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The effect of ovary implants on juvenile hormone production by corpora allata of male *Diploptera punctata*

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Abstract

In the cockroach *Diploptera punctata*, vitellogenic basal oocytes stimulate juvenile hormone production by the corpora allata. Experiments with males were designed to determine whether oocytes must grow vitellogenically in order to stimulate juvenile hormone production. Two ovarioles with vitellogenic basal oocytes were implanted into unoperated and sham-operated males that do not produce vitellogenin, and males with denervated corpora allata, that produce more juvenile hormone, and sometimes more vitellogenin. Males with corpora allata in similar conditions were injected with saline as controls. In males with denervated corpora allata compared to sham-operated and unoperated males, the implanted basal oocytes showed a greater increase in length, protein, and vitellin content. Juvenile hormone synthesis by denervated corpora allata in males with ovariole implants was greater than in controls. In 10 of 50 males with denervated corpora allata in which one or no ovarioles grew, juvenile hormone production was not higher than in controls. This suggests that if sufficient juvenile hormone is not present to produce vitellogenin, or oocytes do not take vitellogenin up, juvenile hormone production is not stimulated. In sham-operated males implanted with ovarioles, no difference was detected in juvenile hormone synthesis compared to controls. However when unoperated males were used a significant increase was detected. This suggests that intact nerves from the brain to the corpora allata restrained juvenile hormone production so that ovarioles could elicit only slight stimulation of the corpora allata, and oocytes continued vitellogenesis but more slowly than in denervated males. Thus the extent of vitellogenesis appears to determine the ability of ovaries to stimulate juvenile hormone production.

Keywords: corpus allatum denervation, oocyte growth, vitellin, vitellogenin

Abbreviation:
KPBS Kingan phosphate-buffered saline
ELISA enzyme-linked immunosorbent assay

Introduction

In adult female *Diploptera punctata*, the ovary in vitellogenic stages stimulates juvenile hormone synthesis by the corpora allata (Rankin and Stay, 1984). Ovarian implants into ovariectomized females demonstrated that the basal follicles are responsible for the signal which must travel humorally since the ovaries are not attached (Rankin and Stay, 1984). However, the nature of the stimulatory factor is unknown. One approach to the isolation of such a factor would be to incubate vitellogenic ovaries *in vitro* and test for the ability of the ovary-conditioned medium to stimulate juvenile hormone synthesis by the corpora allata. To determine conditions for obtaining the stimulatory factor from incubated ovaries, it is necessary to know whether continued vitellogenic growth is required for the release of the factor. The present study was initiated to address this question.

Males were used because ordinarily their corpora allata produce insufficient juvenile hormone for the fat body to produce vitellogenin (Mundall *et al.* 1983). Yet male fat body can produce vitellogenin if provided with sufficient juvenile hormone (Mundall *et al.* 1983), and male corpora allata implanted into allatectomized females can respond to ovarian stimulation (Stay and Woodhead, 1990). Also, if the nerves between the brain and the corpora allata are severed in males, juvenile hormone synthesis increases sufficiently so that one-third of the males produce some vitellogenin (Mundall *et al.* 1983). It was expected that stimulatory ovarioles implanted into males with nerves to the corpora allata intact would not continue vitellogenic growth, and therefore would not stimulate juvenile hormone synthesis, unless they continued to release stimulatory factor without growth. Whereas if continued vitellogenic growth by ovarioles is required for release of stimulatory factor, then stimulatory ovarioles implanted into males with denervated corpora allata would, in only about one-third of the animals, continue vitellogenin uptake and thus stimulate juvenile hormone synthesis

beyond the level resulting from denervation. In this study we measured increase in length, protein, and vitellin content of vitellogenic basal follicles implanted into males with nerves to the corpora allata intact or severed, and determined their effect on rates of juvenile hormone synthesis.

Materials and Methods

Animal maintenance

Males and females were collected from stock cultures on the day of adult ecdysis (day 0), and maintained on Purina lab chow and water at 27° C. Females mate at adult ecdysis and oviposit about day 7. The mean weight of 2-day adult males is 0.130 ± 0.003g (n = 10); that of 2-day adult females is 0.240 ± 0.007g (n = 10).

Surgery

All animals were chilled on ice for 5-10min prior to surgery. Corpora allata of males were denervated or sham-operated on day 1 as previously described (Stay and Tobe, 1977). Ovaries from mated females were dissected in sterile insect saline (Yeager, 1939) with 6.6 mg penicillin and 10.0 mg streptomycin per 100 ml and measured using an ocular micrometer. Ovaries from three stages of development were used. Previtellogenic, vitellogenic, and late vitellogenic ovaries, from days 0, 3, and 5 females, had basal oocyte lengths 0.70, 1.00, and 1.50 mm, respectively. Whole ovaries or ovarioles were injected in saline into the abdomen of host animals with a drawn-out glass pipet. Controls received insect saline. Females were ovariectomized as last instar larvae as previously described (Stay *et al.* 1983)

Juvenile hormone assay and measurement of basal oocyte growth

One, two or three days after ovary implantation, corpora allata were dissected for a radiochemical juvenile hormone assay (Feyereisen and Tobe, 1981). The incubation medium was 50 µl of TC199 without methionine (GIBCO, www.lifetech.com) with 2% Ficoll (Sigma www.sigmaaldrich.com) and [L-methyl-^{14}C] methionine (New England Nuclear, http://las.perkinelmer.com) specific activity 56.0 mCi/mmol, 50µM final concentration. Ovarioles were retrieved and the length of the basal follicle (oocyte including follicle cells), was determined. Lengths are expressed as the mean length of the pair. Hereafter, follicles are referred to as basal oocytes.

Protein assay

Ovarioles were placed in 1.5 ml Eppendorf tubes with 50 µl phosphate buffered saline (PBS; 50 mM sodium phosphate, 0.14 M NaCl, pH 7.2), and frozen at -20° C to break yolk vesicles. After thawing, the tissue was homogenized by uptake and expulsion in a 100 µl syringe (Scientific Glass Engineering, www.sge.com). Homogenization was continued after addition of 150 µl PBS to the tube. The tubes were then centrifuged at 2500 g for 10 min. A protein assay was performed on 100 µl of the supernatant with 2 ml of Bradford reagent (Bradford, 1976).

Vitellin Assay

Vitellin was quantitated by a competitive enzyme-linked immunosorbent assay (ELISA) using a rabbit polyclonal antibody made against *D. punctata* mature egg homogenate. The specificity of the antibody was demonstrated by finding immunoreactivity one peak in immunoelectrophoresis (Stoltzman and Stay, 1997), and lack of immunoreactivity in ELISA in the absence of antibody. Plates of 96 wells (Costar) were coated overnight at 4° C with vitellin in egg homogenate (4 ng/100 µl Kingan's PBS [Kingan, 1989]). Following removal of vitellin solution, wells were washed twice with PBS and treated with 2% goat serum in PBS for 2-4 h at room temperature. Plates were again washed twice with PBS and 100µl of a mixture of anti-vitellin antibody (1:1000) and standards of egg vitellin or unknowns were added and incubated at 4° C overnight. The standards were 0.007 to 1.0 mg vitellin in egg homogenate. After washing 4 times with PBS, wells were treated with 100 µl of goat anti-rabbit alkaline phosphatase (1:500 in PBS plus 2% goat serum) for 2-4 h at room temperature, followed by washing twice with PBS. Wells then were treated with 100µl of phosphatase substrate (Sigma #104) in 10% diethanolamine (40ng/100µl) for 15-20 min. Absorbance of reaction product was measured at 405nm with a Titertek Multiscan plate reader (www.titertek.com).

Statistical analysis

Student's *t* test was used for all statistical analyses. Results are expressed as mean ± standard error of the mean (SEM).

Results

Ovariole dose response

Each ovary of *D. punctata* usually consists of 6 ovarioles. In order to determine the minimun number of ovarioles to implant into males that could elicit detectable stimulation of juvenile hormone synthesis, ovariectomized females were implanted with ovarioles from stimulatory ovaries (basal oocytes 1.00 mm in length). Although one ovariole stimulated juvenile hormone synthesis ($P < 0.05$), two elicited greater stimulation and as much as 3 or 6 ovarioles ($P < 0.01$)(Fig. 1). All basal oocytes grew in length, although those of the whole ovary (6 ovarioles) grew less than the partial ovaries (Fig. 1).

Response of innervated (sham-operated) vs. denervated corpora allata to implanted ovarioles

On the basis of the response of corpora allata of ovariectomized females implanted with different numbers of ovarioles and the fact that males are half the weight of females, in the following experiments, 2 ovarioles were implanted into males. Preliminary experiments demonstrated that cutting nerves between the brain and the corpora allata (denervation) on day one, implantation of ovarioles on day 4 and assays 72 h following implantation was a satisfactory protocol. The implantation of ovarioles with basal oocytes of stimulatory size (1.00 mm in length) resulted in significantly greater juvenile hormone production by denervated corpora allata ($P < 0.001$) but not by innervated corpora allata of sham-operated males compared to saline-injected controls (Fig. 2B). Basal oocytes of implanted ovarioles increased in length in males with both innervated and denervated corpora allata, but the increase was greater in the males with denervated corpora allata (Fig. 2A, $P < 0.001$). This increase in oocyte length includes measurement of the basal oocytes in the 20% (10/50) of the males

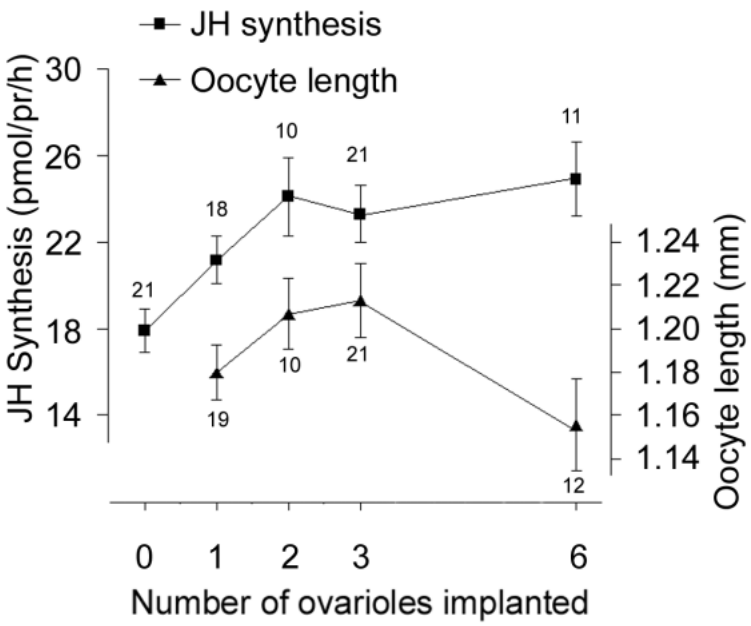

Figure 1. Juvenile hormone (JH) synthesis and length of basal oocytes 48hr after implantation of ovarioles with 1.00mm long basal oocytes into day 2 ovariectomized mated females. Females received saline (0) or 1, 2, 3 ovarioles or a whole ovary (6 ovarioles). Each datum point is the mean of the number of individual measurements shown above or below each point. Bars indicate standard errors of the mean (SEM).

with denervated corpora allata in which only one or neither of the ovarioles increased in length. In some cases the basal oocytes had degenerate yolk and had decreased in length. This could have resulted from damage to ovarioles upon injection. In implants in which a single oocyte grew, the mean basal oocyte length was 1.17 ± 0.02mm (n = 7). juvenile hormone rates were stimulated only in animals in which both of the basal oocytes of ovarioles increased in length; juvenile hormone rates for animals in which one or no ovarioles increased in length were the same as those of saline-injected controls (Fig. 3). Mean juvenile hormone rate for the corpora allata of males in which a single oocyte grew was 19.1 ± 2.3 pmol/pr/h (n = 7) whereas that in which neither oocyte grew was 20.3 ± 6.44 pmol/pr/h (n = 3). From these data it is clear that the ovarioles stimulated juvenile hormone synthesis in males with denervated corpora allata and did so only if two were growing.

Response to ovaries of specific developmental stages on juvenile hormone synthesis by denervated corpora allata

To demonstrate that male corpora allata show differential response to ovaries in different stages of development, a previtellogenic ovary, or 2 ovarioles from a late vitellogenic ovary (basal oocytes, 0.70, 1.50 mm in length, respectively) were implanted into 4-day adult males with corpora allata denervated on day 1. The results after 72h are shown in Figure 4 along with the results of implantation of ovarioles with 1.00 mm long basal oocytes shown in Figure 2 for males with denervated corpora allata. The basal oocytes of previtellogenic ovarioles had increased in length to 0.77 ± 0.01 mm and 38% contained a few yolk vesicles, the basal oocytes of the late vitellogenic ovarioles did not increase in length but did complete chorion formation (Fig. 4A). Rates of juvenile hormone synthesis by corpora allata from males with previtellogenic and late vitellogenic ovariole implants did not show the increase in

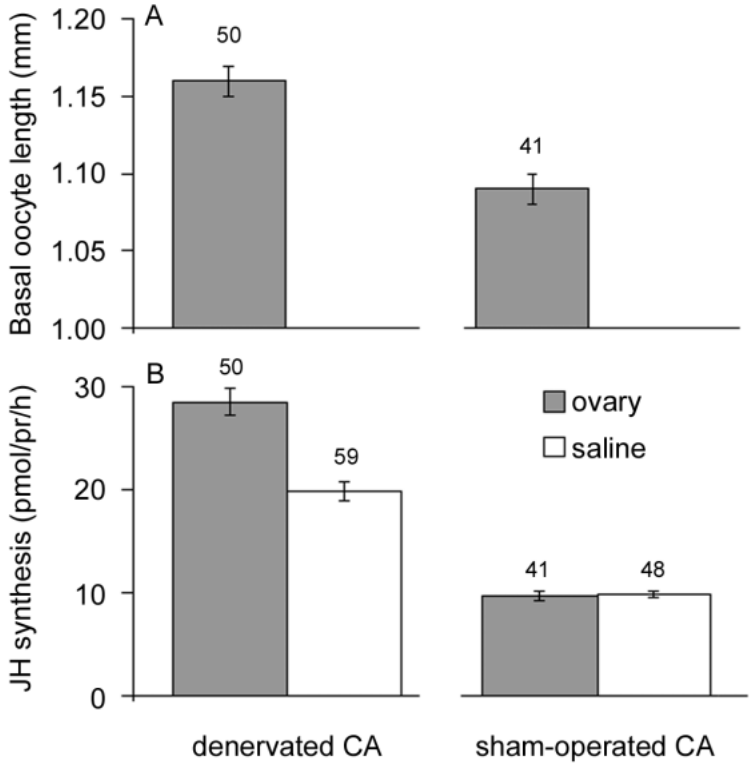

Figure 2. Basal oocyte length of implanted ovarioles (A) and juvenile hormone synthesis (JH) (B) by corpora allata (CA). Measurements were made 72hr after injection of 2 ovarioles (basal oocytes 1.00 mm in length) or saline into 4 day males with normally innervated corpora allata (sham-operated) or corpora allata denervated on day 1. The data for males implanted with ovarioles includes all animals whether or not ovarioles increased in length. Each column shows the mean ± SEM of the number of individual measurements above the column.

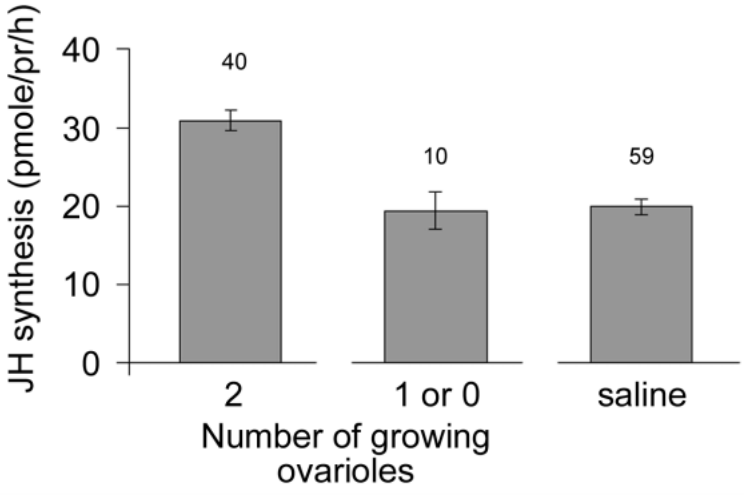

Figure 3. Juvenile hormone (JH) synthesis by the corpora allata of males denervated on day 1, 72 hr after implantation of 2 ovarioles with 1.00mm long basal oocytes. The data in Fig 2B are subdivided into males in which basal oocytes of both ovarioles increased in length (2) and those in which only one or neither increased in length (1 or 0). Controls (saline) for males with denervated corpora allata injected with saline are from Fig 2B. Each column shows the mean ± SEM of the number of individual measurements above each column.

juvenile hormone synthesis that was evident following implantation of ovarioles with vitellogenic basal oocytes 1.00 mm in length ($P < 0.001$) (Fig. 4B). Thus, ovarian stimulation of juvenile hormone synthesis in males appears to be specific to stages of rapid vitellogenic growth.

Protein and vitellin content of ovarioles implanted into males with denervated and innervated (sham-operated) corpora allata

In normal females the increase in length of basal oocytes corresponds to increases in total protein and vitellin content (Mundall *et al.* 1981; Rankin and Stay, 1984). Also, in basal oocytes of ovaries implanted into males with female corpora allata, the increase in length was accompanied by increase in ovarian vitellin (Mundall *et al.* 1979). To confirm that the increase in length of basal oocytes implanted into males with innervated and denervated corpora allata was indeed increase in protein, and not increase in length by swelling, another set of experiments was carried out. Ovaries with basal oocytes 1.00 mm in length were separated into 3 sets of 2 ovarioles. Protein content of 1 set was determined at the time that the other sets were injected into males with denervated or innervated corpora allata. After 72 h the protein content of the implanted ovarioles was determined. Virtually all implants grew in length (Fig. 5). The increase in length of basal oocytes implanted into males with both innervated (sham-operated) and denervated corpora allata was accompanied by an increase in protein. Basal oocyte length was significantly greater in males with denervated corpora allata than in those with innervated (sham-operated) corpora allata ($P < 0.001$) as was the protein content ($P < 0.01$) (Fig. 5).

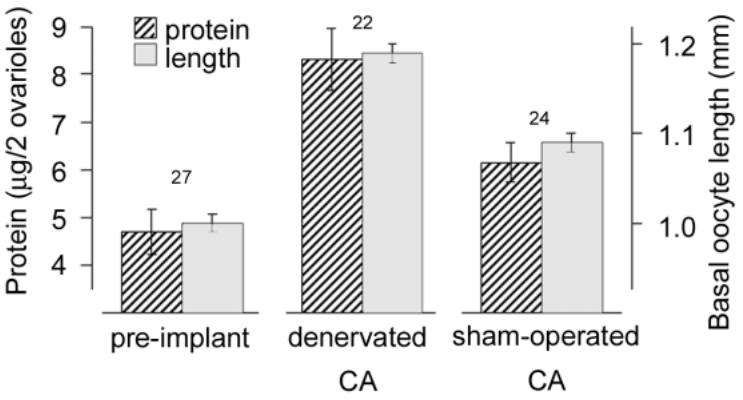

Figure 5. Change in protein content and length of basal oocytes of two ovarioles from the same ovary assayed either at the time of implant of "sister ovarioles" or 72 h after implant into 4 day males with corpora allata (CA) denervated or innervated (sham-operated) on day 1. Each column shows the mean ± SEM of the number of individual measurements above the column.

To confirm that the increase in protein was vitellin and not some other hemolymph proteins, a third set of experiments was performed. The design was the same as for the protein assay except that vitellin was measured by an ELISA with polyclonal antibody against *D. punctata* vitellin. In sham-operated males, both basal oocytes increased in length in 16 of 28 pairs of ovarioles; in males with denervated corpora allata, both basal oocytes increased in length in 14 of 26 pairs of ovarioles. In males with innervated (sham-operated) or denervated corpora allata, vitellin content increased in ovarioles that showed growth in both basal oocytes, compared to vitellin in 2 ovarioles measured at the time of implantation of sister ovarioles into males. The difference was significant in both groups ($P < 0.001$) (Fig. 6). The amount of vitellin in ovarioles in the corpora

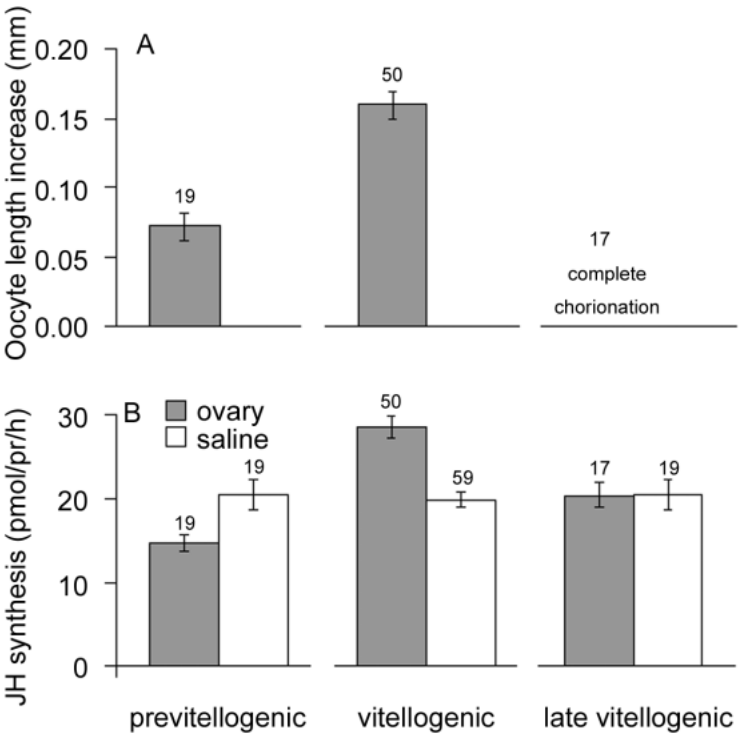

Figure 4. Increase in basal oocyte length (A) and juvenile hormone (JH) synthesis (B) following implantation of a previtellogenic ovary or 2 ovarioles from a vitellogenic or late vitellogenic ovary (basal oocytes 0.70, 1.00, 1.50 mm in length respectively) into 4 day adult males with corpora allata denervated on day 1. Controls received saline. Each column shows the mean ± SEM of the number of individual measurements above the column. The data for 1.00 mm basal oocytes and corresponding saline controls is from Figure 2 males with denervated corpora allata.

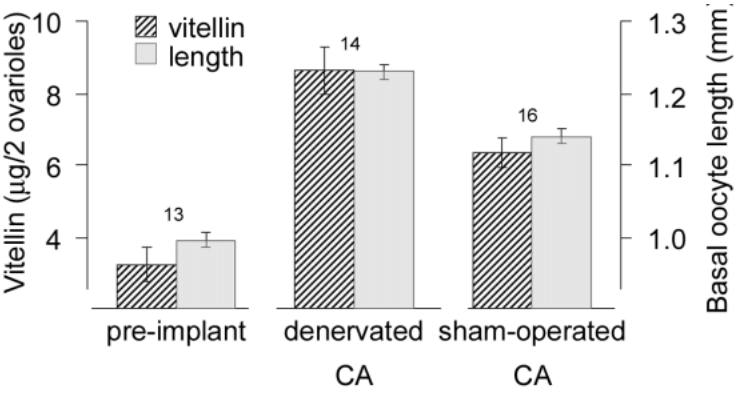

Figure 6. Change in vitellin content and length of basal oocytes of two ovarioles from the same ovary assayed either at the time of implant of "sister ovarioles" or 72 h after implant into 4 day males with corpora allata (CA) denervated or innervated (sham-operated) on day 1. Each column shows the mean ± SEM of the number of individual measurements above the column.

allata-denervated males was significantly greater than that in sham-operated males ($P < 0.05$). The vitellin content of the unimplanted sister pairs of ovarioles was 3.23 ± 0.40 µg vitellin/2 ovarioles (n = 13); that of all implanted ovarioles, whether or not they grew in length, for sham-operated males was 4.57 ± 0.55 µg vitellin/2 ovarioles (n = 28) and for corpora allata-denervated males was 5.71 ± 0.85 µg vitellin/2 ovarioles (n = 26). These values differed significantly from the control sister ovarioles at $P < 0.05$ (sham-operated corpora allata) and $P < 0.01$ (denervated corpora allata).

For the experiments in which increase in length was accompanied by increase in protein and vitellin, rates of juvenile hormone synthesis were also measured. The results are similar to those shown in Fig. 2. In males in which vitellin content of ovarioles was determined, juvenile hormone synthesis by corpora allata of animals with denervated corpora allata was 28.9 ± 2.7 pmol/pr/h (n = 20) whereas that of innervated (sham-operated) corpora allata was 11.7 ± 0.6 pmol/pr/h (n = 25). In males in which protein content of ovarioles was determined, juvenile hormone synthesis by corpora allata of animals with denervated corpora allata was 24.1 ± 1.7 pmol/pr/h (n = 23) while that of innervated (sham-operated) corpora allata was 9.9 ± 0.7 pmol/pr/h (n = 26). In both experiments, denervated corpora allata produced 2.5 times more juvenile hormone than innervated (sham-operated) corpora allata.

Juvenile hormone synthesis in unoperated males on days 1, 2, and 3 after ovariole implantation

The increase in protein in ovarioles implanted into males with innervated (sham-operated) corpora allata is accounted for by an increase in vitellin even though at 72 h after implantation of the ovarioles no increase in juvenile hormone synthesis compared to saline-injected control animals was evident. Since production of vitellin appears to require a higher than normal level of juvenile hormone in males, it was possible that an increase in juvenile hormone synthesis might have occurred earlier than 72 h, or that sham operation could have slightly inhibited rates of juvenile hormone synthesis so that a significant difference could not be detected. Therefore, a fourth set of experiments was performed on unoperated males except for injection of ovarioles or saline, and assays of juvenile hormone synthesis were performed at 24, 48, and 72 h after implantation of ovarioles or injection of saline. No significant difference in juvenile hormone production between animals implanted with ovarioles and controls injected with saline was found at 24 or 48 h. However, the difference in juvenile hormone production was significant at 72 h ($P < 0.05$) (Fig. 7). The vitellin content of the two ovarioles implanted into these unoperated males that increased in length (12 of 13) after 72 h was 6.8 ± 0.6 µg vitellin/2 ovarioles (n=12), which was significantly greater ($P < 0.001$) than the sister ovarioles at the time of implant in Fig. 6.

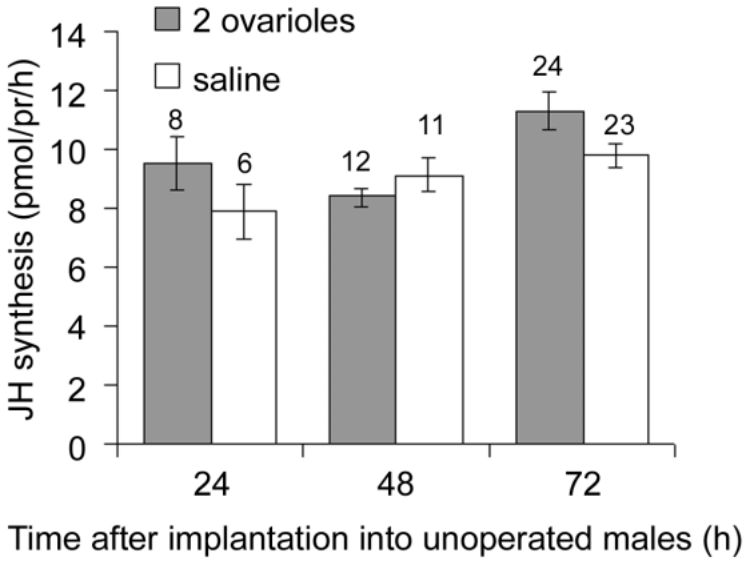

Figure 7. Juvenile hormone (JH) synthesis by the corpora allata of unoperated males 24, 48, and 72 h after implantation of two ovarioles with 1.00mm basal oocytes or injected with saline. Each column shows the mean ± SEM of the number of individual measurements above the column.

Discussion

The results were not as expected. In males with innervated or denervated corpora allata, the basal oocytes of implanted vitellogenic ovarioles were found to increase in length, protein, and vitellin content, in most cases, but their effect on juvenile hormone synthesis depended upon growth of basal oocytes in both ovarioles and upon the condition of the corpora allata. When corpora allata were denervated, the growth of the basal oocytes was greater than that of ovarioles in males with innervated corpora allata and this growth was accompanied by increase in juvenile hormone synthesis beyond that resulting from denervation. On the other hand, the lesser growth of basal oocytes in ovarioles implanted into sham-operated males with innervated corpora allata was not accompanied by a detectable increase in juvenile hormone synthesis. However, in unoperated males implanted with similar ovarioles with vitellogenic basal oocytes, a significant increase in juvenile hormone synthesis was detected although it was much less than the increase in males with denervated corpora allata. The difference between males with innervated and denervated corpora allata is reminiscent of the difference between mated and virgin ovariectomized females implanted with stimulatory ovaries (Woodhead *et al.* 2001). The corpora allata of the virgins, that produce juvenile hormone at one-half the rate of those of mated females, did not, or only slightly increased, juvenile hormone production following implantation of an ovary with 1.00 mm stimulatory basal oocytes, whereas juvenile hormone production was stimulated to a greater extent in the mated females (Woodhead *et al.* 2001). At the time of assay, growth of the oocytes reflects the concentration of juvenile hormone to which they were exposed during the entire experimental period, whereas the production of juvenile hormone by the corpora allata was determined for a 3 h period at the end of the assay. Since in *D. punctata* the titer of juvenile hormone is regulated primarily by its production rather than degradation (Tobe *et al.* 1985), we presume that the increase in juvenile hormone synthesis monitored at the end of the assay reflects an increase in juvenile hormone titer in the hemolymph during the implantation period. A detectable response in production of juvenile hormone by corpora allata of males required 72 h of ovarian influence whereas that in females required 24-48 h (Rankin and Stay, 1984). A longer period of growth of

implanted ovarioles was required for detection of the stimulation of the corpora allata in males probably because male fat body synthesizes less vitellogenin than female fat body (Mundall *et al.* 1983) with the result that the slowly growing oocytes produce less stimulatory factor. An alternative interpretation of the finding that innervated corpora allata of males and those of ovariectomized virgin females are only slightly stimulated is that the innervated corpora allata of males and virgin females are inhibited by the brain and resist stimulation by vitellogenic ovaries. Denervation of corpora allata in males and mating of females frees the corpora allata to be stimulated by the ovary.

The vitellogenic growth of most oocytes (presumably the undamaged oocytes) regardless of the absence of vitellogenin in the hemolymph at the time of implantation suggests that sufficient stimulatory factor was released from the oocytes at the time of implant to stimulate juvenile hormone synthesis and the production of vitellogenin. Therefore, in males with denervated corpora allata, this initial release of stimulatory factor is able to greatly increase juvenile hormone synthesis because there is no inhibition from the brain. With more juvenile hormone, the fat body can produce more vitellogenin which in turn allows for more uptake of vitellogenin by the basal oocyte. It was expected that if vitellogenic uptake was necessary before release of stimulatory factor, then only one-third of the ovarioles implanted into males with denervated corpora allata would be able to grow because it was that proportion of males that would have been expected to have vitellogenin in their hemolymph (Mundall *et al.* 1983). In males with denervated corpora allata implanted with previtellogenic ovaries, 38% appeared to have some vitellogenin in their hemolymph as this proportion of the ovaries showed some yolk spheres in the oocytes after 72 h. In sham-operated males with innervated corpora allata, the initial release of stimulatory factor from vitellogenic oocytes was able to stimulate juvenile hormone synthesis by the corpora allata slightly, but the sham operation possibly inhibits juvenile hormone production sufficiently such that stimulation of juvenile hormone synthesis could not be detected.

Since males with innervated corpora allata have no vitellogenin in their hemolymph, the stimulatory ovarioles must have been able to stimulate juvenile hormone production quickly enough so that vitellogenesis occured before the ovarioles degenerated. Thus it appears that a successful strategy for obtaining stimulatory factor from vitellogenic ovarioles would be to incubate them for short periods of time in medium without vitellogenin. It is not clear, however, whether the ovarioles would require juvenile hormone to release stimulatory factor because all males implanted with stimulatory ovarioles had at least basal levels of juvenile hormone.

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