under a compound microscope. Mitotic rates were calculated by dividing the average number of metaphase nuclei in gland pairs on a given day by the average number of cells in gland pairs on the same day; resulting values were multiplied by 100 to express mitotic rates as the number of metaphase nuclei per 100 cells.

The maximum diameter of enzymatically dissociated CA cells was determined as described previously (Chiang et al., 1989). The volume of a viable corpus allatum was determined with the formula $v = \frac{4}{3}(\pi abc)$, where a, b and c are the radii of the three principal gland axes.

Juvenile hormone release

JH release by CA *in vitro* was measured with a rapid partition radiochemical assay (Pratt and Tobe, 1974; Feyereisen and Tobe, 1981; Holbrook et al.,

1996, 1997). CA were pre-incubated for 90 min in L-15B medium containing 100 μM L-[methyl-^{3}H]-methionine (198 mCi/mmol; New England Nuclear, Wilmington, DE). Gland pairs were then transferred into 20 μl fresh radiolabeled medium in 6×25 mm borosilicate glass culture tubes, where CA were incubated for 6 h at 27°C with orbital shaking. At termination of an assay, 100 μl of non-radiolabeled medium was added to each culture tube and the medium was vortexed at low intensity. Then, 100 μl of mixed medium, without CA, was transferred to a different culture tube and extracted with 250 μl iso-octane. The amount of JH released by CA was determined by spectrometrically counting radioactivity in an aliquot of the isooctane hyperphase.

Analysis of products released by corpora allata

Reverse phase-HPLC (RP-HPLC) was employed to identify products released by CA from embryos that were 32, 46 and 60 days old. Twelve pairs of CA were obtained from embryos at each age, and CA were incubated in L-15B medium with radiolabeled methionine for 6 h at 27°C. Afterward, the medium without CA was isooctane extracted, and extracts from each developmental stage were pooled. Samples were blown to near dryness under nitrogen and reconstituted in 20 μl acetonitrile containing non-radioactive standards of juvenile hormone I (JH I), JH III, and MF (78% *trans-trans*, 22% *cis-trans*). Chromatography was performed at room temperature using a Spectra-Physics® HPLC (San Jose, CA) with a SP8800 ternary pump and a SP8480XR scanning uv detector set at 214 nm. Samples were applied to a 10 μm RP-18 column (Lichrosorb®, Merck) and were eluted for 40 min at a flow rate of 1 ml per min in a linear gradient of 50–100% acetonitrile in water. Eluted fractions were collected at 30 s intervals into scintillation vials and suspended in scintillation cocktail for spectrometry. Blank incubations, without CA, were chromatographed as above.

Ultrastructural techniques

Tissues were fixed, embedded, sectioned and stained as described previously (Cheng and Chiang, 1995). Micrographs were obtained from a Hitachi H-600 electron microscope set at 75 kV.

Statistical analysis

Differences in the diameter of corpus allatum cells at different ages were examined with single-factor analysis of variance (ANOVA). For all means, standard error of the mean (SEM) was used as a measure of variation.

Results

Embryo development

In *D. punctata*, adult females deposit fertilized eggs into a brood sac where embryos are retained until hatch (Hagan, 1951). Because oviposition and parturition occurred 7.8 ± 0.1 days and 73.2 ± 0.1 days after mating (Fig. 1), the total length of embryogenesis was about 65 days.

Embryo growth and corpus allatum development

Embryos of *D. punctata* undergo considerable growth during gestation (Roth and Willis, 1955; Stay and Coop, 1973). Between days 28 (43% development) and 46 (71% development), the mean wet weight of embryos more than tripled from 1.1 ± 0.04 mg to 3.9 ± 0.16 mg (Fig. 2A). Embryos continued to grow at a similar rate until hatch so that neonates weighed 13.1 ± 0.20 mg on day 0 of the first stadium.

Corpus allatum cell number and body weight increased coincidentally during most of embryogenesis (Fig. 2A). The two variables did, however, differ in pattern and rate of increase. Between days 28 and 56 (86% development), when mean embryo weight increased almost 8-fold from 1.1 ± 0.04 mg to 8.3 ± 0.34 mg, mean cell number per corpus allatum rose by only about threefold from 131 ± 9 cells to 433 ± 12 cells. Between days 56 and 60 (92% development), mean corpus allatum cell number rose by 60% to 691 ± 29, while mean embryo weight climbed by half that rate from 8.3 ± 0.34 mg to 10.9 ± 0.43 mg (Fig. 2A). After day 60, CA cell number remained constant as embryos continued to increase in weight.

To determine whether the rapid increase in CA cell number late in embryogenesis was due to elevation of mitotic rates, we examined the temporal pattern of mitosis within embryonic CA. When corpus allatum cell number was increasing slowly, between days 32 and 54, the number of mitoses per CA pair was low and the mitotic rate, the number of metaphase nuclei per 100 cells after 4 h of colchicine treatment, never exceeded 0.8 (Fig. 2B). After day 54, when cell number began to increase more rapidly, rates of mitosis increased, and CA contained 1.2 and 1.0 mitoses per 100 cells on days 56 and 58. After day 58, the number of mitoses per gland pair remained relatively constant, and rates of mitosis decreased to 0.7 and 0.6 on days 60 and 62, respectively.

Hormonal products of embryonic CA

Because the identity of hormones produced by the CA has been shown to vary during embryonic development in *N. cinerea* (Bürgin and Lanzrein,
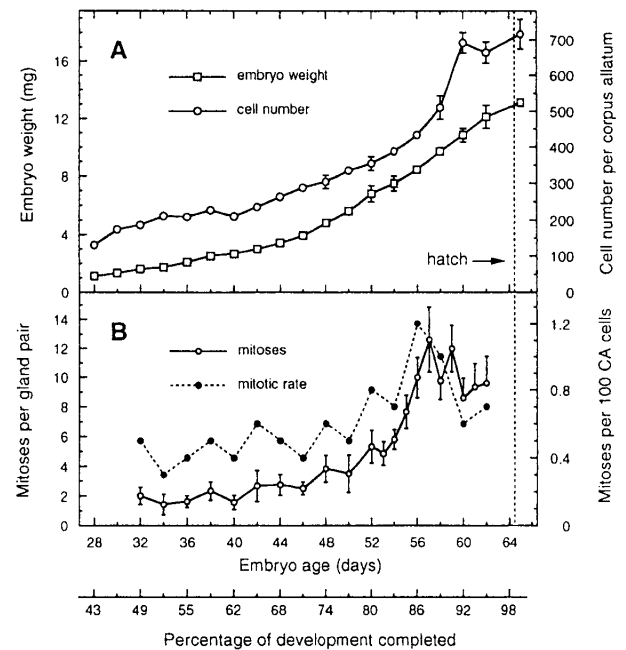
Fig. 2. Corpus allatum development in relation to embryo growth in *D. punctata*. Changes in embryo wet weight (A), corpus allatum cell number (A), CA cell mitosis (B), and mitotic rate (B) were examined during embryogenesis. A monolayer technique was employed to count both the number of cells in a corpus allatum and the number of metaphase nuclei in gland pairs incubated for 4 h in 0.01% colchicine. Error bars represent $\pm$SEM. For embryo weight and corpus allatum cell number, data points were the result of 5–14 and 10–13 measurements, respectively. For quantification of mitotic nuclei, 3–8 gland pairs were examined at each age.
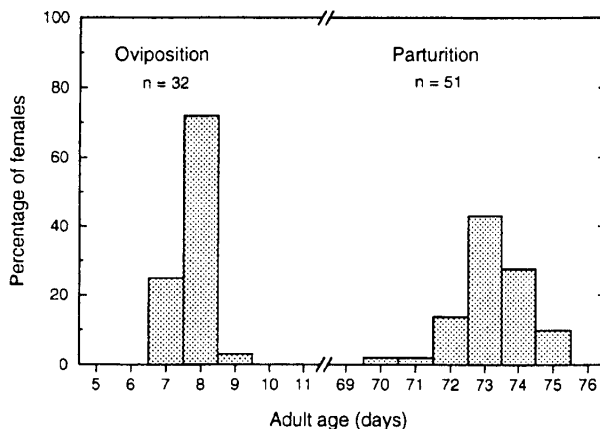
Fig. 1. Oviposition and parturition in adult females of *D. punctata*. Adult females were mated on day 0 and maintained thereafter in groups of 10–20 at 27°C. Separate cohorts were inspected daily for oviposition or parturition. A female was considered to have oviposited if her genital chamber and (or) brood sac contained eggs. Bars show the percentage of females ovipositing or giving birth on a given day.

1988), it was necessary to characterize CA products at more than one embryo age in *D. punctata*. When isooctane extracts of incubation media were examined with RP-HPLC, approximately 90% of CA products at 49, 71, and 92% embryo development time (Fig. 3) were found to coelute at 16.5 min with a synthetic JH III standard and with the major product, JH III (Tobe and Stay, 1977), of CA from adult females (Fig. 3). Although 2% of radiolabeled products of adult CA coeluted with MF, embryonic CA did not release MF, or JH I, at any of the three developmental stages.

Juvenile hormone production by embryonic corpora allata

Rates of JH release by CA *in vitro* fluctuated between day 28 (43% development) and hatch (Fig. 4A). On day 28, gland pairs produced 0.27 ± 0.04 pmol

JH h^{-1}, and by day 42 (65% development) CA activity had steadily risen to 1.89 ± 0.16 pmol JH h^{-1}. From day 42 to 56, JH release rates remained near 2 pmol h^{-1}, and on day 58 CA activity fell to 1.43 ± 0.17 pmol JH h^{-1} and remained near this level until hatch. These results showed a cycle of gland activity in embryos.

Direct measurements of JH synthesis did not account for the tremendous growth in weight of embryos or increase in cell number of CA that occurred during embryogenesis. We therefore show CA activity relative to both these parameters (Fig. 4B). When expressed as JH release per mg embryo wet weight, CA activity rose distinctly from 0.24 pmol h^{-1} mg^{-1} on day 28 to a peak of 0.63 pmol h^{-1} mg^{-1} on
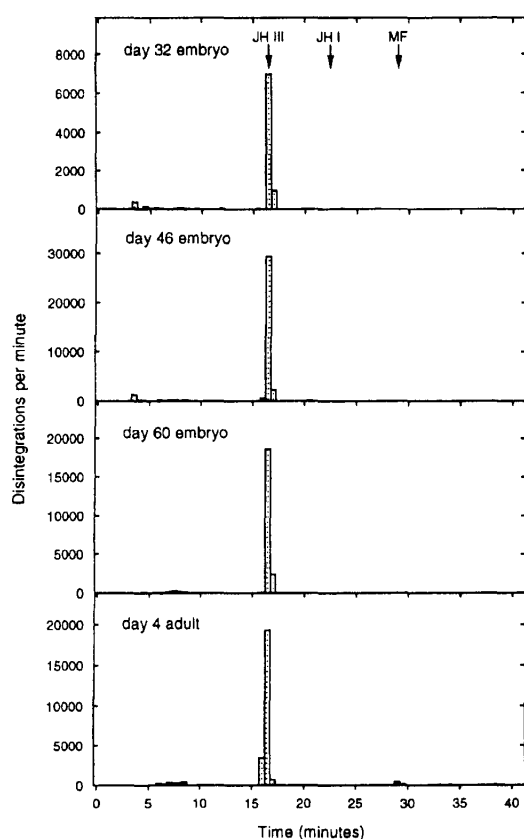

Fig. 3. Hormonal products released by embryonic CA of *D. punctata*. Reverse phase-HPLC was used to analyze isooctane extracts of media in which CA from 32-, 46- or 60-day-old embryos had been incubated for 6 h at 27°C. Hormonal products of CA from 4-day-old adult females, mated on day 0, were also examined to confirm the identity of compounds released by embryonic CA. All samples were co-chromatographed with cold standards of JH I, JH III and MF, the retention times of which are indicated by the arrows.
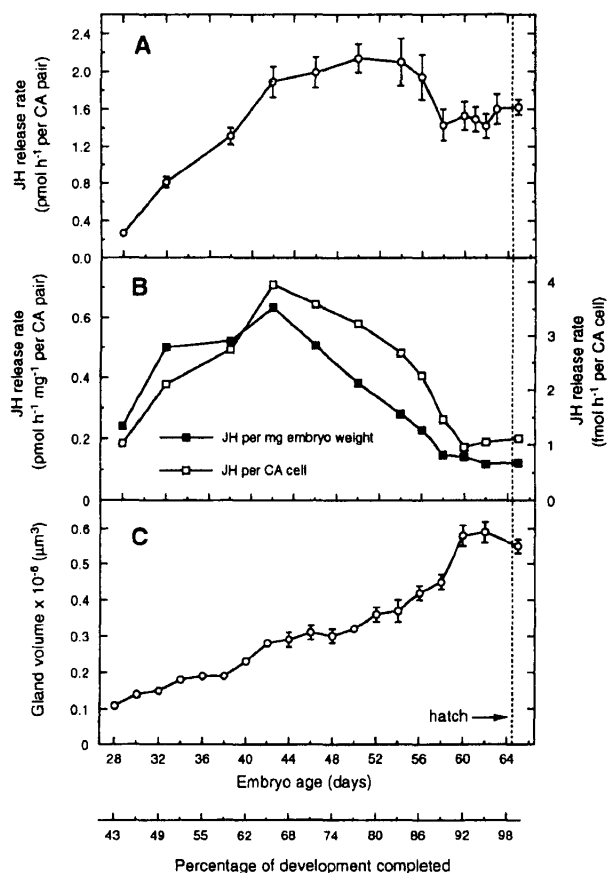

Fig. 4. Changes in CA activity and volume in embryos of *D. punctata*. (A) Rates of JH release by embryonic CA (n = 7–14) *in vitro* were determined with a radiochemical assay. (B) JH production was expressed relative to embryo weight by dividing the mean rate of JH release on a given day by the mean weight of embryos on that day. A similar adjustment was made for changes in corpus allatum cell number; mean rates of JH release were divided by the mean number of cells in gland pairs. (C) Gland volume (n = 9–18) was determined by measuring dimensions of viable CA. Error bars in graphs represent ±SEM.

day 42 and thereafter fell gradually to less than 0.15 pmol h^{-1} mg^{-1} before hatch (Fig. 4B). A nearly identical pronounced cycle of activity was evident when JH release rates were expressed on a per-CA-cell basis (Fig. 4B). This correction better showed the relative activities of individual cells at different developmental stages and therefore allowed more precise comparison of the temporal relationship among changes in activity, size and proliferative rates of CA cells.

Morphometric development of embryonic corpora allata

To determine whether changes in CA volume and activity were related in *D. punctata* embryos, we measured gland volume throughout the cycle of JH synthesis. From day 28 to 42, a near tripling of gland volume from $0.11 \pm 0.01 \times 10^6 \mu m^3$ to $0.28 \pm 0.01 \times 10^6 \mu m^3$ (Fig. 4C) corresponded with an elevation in the rate of JH release from 1.0 fmol h^{-1} per cell to its peak of 4.0 fmol h^{-1} per cell (Fig. 4B). After day 42, however, gland volume continued to increase even as CA activity, on a per-cell basis, slowly declined. Thus, in embryos changes in gland volume did not always reflect changes in rates of JH synthesis.

The size of CA cells remained constant throughout the cycle of JH synthesis. Mean cell diameter did not differ ($P = 0.43$, single-factor ANOVA) on days 32 ($11.7 \pm 0.2 \mu m$, $n = 58$), 46 ($11.6 \pm 0.3 \mu m$, $n = 100$) and 60 (11.3 ± 0.1, $n = 100$), suggesting that alterations in number, but not in size, of CA cells were mainly responsible for changes in embryonic CA volume. Both gland volume (Fig. 4C) and cell number (Fig. 2A) rose slowly from day 28 to 56, increased rapidly from day 56 to 60, and subsequently remained constant.

Ultrastructure of embryonic CA

Constant cell size throughout embryogenesis implied that fluctuations in gland activity were not accompanied by alterations in cell structure. To test this conjecture, we investigated ultrastructural changes in CA cells coincident with declining JH synthesis between days 46 and 60. In 46-day-old embryos, in which CA activity was near peak, CA cells contained abundant ring-like and branched mitochondria and were replete with ribosomes and microtubules (Fig. 5A). In 60-day-old embryos, in which CA activity was low, CA cells contained well-defined Golgi and appeared to contain more autophagic vacuoles than cells of younger embryos (Fig. 5B). Nevertheless, CA cells in 60-day-old embryos, as in younger counterparts, were large and contained bountiful cytoplasm, microtubules, ribosomes, and mitochondria. The mitochondria in older embryos were, however, mostly rod-shaped or globular.

Discussion

Hormonal products of embryonic corpora allata

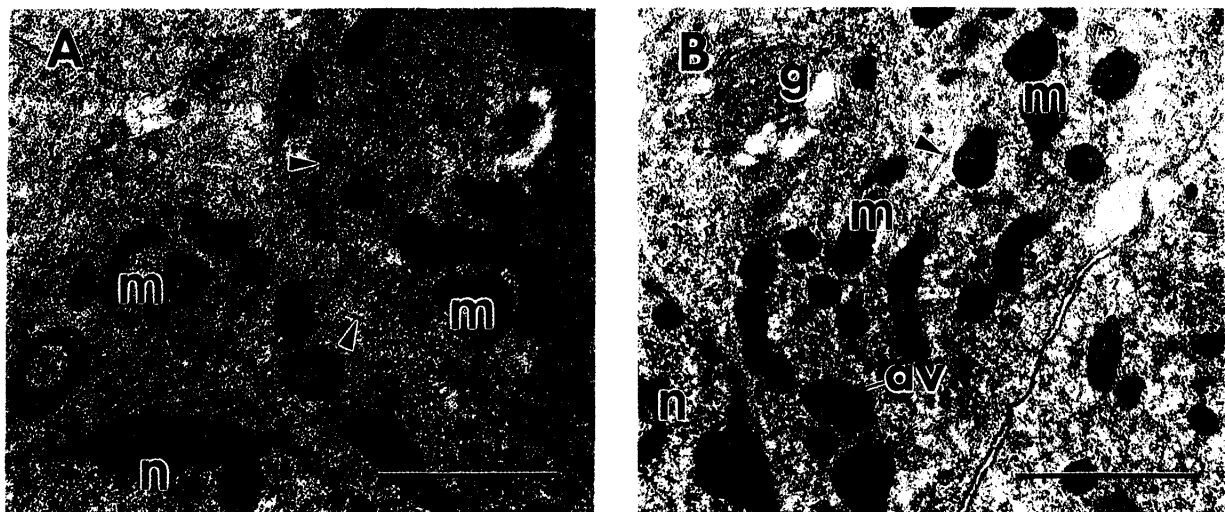For many years, embryos have been known to contain compounds with JH activity in bioassays

Fig. 5. Ultrastructure of CA with high and low rates of JH synthesis. Bar: 1 μm. (A) Highly active CA from a 46-day-old embryo. (B) CA of low activity from a 60-day-old embryo. Arrow heads denote microtubules. av, autophagic vacuole; g, Golgi apparatus; m, mitochondria; n, nucleus.

(Novak, 1951; Gilbert and Schneiderman, 1961; Dorn, 1975; Imboden et al., 1978; Roussel and Schneider, 1979), and in recent studies different JHs have been isolated and identified in embryos of many insect species (Bergot et al., 1981; Brüning et al., 1985; Temin et al., 1986; Cusson et al., 1991; Short and Edwards, 1992). In larval and adult cockroaches, including *N. cinerea* and *D. punctata*, JH III is the predominant hormonal product of the CA (Tobe and Stay, 1977; Tobe and Stay, 1985). Thus, it was surprising when Lanzrein et al. (1984) discovered that embryonic CA of *N. cinerea* produced large amounts of MF, an unepoxidized precursor of JH III. A contrasting result was later obtained in *D. punctata*, in which embryonic CA were shown to release primarily JH III and very little MF (Cusson et al., 1991). Nevertheless, Cusson et al. (1991) examined CA products only during late embryogenesis, when MF production was typically low in *N. cinerea*. We therefore reinvestigated CA products in *D. punctata* by examining three developmental stages at which CA of *N. cinerea* synthesized low to high amounts of MF. Our finding that *D. punctata* CA synthesized only JH III at 71% and 92% development confirmed prior results showing that CA of late cockroach embryos produced mostly JH III and little MF (Bürgin and Lanzrein, 1988; Cusson et al., 1991). Surprisingly, however, CA of *D. punctata* produced only JH III at 49% development, an early stage at which the eyes of embryos are only lightly pigmented (Stay and Coop, 1973). At a comparable developmental stage in *N. cinerea*, the CA synthesized several-fold more MF than JH III (Bruning et al., 1987; Bürgin and Lanzrein, 1988).

At this time, it can only be speculated why CA of *N. cinerea* release MF and those of *D. puntata* do not. Bürgin and Lanzrein (1988) hypothesized that the well-developed chorion encompassing *N. cinerea* embryos inhibited oxygen penetration into embryonic tissues and thusly reduced the availability of oxygen for conversion of MF to JH III. In concordance with this hypothesis, *N. cinerea* embryos contained significant JH III only after chorion break-up late in embryogenesis (Bruning et al., 1985). Our results lend further support to the hypothesis of Bürgin and Lanzrein. In embryos of *D. punctata*, the chorion is very thin and often ruptured and likely permits free air exchange between the embryo and its surrounding environment. Therefore, embryonic CA of *D. punctata* ought to be well-oxygenated, and conversion of MF to JH III should be promoted. It nevertheless remains puzzling why CA of *N. cinerea* embryos release substantial MF *in vitro* (Bürgin and Lanzrein, 1988) when such conditions should foster tissue oxygenation.

Patterns of corpora allata activty in embryos

The first attempt to elucidate a pattern of JH synthesis in embryos was undertaken by Kikukawa and Tobe (1987), who reported that CA activity in *D. punctata* rose between 44% and 60% development from 0.57 to 1.61 pmol JH h^{-1} per CA pair and thereafter fell to 0.20 pmol JH h^{-1} per CA pair at 68% development. Although our current results and those from a previous report (Holbrook et al., 1996) confirm the early rise in JH sythesis noted by Kikukawa and Tobe, we have not found a sharp drop in gland activity at 68% development. Rather, JH release rates remained above 1.4 pmol h^{-1} per CA pair from 65% development (day 42) to hatch (Fig. 4A).

The most complete analysis to date on embryonic CA activity was performed in *N. cinerea*, in which biosynthetic output of the CA was reported to undergo a slow decline from dorsal closure to hatch (Bürgin and Lanzrein, 1988). In contrast, we have found that gland activity both rises and falls after dorsal closure in embryos of *D. punctata* (Holbrook et al., 1996; Fig. 4A). This single cycle of JH synthesis was more evident when CA activity was corrected for changes in embryo weight (Fig. 4B). Embryos of *D. punctata* increase by about 50-fold in both wet and dry weight during gestation (Stay and Coop, 1973; Fig. 2A), whereas those of *N. cinerea* increase in wet weight by only 2.5-fold and decrease slightly in dry weight (Imboden et al., 1978). On a per-wet-weight basis, rates of JH release in *D. punctata* nearly tripled from 0.24 pmol h^{-1} mg^{-1} on day 28 to 0.63 pmol h^{-1} mg^{-1} on day 42, after which CA activity underwent a gradual decline to 0.14 pmol h^{-1} mg^{-1} on day 58.

Results from our current study do not preclude the existence of more than one cycle of JH synthesis in embryos of *D. punctata*. With our current techniques, we were unable to obtain CA from embryos which had completed less than 43% of embryogenesis. Since CA differentiation precedes definitive dorsal closure (Haget, 1977), which occurs at about 20% embryo development time in *D. punctata* (Stay and Coop, 1973), we did not measure CA activity for nearly a quarter of embryogenesis. In the phasmid, *Carausius morosus*, ultrastructural modifications of CA cells suggested a cycle of JH synthesis near dorsal closure (Haget et al., 1981). We are currently investigating whether CA become active in early embryos of *D. punctata*.

Development of corpora allata during embryogenesis

Developmental changes in the number of cells in embryonic CA have been reported in the hemipterans *O. fasciatus* and *Dysdercus cingulatus*. In these

species, corpus allatum cell number increased early before definitive dorsal closure (Dorn, 1972; Jacob and Prabhu, 1985) but thenceforth remained constant until hatch. Although we did not examine early organogenesis of the CA in *D. punctata*, our results show clear differences between the beetle cockroach and Hemiptera. In *D. punctata*, corpus allatum cell number increased for much of embryogenesis, rising most rapidly between 86% and 92% development (Fig. 2A). A similar rapid increase in CA cell number occurred late in embryogenesis of *L. migratoria* (Aboulafia-Baginsky et al., 1984), suggesting that CA share a common pattern of development in embryos of orthopteroid insects.

For the first time, we have described in detail the relationship between changes in CA activity and CA cell number in embryos. Our results demonstrate great similarity between embryonic and larval CA development. In larvae of *D. punctata*, a cycle of JH synthesis occurs in each stadium, except for the last (Kikukawa and Tobe, 1986). When larval CA are activating or at peak activity, CA cell number remains constant or increases only slightly (Szibbo et al., 1982; Chiang et al., 1996a). After CA activity declines, a cycle of mitosis occurs within larval CA and cell number increases (Chiang et al., 1996a). In embryos, as in first through penultimate stadium larvae, we noted a single broad cycle of JH synthesis. While embryonic CA were activating or highly active (Fig. 4B), between days 28 and 56, CA cell number increased by less than 5% per day and mitotic rates were low. When rates of JH release reached low levels after day 56, rates of mitosis peaked and corpus allatum cell number rose by almost 15% per day until day 60. These results suggest that a high JH titer may restrain CA development in both embryos and larvae. This conjecture is supported by recent experiments in which topical application of second instars with fenoxycarb, a JH analog, suppressed CA cell mitosis and disrupted normal increase in CA cell number for up to 20 days (Chiang et al., 1996a).

The relationship between CA development and insect growth also displays striking similarity between embryos and larvae. In both stages, CA cells proliferate most rapidly shortly before a molt, be it larval-larval or embryo-larval (eclosion). For example, in second instars of *D. punctata*, mitotic activity is highest within CA on day 12 of the stadium, which lasts about 15 days (Szibbo et al., 1982; Chiang et al., 1996a), whereas in embryos, mitosis is maximal 56 to 58 days into embryogenesis (Fig. 2B), which lasts 65 days (Fig. 1). Because a peak in ecdysteroid titer coincides with onset of CA cell mitosis in second instars (Kikukawa and Tobe, 1986; Chiang et al.,

1996a), it has been speculated that ecdysteroids may in part modulate CA cell proliferation (Chiang et al., 1996a). Results from embryos provide additional support for this hypothesis. Although ecdysteroid titers have not been measured in *D. punctata* embryos, in *N. cinerea* ecdysteroid levels peak at about 75–90% embryo development time (Lanzrein et al., 1984). During this time in *D. punctata*, CA cells exhibit high rates of mitosis, and CA cell number increases rapidly (Fig. 2).

Morphometric and ultrastructural changes in embryonic corpora allata

Before the advent of assays for quantification of JH synthesis, measurements of CA volume were often performed to identify periods of CA activity (Cassier, 1990). In many cases, for example in adult females of *D. punctata* (Szibbo and Tobe, 1981), glandular expansion and subsequent contraction were proven to correspond with rising and falling rates of JH synthesis. We have now, for the first time, explored the size-activity relationship in embryonic CA of *D. punctata*. During mid-embryogenesis CA volume and activity increased simultaneously, but gland volume continued to rise in late embryos even as JH release rates declined. Therefore, a cycle in gland volume did not correspond with the cycle of JH synthesis. We recently obtained a similar result in second instars of *D. punctata* (Chiang et al., 1996a). Rapid growth of the CA in the first two-thirds of the stadium closely paralleled rising JH synthesis and was due to enlargement of CA cells. Continued glandular growth in the last third of the stadium coincided with diminution of CA activity and was due to cell proliferation. Unlike in larvae, however, in embryos changes in CA volume appear due only to cell proliferation because both parameters increased synchronously for most of embryogenesis while CA cells did not change in size.

Constant cell size in embryonic CA was surprising because the diameter of CA cells changes considerably during cycles of JH synthesis in larvae and adults (Chiang et al., 1989, 1996a, 1997; Chiang and Schal, 1994). Typically, cells increase in size when rates of JH synthesis rise, reach maximal size at peak gland activity, and become smaller when JH production decreases. Two factors could account for lack of cyclicity in cell size in embryos. First, although gland activity does change on a per-cell basis during embryogenesis, all glands are in fact at least moderately active. At 49% and 93% development, before and after peak gland activity, embryonic CA display per-cell rates of JH synthesis at least three

times higher than early second instar CA, which contain at least double the number of cells (Chiang et al., 1996a). Second, in late embryos CA may at times synthesize large amounts of JH even though mean rates of JH production are moderately low. From 89% development to hatch, CA activity was highly variable, ranging from 0.69 to 2.69 pmol JH h^{-1}. CA cells may remain large in order to maintain the capacity to synthesize high amounts of JH in rapid surges, perhaps during cuticular pigmentation, which occurs a few days before hatch in *D. punctata* and which is modulated by JH in some insects (Hiruma et al., 1984; Das and Gupta, 1977). The fact that CA at 92% development can be stimulated with farnesol, a late JH precursor, to produce amounts of JH similar to those by highly active CA at 71% development (Holbrook et al., 1996) lends further support to the hypothesis that late embryonic CA retain great synthetic capacity.

In previous studies on embryos of *O. fasciatus* (Dorn, 1975) and *C. morosus* (Haget et al., 1981), changes in CA ultrastructure were examined in order to characterize fluctuations in CA activity. In both species, the amounts of cellular organelles (mitochondria, Golgi, endoplasmic reticulum) within CA did not change greatly over time, but peaks of JH synthesis were assumed to correspond with the appearance of swirled endoplasmic reticulum. Nevertheless, since JH synthesis was not measured in either species, the relationship between changes in CA ultrastructure and activity could only be conjectured. Dorn (1975) did find, however, that JH activity in whole embryos, as measured with the *Tenebrio* assay, correlated directly with alterations in ultrastructural parameters presumably associated with JH synthesis. In concert with previous reports, we found that CA structure changed little in embryos. Cells in CA of both high and low activity from 46- and 60-day-old embryos, respectively, contained abundant cytoplasm, ribosomes, microtubules and mitochondria. This result is suprising because cell structure and organelle content change considerably during rising and falling JH synthesis in larvae and adults (Johnson et al., 1985; Johnson et al., 1993; Cheng and Chiang, 1995). Our results do, however, suggest an increase in Golgi and autophagic vacuoles in low-activity CA of older embryos. A similar increase in these structures is seen in adult CA when JH synthesis falls after a peak. Nevertheless, a more extensive examination of CA ultrastructure in *D. punctata* embryos is necessary and is indeed underway. This effort, which benefits from our establishment of the embryonic CA activity pattern, will for the first time determine precisely ultrastructural changes associated with known fluctuations in JH synthesis in embryos.

References

Aboulafia-Baginsky, N., Pener, M.P. and Staal, G.B., Chemical allatectomy of late *Locusta* embryos by a synthetic precocene and its effect on hopper morphogenesis. J. Insect Physiol., 30 (1984) 839–852.

Baker, F.C., Lanzrein, B., Miller, C.A., Tsai, L.W., Jamieson, G.C. and Schooley, D.A., Detection of only JH III in several life stages of *Nauphoeta cinerea* and *Thermobia domestica*. Life Sci., 35 (1984) 1553–1560.

Bergot, B.J., Baker, F.C., Cerf, D.C., Jamieson, G. and Schooley, D.A., Qualitative and quantitative aspects of juvenile hormone titers in developing embryos of several insect species: Discovery of a new JH-like substance extracted from eggs of *Manduca sexta*. In: Juvenile Hormone Biochemistry, G.E. Pratt and G.T. Brooks (eds.), Elsevier/North-Holland Biomedical Press, New York, 1981, pp. 33–45.

Brüning, E., Saxer, A. and Lanzrein, B., Methyl farnesoate and juvenile hormone III in normal and precocene treated embryos of the ovoviviparous cockroach *Nauphoeta cinerea*. Int. J. Invert. Reprod. Develop., 8 (1985) 269–278.

Brüning, E., Lanzrein, B., Function of juvenile hormone III in embryonic development of the cockroach, *Nauphoeta cinerea*. Int. J. Invert. Reprod. Develop., 12 (1987) 29–44.

Bürgin, C. and Lanzrein, B., Stage-dependent biosynthesis of methyl-farnesoate and juvenile hormone III and metabolism of juvenile hormone III in embryos of the cockroach, *Nauphoeta cinerea*. Insect Biochem., 18 (1988) 3–9.

Cassier, P., Morphology, histology, and ultrastructure of JH-producing glands in insects. In: Morphogenetic Hormones of Arthropods, Vol. 1, Pt. 2, A.P. Gupta (ed.), Rutgers University Press, New Brunswick, NJ, 1990, pp. 83–194.

Cheng, H-W. and Chiang, A-S., Autophagy and acid phosphatase activity in the corpora allata of adult mated females of *Diploptera punctata*. Cell Tiss. Res., 281 (1995) 109–117.

Chiang, A.-S. and Schal, C., Cyclic volumetric changes of corpus allatum cells in relation to juvenile hormone biosynthesis during ovarian cycles in cockroaches. Arch. Insect Biochem. Physiol., 27 (1994) 53–64.

Chiang, A.-S., Gadot, M. and Schal, C., Morphometric analysis of corpus allatum cells in adult females of three cockroach species. Mol. Cell. Endocrinol., 67 (1989) 179–184.

Chiang, A.-S., Holbrook, G.L. and Schal, C., Development-activity relationships in nymphal corpora allata of Diploptera punctata. Physiol. Entomol., 21 (1996a) 268–274.

Chiang, A.-S., Holbrook, G.L., Cheng, H-W. and Schal, C., Neural control of cell size in the corpora allata during the reproductive cycle of the cockroach Diploptera punctata (Dictyoptera: Blaberidae). Invert. Reprod. Develop., in press.

Chiang, A-S, Tsai, W-H., Holbrook, G.L. and Schal, C., Control of cell proliferation in the corpora allata during the reproductive cycle of the cockroach Diploptera punctata. Arch. Insect Biochem. Physiol., 32 (1996b) 299–313.

Cusson, M., Yagi, K.J., Ding, Q., Duve, H., Thorpe, A., McNeil, J.R. and Tobe, S.S., Biosynthesis and release of juvenile hormone and its precursors in insects and crustaceans: The search for a unifying arthropod endocrinology. Insect Biochem., 21 (1991) 1–6.

Das, Y.T. and Gupta, A.P., Nature and precursors of juvenile hormone-induced excessive cuticular melanisation in German cockroach. Nature, 268 (1977) 139–140.

Dorn, A., Die endokrinen drusen im embryo von Oncopeltus fasciatus Dallas (Insecta, Heteroptera). Morphogenese, funktionsaufnahme, beeinflussung des gewebewachstums und beziehungen zu den embryonalen häutungen. Z. Morphol.Tiere, 71 (1972) 52–104.

Dorn, A., Struktur und funktion des embryonalen corpus allatum von Oncopeltus fasciatus Dallas (Insecta, Heteroptera). Verh. Dtsch. Zool. Ges., 67 (1975) 85–89.

Engelmann, F., Die steuerung der ovarfunktion bei der ovoviviparen schabe Leucophaea maderae (Fabr.). J. Insect Physiol., 1 (1957) 257–278.

Feyereisen, R. and Tobe, S.S., A rapid partition assay for routine analysis of juvenile hormone release by insect corpora allata. Anal. Biochem., 111 (1981) 372–375.

Gilbert, L.I. and Schneiderman, H.A., The content of juvenile hormone and lipid in Lepidoptera: Sexual differences and developmental changes. Gen. Comp. Endocrinol., 1 (1961) 453–472.

Hagan, H.R., Embryology of the Viviparous Insects, Ronald Press, New York, 1951.

Haget, A., L'embryologie des insectes. In: Traite de Zoologie, Vol. 8, Pt. 5B, P.P. Grassé (ed.), Masson, New York, 1977, pp. 2–387.

Haget, A., Ressouches, A. and Rogueda, J., Chronological and ultrastructural observations on the activities of the embryonic corpus allatum in Carausius morosus Br. (Phasmida: Lonchodidae). Int. J. Insect Morphol. Embryol., 10 (1981) 65–81.

Hiruma, K., Matsumoto, S., Isogai, A. and Suzuki, A., Control of ommochrome synthesis by both juvenile

hormone and melanization hormone in the cabbage armyworm, Mamestra brassicae. J. Comp. Physiol., 154 (1984) 13–21.

Holbrook, G.L., Chiang, A-S. and Schal, C., Allatostatin inhibition and farnesol stimulation of corpus allatum activity in embryos of the viviparous cockroach, Diploptera punctata. Arch. Insect Biochem. Physiol., 32 (1996) 341–352.

Holbrook, G.L., Chiang, A-S. and Schal, C., Improved conditions for culture of biosynthetically active cockroach corpora allata. In Vitro Cell. Dev. Biol.–Anim., 33 (1997) 452–458.

Imboden, H., Lanzrein, B., Delbecque, J.P. and Lüscher, M., Ecdysteroids and juvenile hormone during embryogenesis in the ovoviviparous cockroach Nauphoeta cinerea. Gen. Comp. Endocrinol., 36 (1978) 628–635.

Jacob, M. and Prabhu, V.K.K., Development, endocrine organs and moulting in the embryos of Dysdercus cingulatus Fabr. (Heteroptera, Pyrrhocoridae). Proc. Indian Acad. Sci. (Anim.Sci.), 94 (1985) 489–501.

Johnson, G.D., Stay, B. and Rankin, S.M., Ultrastructure of corpora allata of known activity during the vitellogenic cycle in the cockroach Diploptera punctata. Cell Tiss. Res., 239 (1985) 317–327.

Johnson, G.D., Stay, B. and Chan, K.K., Structure-activity relationships in corpora allata of the cockroach Diploptera punctata: roles of mating and the ovary. Cell Tiss. Res., 274 (1993) 279–293.

Johnson, R.A. and Hill, L., The activity of the corpora allata in the fourth and fifth larval instars of the migratory locust. J. Insect Physiol., 19 (1973) 1921–1932.

Kikukawa, S. and Tobe, S.S., In vitro biosynthesis of juvenile hormone III by the embryonic corpora allata of the cockroach, Diploptera punctata. In: Proc., First Congress of the Asia and Oceania Society for Comparative Endocrinology, 1987, pp. 232–233.

Kikukawa, S. and Tobe, S.S., Juvenile hormone biosynthesis in female larvae of Diploptera punctata and the effect of allatectomy on haemolymph ecdysteroid titre. J. Insect Physiol., 32 (1986) 981–986.

Kurtti, T.J. and Brooks, M.A., The dissociation of insect embryos for cell culture. In Vitro, 12 (1976) 141–146.

Lanzrein, B., Imboden, H., Bürgin, C., Brüning, E. and Gfeller, H., On titers, origin, and functions of juvenile hormone III, methyl farnesoate, and ecdysteroids in embryonic development of the ovoviviparous cockroach Nauphoeta cinerea. In: Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones, J. Hoffmann and M. Porchet (eds.), Springer-Verlag, Berlin, 1984, pp. 454–465.

Munderloh, U.G. and Kurtti, T.J., Formulation of medium for tick cell culture. Exp. Appl. Acarol., 7 (1989) 219–229.

Novak, V.J.A. The metamorphosis hormones and morphogenesis in Oncopeltus fasciatus Dal. Vest. Cesk. Spol. Zool., 15 (1951) 1–48.

Pratt, G.E. and Tobe, S.S., Juvenile hormones radio-biosynthesised by corpora allata of adult female locusts in vitro. Life Sci., 14 (1974) 575–586.

Roth, L.M. and Willis, E.R., Intra-uterine nutrition of the

"beetle roach" *Diploptera dytiscoides* (Serv.) during embryogenesis, with notes on its biology in the laboratory (Blattaria: Diplopteridae). Psyche, 62 (1955) 55–68.

Roussel, J.-P. and Schneider, M., Mise en évidence, a l'aide d'un dosage biologique, d'hormones juvéniles au cours du développement embryonnaire de *Locusta migratoria*. C.R. Acad. Sci., 289D (1979) 29–31.

Scharrer, B. and von Harnack, M., Histophysiological studies on the corpus allatum of *Leucophaea maderae*. I. Normal life cycle in male and female adults. Biol. Bull., 115 (1958) 508–520.

Short, J.E. and Edwards, J.P., Levels of juvenile hormone III during embryonic development in the oriental cockroach *Blatta orientalis* (L.) (Dictyoptera: Blattidae). In: Insect Juvenile Hormone Research Fundamental and Applied Approaches. B. Mauchamp, F. Couillaud and J.C. Baehr (eds.), Institut National de la Recherche Agronomique, Paris, 1992, pp. 19–25.

Stay, B. and Coop, A., Developmental stages and chemical composition in embryos of the cockroach, *Diploptera punctata*, with observations on the effect of diet. J. Insect Physiol., 19 (1973) 147–171.

Szibbo, C.M. and Tobe, S.S., Cellular and volumetric changes in relation to the activity cycle in the corpora allata of *Diploptera punctata*. J. Insect Physiol., 27 (1981) 609–613.

Szibbo, C.M., Rotin, D., Feyereisen, R. and Tobe, S.S., Synthesis and degradation of C_{16} juvenile hormone (JH III) during the final two stadia of the cockroach, *Diploptera punctata*. Gen. Comp. Endocrinol., 48 (1982) 25–32.

Temin, G., Zander, M. and Roussel, J.-P. Physico-chemical (GC-MS) measurements of juvenile hormone III titres during embryogenesis of *Locusta migratoria*. Int. J. Invert. Reprod. Develop., 9 (1986) 105–112.

Tobe, S.S., Clarke, N., Stay, B. and Ruegg, R.P., Changes in cell number and activity of the corpora allata of the cockroach *Diploptera punctata*: a role for mating and the ovary. Can. J. Zool., 62 (1984) 2178–2182.

Tobe, S.S. and Stay, B., Corpus allatum activity in vitro during the reproductive cycle of the viviparous cockroach, *Diploptera punctata* (Eschscholtz). Gen. Comp. Endocrinol., 31 (1977) 138–147.

Tobe, S.S. and Stay, B., Structure and regulation of the corpus allatum. Adv. Insect Physiol., 18 (1985) 305–432.

Tsai, W-H., Holbrook, G.L., Schal, C., and Chiang, A-S. *In vitro* growth of corpora allata from *Diploptera punctata*. In Vitro Cell. Dev. Biol.–Anim., 31 (1995) 542–547.