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# Allatostatin Inhibition and Farnesol Stimulation of Corpus Allatum Activity in Embryos of the Viviparous Cockroach, *Diploptera punctata*

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Juvenile hormone (JH) biosynthesis by corpora allata (CA) from embryos of the cockroach *Diploptera punctata* was measured at four stages during the latter half of embryogenesis. Individual glands from 32-day-old embryos that had completed 49% of embryonic development synthesized 0.3 pmol JH III $h^{-1}$. By day 46 (70% development) gland activity rose to 1.1 pmol JH $h^{-1}$, but on subsequent days JH synthetic rates declined, measuring only 0.8 pmol $h^{-1}$ on day 56 (86% development) and 0.5 pmol $h^{-1}$ on day 60 (92% development). Differences in JH biosynthesis by CA from different-aged embryos were more evident when gland activity was corrected for either corpus allatum cell number, which increased progressively from fewer than 200 cells per gland on day 32 to almost 700 cells per gland on day 60, or embryo mass, which increased from 1.6 mg per embryo on day 32 to 10.8 mg per embryo on day 60. JH biosynthetic rates were significantly inhibited in a medium containing $10^{-8}$ M Dip-allatostatin 7 which suppressed CA activity by 68, 83, 76, and 51% on days 32, 46, 56, and 60, respectively. In all embryonic stages JH production was significantly stimulated by incubation of glands with 200 μM farnesol, a late precursor in the JH biosynthetic pathway. 

**Key words: cockroach, embryos, corpora allata, juvenile hormone, allatostatin, farnesol**

## INTRODUCTION

Regulation of corpus allatum activity has been examined extensively in larval and adult insects (Tobe and Stay, 1985; Stay et al., 1994). To date, how-

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ever, no information is available on regulation of embryonic corpora allata (CA*), despite the fact that JH is present in embryos (Dorn, 1990) and that embryonic CA synthesize JH (Kikukawa and Tobe, 1987; Bürgin and Lanzrein, 1988). The dearth of information on embryonic CA regulation is likely due to the small size of most insect embryos, which makes such embryos difficult subjects for endocrinological studies. This difficulty is overcome in *Diploptera punctata,* a viviparous cockroach in which internally brooded embryos undergo substantial growth and become very large during embryogenesis (Stay and Coop, 1973). The ability to dissect CA from embryos of this species coupled with the availability of allatostatic neuropeptides, such as Dip-allatostatins from *D. punctata* (Woodhead et al., 1989, 1994; Pratt et al., 1989), and of late JH precursors, such as farnesol, provides an opportunity to gain information on regulation of embryonic CA through in vitro experiments.

Allatostatins, rapid and reversible neuropeptide inhibitors of JH biosynthesis, have been identified and described in three cockroach species, *D. punctata* (Woodhead et al., 1989, 1994; Pratt et al., 1989), *Periplaneta americana* (Weaver et al., 1994) and *Blattella germanica* (Bellés et al., 1994). While allatostatins are known to be present in larval and adult cockroaches and to inhibit activity of CA in these postembryonic stages (Woodhead et al., 1989, 1994; Pratt et al., 1989, 1990; Stay et al., 1991; Weaver et al., 1994; Bellés et al., 1994), the role of allatostatins in embryonic CA regulation remains unknown. Nevertheless, a function for allatostatins in embryos is suggested by the fact that lateral neurosecretory cells, which likely produce allatostatins in adults (Stay et al., 1992), are biosynthetically active (Khan and Fraser, 1962) and allatostatin immunopositive during mid to late embryogenesis (Stay and Woodhead, 1994).

It has been the objective of our research to identify similarities and (or) differences in the regulation of CA activity in embryos and in larvae and adults of *D. punctata.* To this end, we have examined the response of embryonic CA to Dip-allatostatin 7, which inhibits both larval and adult CA activity (Stay et al., 1991). In addition, we have explored rate limitation in the JH biosynthetic pathway in embryos by measuring the extent to which JH production by embryonic CA is stimulated with farnesol, a late precursor in JH biosynthesis.

## MATERIALS AND METHODS

### Insect Rearing

The *D. punctata* colony was maintained at 27 ± 0.3°C and was provided Purina rat chow and water ad libitum. Newly eclosed adult females were mated and thereafter kept in groups of 10 to 20 under conditions similar to the colony. Under these conditions oviposition and parturition occurred 7.8 ± 0.1 (SEM) days (n = 16) and 73.2 ± 0.2 days (n = 29) after mating. Thus, embryonic development lasted about 65 days. Embryo broods were obtained

*Abbreviations used: CA = corpora allata; FA = farnesoic acid; FEAR = fractional endocrine activity ratio; JH = juvenile hormone; RP-HPLC = reverse phase high pressure liquid chromatography; TLC = thin layer chromatography.

from pregnant females by application of gentle pressure to females' abdomens. Individual embryos were partitioned under cockroach saline (Kurtti and Brooks, 1976) at 360 mOsM.

### Corpus Allatum Cell Number and Activity

In *D. punctata*, dorsal closure occurs in 12-day-old embryos that have completed 19% of embryonic development (Stay and Coop, 1973). Although CA differentiation in insects precedes dorsal closure (Dorn, 1990), we were unable to consistently locate embryonic CA in *D. punctata* until day 28 (43% development) when individual glands had completed migration and become paired dorsal to the esophagus. Thus, our study focused on the latter half of embryogenesis. To obtain CA, embryos were decapitated and heads were transferred into cockroach saline where CA were separated from adjacent tissues.

Corpus allatum cell number was determined with a monolayer technique (Chiang et al., 1989). Briefly, individual glands were partially digested in 0.1% collagenase, transferred into a drop of nuclear stain solution (0.1% safranin and 0.1 M citric acid) on the surface of a gelatin-subbed slide, and spread into a monolayer beneath a coverslip. Cell number was determined by counting all red-stained nuclei with the aid of a grid in the ocular of a compound microscope.

JH biosynthesis by CA in vitro was quantified with a modified partition radiochemical assay (Tobe and Pratt, 1974; Pratt and Tobe, 1974; Feyereisen and Tobe, 1981). Individual glands were preincubated for 90 min in L-15B medium (Munderloh and Kurtti, 1989) containing 100 μM L-[methyl-$^3$H]methionine (specific activity of 194 mCi/mmol; New England Nuclear, Wilmington, DE) as the only methionine source. After preincubation, individual glands were transferred into 20 μl of fresh medium containing tritiated methionine in 6 ×25 mm glass culture tubes. After 6 h of incubation at 27°C the medium in each tube, along with the gland, was extracted with 80 μl isooctane. To quantify JH biosynthesis by CA, a 40 μl aliquot of the isooctane hyperphase was analyzed by liquid scintillation spectrometry. When JH release, rather than JH biosynthesis, was measured, medium was extracted without glands. Over 95% of radiolabeled products synthesized by embryonic CA coeluted with synthetic JH III and the primary product of adult CA in TLC and RP-HPLC (unpublished results).

### Allatostatin and Farnesol

Dip-allatostatin 7 (Ala-Pro-Ser-Gly-Ala-Gln-Arg-Leu-Tyr-Gly-Phe-Gly-Leu-$NH_2$) and *trans,trans*-farnesol (96% pure) were purchased, respectively, from Sigma (St. Louis, MO) and Aldrich (Milwaukee, WI). Dip-allatostatin 7 (Dip-allatostatin) was maintained in a $10^{-5}$ M stock solution in distilled water at –20°C. To prepare $10^{-8}$ M Dip-allatostatin 7, the stock was diluted to $10^{-6}$ M with distilled water, and 10 μl of this solution with 5 μl 1% BSA in distilled water was blown to dryness under nitrogen. The Dip-allatostatin and BSA were resuspended in 10 μl of 1.0 N HCl (Stay et al., 1991). L-15B medium (980 μl) was added to the acid solution and the pH of the medium was adjusted to 7.2 with 1.0 N NaOH. For control experiments L-15B medium was

modified as above but without Dip-allatostatin. To prepare 200 μM farnesol in L-15B medium, 10 μl of 20 mM farnesol in methanol was air dried in a test tube, after which 1 ml of L-15B medium was added to the tube. The medium with farnesol was extensively vortexed before use in the radiochemical assay.

A split gland design was employed to measure Dip-allatostatin inhibition and farnesol stimulation of JH biosynthesis. CA pairs were split and one gland from each pair was incubated in L-15B medium while the contralateral gland was incubated in L-15B medium with either $10^{-8}$ M Dip-allatostatin 7 or 200 μM farnesol. In both Dip-allatostatin and farnesol experiments, JH biosynthesis was determined for each member of gland pairs. Percent inhibition of CA activity with Dip-allatostatin 7 was determined from the formula [1-(treated rate/untreated rate)] ×100 (Stay et al., 1991).

## RESULTS

### Corpus Allatum Development and Activity in *D. punctata* Embryos

Embryo mass increased by almost 7-fold between 49% (day 32) and 92% (day 60) embryonic development time (Table 1). During this period cell number per single corpus allatum increased progressively from 187 ± 8 cells on day 32 to nearly 700 cells on day 60.

JH biosynthetic rates rose and then declined during the latter half of embryogenesis (Table 1). On day 32 individual glands displayed the lowest rate of JH biosynthesis (0.3 pmol $h^{-1}$) of any measured day. The highest rate of JH production, 1.1 ± 0.1 pmol $h^{-1}$ per corpus allatum, occurred on day 46. On days 56 and 60 JH biosynthetic rates became sequentially lower.

Although direct measurements of JH biosynthesis revealed that CA from 32-, 46-, 56-, and 60-day-old embryos produced different amounts of JH, differences in CA activity were more evident upon correction of hormonal synthesis by the number of cells within glands. This arithmetic extrapolation makes the untested assumption that all CA cells contribute equally to total glandular output. Nevertheless, determining JH produced per cell is, at the very least, an acceptable parameter for measuring relative JH biosynthesis, since single cells can be considered functional biosynthetic units within CA. On a per-cell basis JH biosynthesis was highest on day 46, intermediate on days 32 and 56, and lowest on day 60 (Table 1). Correction of JH biosynthesis for embryo mass confirmed that CA from 46-day-old embryos were most active while those from 60-day-old embryos were least active.

### Biosynthetic Symmetry in Embryonic Corpora Allata

Hormonal output by the two members of a corpus allatum pair was highly positively correlated ($r$ = 0.87, Pearson's product-moment correlation coefficient), and the y-intercept (0.02) and slope (1.03) of the regression line indicated that glands within a pair produced similar amounts of JH (Fig. 1). The difference in JH release exceeded 25% in only 5 of 34 gland pairs, and a greater than twofold difference occurred in only one gland pair. Although we measured hormone release rather than hormone biosynthesis, our results clearly

**TABLE 1. Juvenile Hormone Biosynthesis in Relation to Embryo and Corpus Allatum Development***

| Embryo age (stage) | Fresh embryo mass (mg)[a] | Cell number per corpus allatum | JH biosynthesis (pmol $h^{-1}$ per gland) | JH biosynthesis (fmol $h^{-1}$ per cell)[b] | JH biosynthesis (fmol $h^{-1}$ per mg)[c] |
|---|---|---|---|---|---|
| 32 days (49%) | 1.6 ± 0.04 (13) | 187 ± 8 (10) | 0.3 ± 0.05 (13) | 1.6 | 187.5 |
| 46 days (70%) | 3.9 ± 0.16 (12) | 288 ± 13 (12) | 1.1 ± 0.10 (13) | 3.8 | 282.1 |
| 56 days (86%) | 8.4 ± 0.26 (12) | 433 ± 12 (12) | 0.8 ± 0.12 (14) | 1.8 | 95.2 |
| 60 days (92%) | 10.8 ± 0.38 (14) | 691 ± 29 (13) | 0.5 ± 0.09 (20) | 0.7 | 46.3 |

*Results for embryo mass, corpus allatum cell number, and JH biosynthesis are expressed as means ± SEM with the number of individual glands or embryos indicated in parentheses.

[a]Embryos were obtained from adult females 32, 46, 56, and 60 days after oviposition. Embryo stage, a percent measure of development completed by embryos, was calculated by dividing embryo age by the mean length of embryonic development.

[b]JH biosynthesis per cell was determined by dividing the mean rate of hormone synthesis of glands from a given age by the mean number of cells in glands from the same sage.

[c]JH biosynthesis per mg body mass was calculated by dividing embryo mass into the mean JH biosynthetic rate of individual glands.

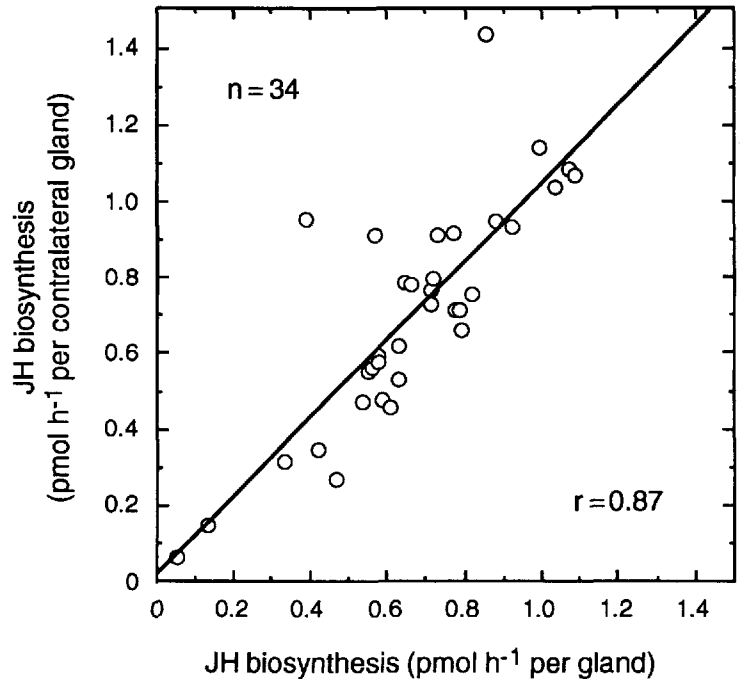


Fig. 1. Symmetry in JH III release between members of corpus allatum pairs. Thirty-four gland pairs from embryos of various ages were split, and JH release rates were determined for each member of every pair. Individual glands within a pair were randomly assigned to two different groups and JH release by one gland was plotted against JH release by the other gland of a pair.

indicate biosynthetic symmetry since JH biosynthesis and release are directly related in cockroach CA (Tobe and Stay, 1985). Symmetry of JH synthesis within embryonic CA pairs justified our use of a split-gland design in subsequent experiments wherein one gland of a pair received farnesol or Dip-allatostatin treatment while the contralateral gland served as a control.

### Corpus Allatum Response to Farnesol and Dip-Allatostatin 7

At each of the four embryonic stages, JH biosynthesis was significantly increased ($P < 0.05$, paired $t$-test) by incubation of CA in medium containing 200 μM farnesol (Fig. 2). This suggested that late enzymes in the JH III biosynthetic pathway were always in excess during the latter half of embryogenesis. While the level of JH produced by farnesol-treated glands varied with both basal activity of CA and embryo age, the degree of stimulation, 2.6- to 3-fold, was similar for all embryonic stages. To allow direct comparison of the effect of farnesol on embryonic CA with the effect of late JH precursors on larval and adult CA (Feyereisen et al., 1981a,b; Yagi et al., 1991), we calculated a fractional endocrine activity ratio (FEAR) for each gland pair by dividing the spontaneous rate of JH biosynthesis of the control gland by the stimulated rate of JH biosynthesis of the contralateral farnesol-treated gland (Pratt and Tobe, 1976). Mean FEAR values ranged from 0.34 ± 0.06

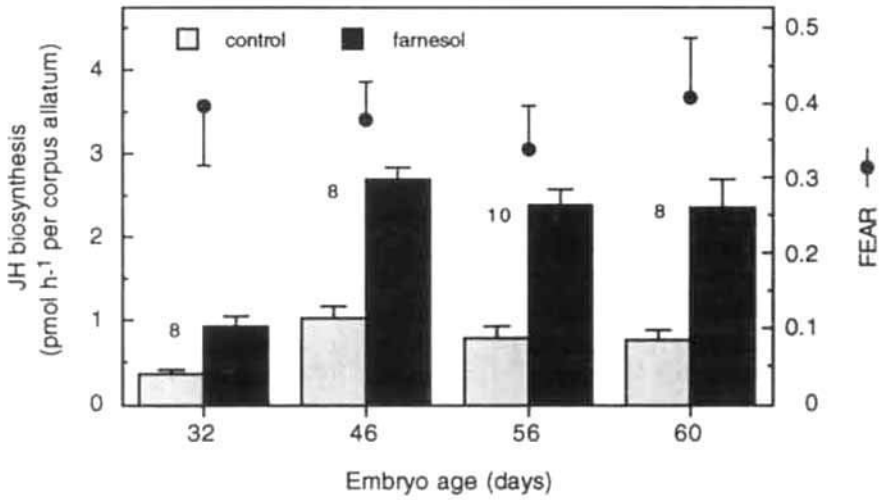


Fig. 2. Farnesol stimulation of JH III biosynthesis. Corpus allatum pairs were obtained from 32-, 46-, 56-, and 60-day-old embryos. Gland pairs were split and one gland from each pair was incubated in L-15B medium while the contralateral gland was incubated in L-15B medium with 200 μM farnesol. Bars represent mean rates of JH biosynthesis for glands incubated with and without farnesol. A fractional endocrine activity ratio (FEAR) was determined for every gland pair by dividing the spontaneous activity of the control gland by the stimulated activity of the contralateral farnesol-treated gland. Mean FEAR values, represented by circles, were calculated for each embryo age. Error bars represent one SEM. The number of examined gland pairs is shown for each age.

(day 56) to 0.41 ± 0.08 (day 32) but did not significantly differ at any embryonic stage ($P > 0.05$, ANOVA, Games-Howell multiple comparison of means, arcsin transformation of data; Fig. 2). A relatively constant and high degree of farnesol-stimulated JH III production at all four stages suggested that the activity of late enzymes in JH III biosynthesis is modulated coordinately with changes in early rate-limiting steps.

Because a low FEAR (i.e., high farnesol stimulation of JH biosynthesis) indicates the presence of substantial early rate limitation, we speculated that JH production by embryonic CA would be modulated by allatostatins, which are known to inhibit CA activity by affecting early steps in the JH biosynthetic pathway (Pratt et al., 1989, 1991; Woodhead et al., 1994). We found that CA activity was significantly suppressed ($P < 0.05$, paired $t$-test) at all embryonic stages by $10^{-8}$ M Dip-allatostatin 7 (Fig. 3), but the extent of inhibition varied among embryos of different ages. Glands of highest activity from day 46 embryos were the most sensitive to Dip-allatostatin 7, being inhibited by 83 ± 4%. In contrast, glands of lowest activity (on a per-cell or per-weight basis) from day 60 embryos were the least sensitive to Dip-allatostatin 7, and the level of inhibition on day 60 (51 ± 11%) was significantly lower ($P < 0.05$, unpaired $t$-test on arcsin transformed data) than on day 46. CA from the remaining embryonic stages displayed intermediate levels of inhibition with Dip-allatostatin 7, 76 ± 10% on day 32 and 68 ± 8% on day 56.

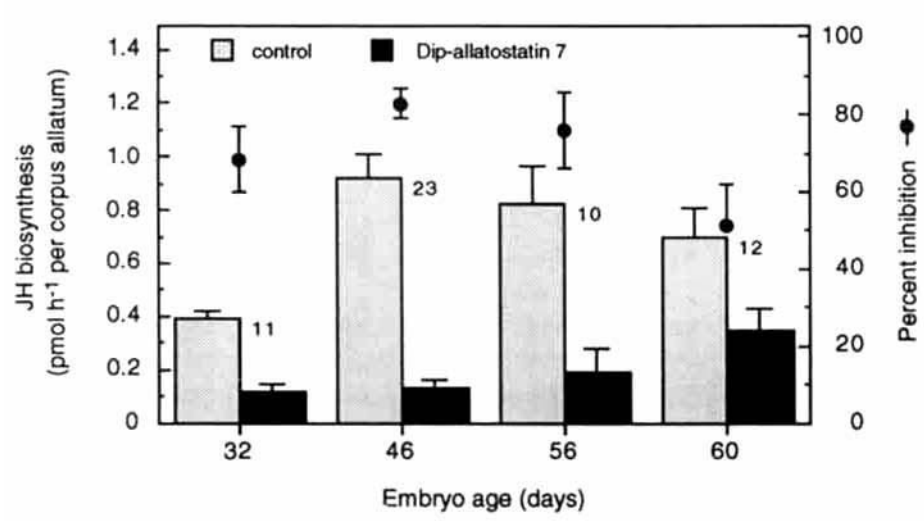


Fig. 3. Dip-allatostatin inhibition of JH III biosynthesis. Corpus allatum pairs were obtained as in Figure 2. Gland pairs were split and one gland from each pair was incubated in L-15B medium containing 0.05% BSA and $10^{-8}$ M Dip-allatostatin 7. The contralateral gland served as a control and was incubated in the same medium with BSA but without Dip-allatostatin. Bars represent mean rates of JH biosynthesis and error bars represent one SEM. Circles show the mean percent inhibition of CA activity at each embryo age. Error bars for data points represent SEM. The number of examined gland pairs is shown for each age.

## DISCUSSION

### Activity of Embryonic Corpora Allata

Hormone synthesis by embryonic CA has been explored previously in two cockroach species, *Nauphoeta cinerea* and *D. punctata*. In *N. cinerea*, Bürgin and Lanzrein (1988) found that total hormonal output of CA underwent a continuous, yet highly variable, decline from dorsal closure to hatch. In *D. punctata*, Kikukawa and Tobe (1987) reported a pattern of increasing JH biosynthesis between 44 and 60% embryonic development time with gland pairs producing JH at a peak rate of 1.61 pmol $h^{-1}$ at 60% development; subsequently CA activity declined sharply to 0.20 pmol $h^{-1}$ per gland pair at 68% development. Our results differed from those of both Bürgin and Lanzrein (1988) and Kikukawa and Tobe (1987). CA appeared to display a broad cycle of activity with JH biosynthesis peaking at 70% development (Table 1), the stage at which Kikukawa and Tobe (1987) found a collapse in CA activity. While only a few days of embryonic development have been examined both in our study and that of Kikukawa and Tobe (1987), preliminary results from examination of CA activity at 4 day intervals throughout the latter half of embryogenesis confirm our present conclusions (unpublished results by the authors).

In cockroaches the CA are paired organs and the two members of a pair display a high degree of functional symmetry. For example, JH biosynthetic rates by individual glands of a pair differ by less than twofold in 72% of CA pairs from adult females of *P. americana* (Weaver, 1979) and in 80% of gland pairs from adult females of *B. germanica* (Chiang and Schal, 1991). To date,

however, CA from *D. punctata* have been shown to be the most functionally symmetric of all cockroach CA. In adult females of *D. punctata,* nine out of ten CA pairs contained glands with activities differing by less than a factor of two (Szibbo and Tobe, 1981). We now show that embryonic CA of *D. punctata* are even more symmetric than those from adults. In embryos JH production by the two glands of a pair differed by less than twofold in 33 of 34 gland pairs and by less than 25% in 29 of 34 gland pairs (Fig. 1). This exceptional symmetry justified the use of split gland experiments in which one gland of a pair served as a control for the contralateral gland which received either farnesol or allatostatin treatment.

**Regulation of Embryonic Corpora Allata**

In both larval and adult cockroaches late JH precursors have been shown to enhance CA activity in vitro (Feyereisen et al., 1981a,b; Aclé et al., 1989; Gadot et al., 1989; Yagi et al., 1991). We have now found that JH biosynthesis by CA from embryos is significantly enhanced with farnesol (Fig. 2), the second antepenultimate precursor in the JH biosynthetic pathway. A similar result with farnesol had previously been obtained with adult CA of *D. punctata* (Feyereisen et al., 1981a). Thus, in both embryos and adults of this species none of the last four enzymes (farnesol dehydrogenase, farnesal dehydrogenase, *O*-methyl transferase and *10,11*-epoxidase) in JH biosynthesis is rate-limiting, and rate limitation occurs early in the JH biosynthetic pathway prior to conversion of farnesol to farnesal by farnesol dehydrogenase.

In *D. punctata,* the degree to which late JH precursors stimulate JH biosynthesis of CA in vitro varies with both the basal activity of the CA and the physiological stage of the insect from which the CA were obtained. For example, in adult females farnesol and FA only slightly enhance JH production by peak active CA from vitellogenic females (FEAR = 0.6 to 1) but substantially stimulate JH biosynthesis by far less active CA from ovipositing females (FEAR = 0.2 to 0.4) (Feyereisen et al., 1981a,b). In the last stadium, as in the adult stage, highly active CA from early-stadium larvae are stimulated with FA to a lesser extent (FEAR > 0.8) than low activity CA from late-stadium larvae (FEAR = 0 to 0.2) (Yagi et al., 1991). Our results with embryonic CA contrast with those from larvae and adults. While spontaneous rates of JH biosynthesis changed dramatically during embryogenesis, especially on a per cell or weight basis, the FEAR remained surprisingly constant at 0.34 to 0.41, indicating that stimulated rates of JH biosynthesis were always about threefold basal rates. This suggested that throughout the second half of embryogenesis changes in early rate limiting step(s) are accompanied by proportional changes in the amount and (or) activity of late enzymes in the JH biosynthetic pathway. A possible implication of this tight correlation is that the activities of all enzymes in JH biosynthesis, including late enzymes, may be coordinately modulated during embryogenesis. Such an occurrence would lend support to the hypothesis that coordinated alterations in glandular and cellular machinery (cell size, organelle number and size, enzymes, etc.) associated with JH biosynthesis are of great importance in regulating the capacity of embryonic CA to synthesize JH.

Allatostatins have been isolated from both larval and adult brains of *D.*

*punctata* and have been shown to inhibit activity of CA from these developmental stages (Paulson et al., 1987; Woodhead et al., 1989; Pratt et al., 1989, 1990; Stay et al., 1991; Woodhead et al., 1994). In a recent report Dip-allatostatin immunoreactivity was localized in brains of *D. punctata* embryos (Stay and Woodhead, 1994), suggesting a role for these peptides in embryonic CA regulation. We now report that Dip-allatostatin 7 at $10^{-8}$ M suppresses JH production of embryonic CA by 50 to 80% (Fig. 3). Comparable levels of inhibition at the same Dip-allatostatin 7 concentration have previously been reported for CA from both larvae and adults of *D. punctata* (Stay et al., 1991).

The profile of response of CA to allatostatins varies both within and between developmental stages. For example, within the penultimate stadium of *D. punctata* peak active glands at mid-stadium are inhibited by 60% with $10^{-8}$ M Dip-allatostatin 7, while late in the stadium, after gland activity has declined, Dip-allatostatin inhibits JH biosynthesis by only 30% (Stay et al., 1991). In adult reproductive females of *D. punctata* CA also show a pattern of changing response to allatostatins, but the pattern differs from that in penultimate stadium larvae. Peak active glands from females at the middle of the gonotrophic cycle are relatively insensitive to $10^{-8}$ M Dip-allatostatin 7, but glands of low activity from ovipositing females are inhibited by more than 90% (Pratt et al., 1990). Our results indicate that CA from embryos display a profile of response to Dip-allatostatin 7 more similar to that of CA from penultimate stadium larvae than from adult females. In both larvae and embryos, highly active glands display great sensitivity to Dip-allatostatin 7 while CA that produce JH at low rates show significantly less Dip-allatostatin sensitivity. These results prompt speculation that Dip-allatostatins may play different roles in regulating function of pre-adult and adult CA.

This is the first study to explore potential mechanisms regulating CA activity in embryos. Further studies with embryonic CA may promote understanding of CA function in all developmental stages. For example, the embryonic system may prove beneficial to studies on rate limitation in JH biosynthesis. Poor penetration of early JH precursors into CA tissues is a major shortcoming in studies on rate limitation (Feyereisen et al., 1981a) but precursor uptake may be promoted by the very small size of embryonic CA. Studies on long-term modulation of CA activity may also benefit from the use of embryonic CA, since embryonic tissues are often very amenable to extended culture in vitro. In any case, embryonic endocrinology of the CA has been understudied and deserves much greater attention.

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