

Juvenile Hormone and the Development of Ovarian Responsiveness to a Brain Hormone in the Mosquito, *Aedes aegypti*

J. P. SHAPIRO¹ AND H. H. HAGEDORN

Department of Entomology, Cornell University, Ithaca, New York 14853

Accepted May 7, 1981

This study examined the responsiveness of developing ovaries to extracts of heads of the mosquito *Aedes aegypti* and regulation of that responsiveness by juvenile hormone (JH). Ovaries of 60-hr-old mosquitoes responded to saline extracts of heads *in vitro* by secreting ecdysone. Ovaries isolated from 12-hr-old females did not respond significantly to extracts, but developed responsiveness *in vivo* during the previtellogenic phase of growth, 24-60 hr after emergence. Ovaries in abdomens isolated from females (and thus from the source of JH, the corpora allata (CA)) soon after emergence did not grow or become responsive. However, topical application of JH-I or an analog (methoprene) to isolated abdomens induced growth and responsiveness in ovaries, methoprene with greater effect than JH-I. Ovarian growth and responsiveness were functions of the dose of JH-I applied to abdomens. The system predicted results *in vivo*, as 20-OH-ecdysone content of females CA ablated after emergence increased by only 39 pg-eq when those animals were blood fed, whereas 20-OH-ecdysone in sham-operated or unoperated animals increased 188 pg-eq upon blood feeding.

Egg development in the adult female mosquito *Aedes aegypti* proceeds through two developmental phases, a previtellogenic and a vitellogenic phase. While previtellogenic development begins upon or before emergence, vitellogenic development is initiated by the blood meal. Hormones regulate both phases of development.

Prior to emergence, and again after a blood meal, incipient follicles form within, and separate from the germarium of each ovariole in the ovary, beginning previtellogenic development. Follicle separation is probably coincident with peak titers of the hormone 20-hydroxyecdysone (20-OH-ecdysone), and has been induced by injection of that hormone (Beckemeyer and Lea, 1980). Further follicular development proceeds under the influence of juvenile hormone (JH) secreted by the corpora allata (CA), a pair of glands located in the prothorax of the mosquito. Ablation of CA at

emergence abolished follicular growth beyond 52 μm , while reimplantation of CA or treatment with JH analogs restored growth to over 100 μm (Gwadz and Spielman, 1973). Similar results were obtained using abdomens isolated from animals (and CA) at emergence (Hagedorn *et al.*, 1977). Ovaries that have attained 100- μm follicle length, the resting stage, grow no further until vitellogenic development is initiated by blood feeding. The ingested blood meal stimulates release of egg development neurosecretory hormone (EDNH) from the medial neurosecretory cells (MNC) of the brain or from the corpora cardiaca, the neurohemal organs of the MNC (Lea, 1967, 1972). Recently, aqueous extracts of mid-brains and heads were found to stimulate ovaries to secrete ecdysone *in vitro* (Hagedorn *et al.*, 1979). We hypothesized that the active factor(s), presumably EDNH, stimulated resting stage ovaries to secrete ecdysone, which, when converted to 20-OH-ecdysone, would stimulate vitellogenin synthesis by the fat body. The latter process has been well demonstrated *in vitro*

¹ Present address: Department of Biochemistry, University of Arizona, Tucson, Ariz. 85724.

containers at 27°, 70–80% RH, with 3% sucrose solution. Sham-operated animals were treated by opening the neck membrane and grasping the commissure briefly without disrupting the CA. Animals were fed on a human forearm 60–72 hr after emergence, returned to the rearing room, and frozen 20 hr after feeding. Mortality was 5–40% for sham-operated and CA-ablated animals and 80% for CA-ablated, methoprene-treated animals.

Individual animals were extracted for ecdysteroids in motor-driven tapered glass homogenizers in 30 μ l of cold 50% methanol, and homogenizers rinsed with 30 μ l, then 40 μ l of 50% methanol. The combined homogenates and rinses were heated just to boiling for 1 min, then centrifuged 30 min at 18,000g (0°). Supernatants were transferred to clean tubes and pellets reextracted with 50 μ l of 50% methanol, centrifuged 20 min, and supernatant pooled with the first supernatant. Methanol was removed *in vacuo* at 50° for 10 hr, borate buffer (Borst and O'Connor, 1974) added, and tubes vortexed thoroughly. Extracts were assayed by RIA with 20-OH-ecdysone standards because blood-fed females contain predominantly that ecdysteroid (Hagedorn *et al.*, 1975). Extraction efficiency was 86% by radiotracer analysis.

Radioimmunoassay. Ecdysteroids secreted into saline media or extracted from whole animals were assayed by RIA (Borst and O'Connor, 1974; Chang and O'Connor, 1979) using Horn antiserum I-2 (Horn *et al.*, 1976). The lot of antiserum supplied to us bound ecdysone and 20-OH-ecdysone with equal affinity, so standards of either ecdysteroid could be used to compete against [³H]ecdysone. Ecdysone standards were run with samples of medium, 20-OH-ecdysone standards with extracts of whole animals. The 100- μ l samples, dissolved in saline (culture medium) or borate buffer (extracts), were assayed in 6 \times 50-mm culture tubes using [³H]ecdysone (purified by thin-layer chromatography; sp act, 54 Ci/mmol; 10,000–13,000 dpm/tube). Incubations were for 18–24 hr at 4–6°. Standards of ecdysone in saline or 20-OH-ecdysone in borate buffer were included in triplicate in each assay, ranging from 16 to 1000 pg. Radioactivity was counted in a Beckman LS-3100 liquid scintillation counter for 10 min/tube, after addition of 500 μ l of RIAfluor (New England). Experimental values were read from standard curves plotted on semi-log paper.

RESULTS

Development of Ovarian Responsiveness

Ovaries were isolated from female *A. aegypti* at various times after emergence and incubated in saline extract of whole heads for 18 hr to determine their competence to secrete ecdysone in response to EDNH in the extracts. Only after 18 hr *in*

vivo were ovaries responsive *in vitro*, and maximal response was obtained from ovaries isolated 60 hr after emergence (Fig. 1). While 80% of responsiveness developed between 24 and 48 hr after emergence, a curve of ovarian follicle growth (from Hagedorn *et al.*, 1977) shows that ovaries continued to grow through 60 hr.

The Effect of JH on Growth and Responsiveness

The ability of JH-I or an analog (methoprene) to induce growth and responsiveness in ovaries was tested by applying the compound or vehicle (acetone) to abdomens isolated soon after emergence, removing ovaries 60 hr later (the period required for development *in vivo*), and measuring follicle length and ecdysone secretion of ovaries in response to saline extract of heads.

Since abdomens were isolated from the source of JH (the CA), ovaries in abdomens treated only with acetone showed little or no growth (Table 1, follicle length). Follicle length of these ovaries was comparable to that of ovaries from animals 12 hr after emergence (compare with Fig. 1). Growth was restored by application of JH-I or methoprene. Ovaries from isolated abdomens treated with acetone only did not respond to an extract of heads, while responsiveness was restored by application of JH-I or methoprene to abdomens (Table 1, ecdysone secreted). Ovaries from JH-treated abdomens responded specifically to extract, as shown by comparison of ovaries incubated in saline with those incubated in extract. Only ovaries from abdomens treated with methoprene secreted detectable quantities of ecdysone into saline. Nonetheless, secretion by these ovaries increased sixfold upon incubation in extract, exceeding that of other treatments by 43%. Follicular growth of ovaries from methoprene-treated abdomens also exceeded growth of ovaries from intact controls ($P < 0.025$) as well as growth of ovaries from JH-I-treated abdomens ($P < 0.005$).

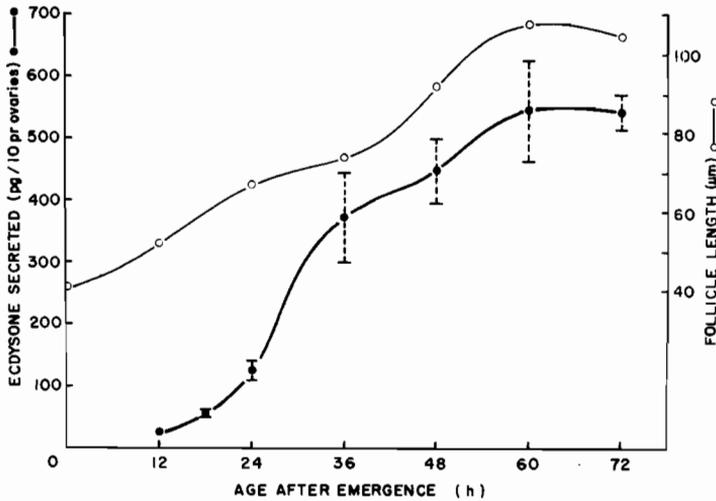


FIG. 1. Ecdysone secreted by ovaries into a saline extract of heads as a function of the postemergent age of ovarian donors. Ovaries isolated from sugar-fed mosquitoes of increasing age after emergence were incubated in 2.8 head-eq/50 µl concentration of head extract for 18 hr. Ecdysone in the extract after incubation (●—●) was measured by RIA (mean ± SEM of triplicates). Plot of follicular growth (○—○) is from Hagedorn *et al.* (1977).

Dose Dependence of JH-Induced Growth and Responsiveness

To test whether growth and responsiveness of ovaries in isolated abdomens developed in proportion to the dose of JH, increasing doses of JH-I were applied to abdomens, and ovaries were isolated and incubated with extract 60 hr later. Follicle

length increased with dose of JH-I when 5 to 500 ng were applied (Table 2). Responsiveness of these ovaries to extract also increased, although not in direct proportion to follicle length. While follicles gained 75% of maximal growth with a 50-ng dose of JH-I, ecdysone secretion was only 50% of the maximum.

TABLE 1
FOLLICLE LENGTH OF OVARIES FROM ABDOMENS TREATED WITH JH-I OR METHOPRENE, AND ECDYSONE SECRETED UPON INCUBATION IN SALINE OR EXTRACT OF HEADS

Ovary donors	Topical treatment	Follicle length (µm)	Ecdysone secreted (pg-eq/10 pr)	
			Into saline	Into extract
Intact females, 60 hr old	None	97 ± 1	<16	273 ± 36
Abdomens isolated at emergence	Acetone	52 ± 1	<16	28 ± 8
	JH-I	96 ± 1	<16	268 ± 12
	Methoprene	100 ± 1	62 ± 6	385 ± 20

Note. Abdomens were isolated within 1 hr of emergence, treated with 500 ng/abdomen JH-I, 5 ng/abdomen methoprene, or 5 µl acetone vehicle, and incubated 60 hr. Ovaries were isolated and incubated 6 hr, 10 pr/50 µl of saline or extract (concentration, 3 head-eq/50 µl). Follicle lengths of 25 follicles per sample were measured and medium was assayed for ecdysone by RIA. Mean ± SEM of five samples.

TABLE 2
FOLLICLE LENGTH OF OVARIES FROM ABDOMENS
TREATED WITH INCREASING DOSES OF JH-I,
AND ECDYSONE SECRETED UPON INCUBATION
IN EXTRACT OF HEADS

Treatment	Follicle length (μm)	Ecdysone secreted (pg-eq/10 pr)
Acetone, 0.5 μl	46 \pm 1	<16
JH-I		
5 ng	66 \pm 2	53 \pm 4
50 ng	83 \pm 0	100 \pm 12
500 ng	95 \pm 2	198 \pm 16

Note. Conditions as in Table 1, except dose of JH-I. Mean \pm SEM of triplicates.

The Effect of CA Ablation on Responsiveness

In the previous experiments, isolation of abdomens was intended to approximate isolation of ovaries from the source of JH, the CA, and exposure of ovaries to extracts of heads *in vitro* was intended to approximate exposure to EDNH after a blood meal. We performed equivalent manipulations in the whole animal to determine whether predictions from the isolated systems were borne out *in vivo*. When we ablated CA soon after emergence and blood fed animals 60–72 hr later, 67% less 20-OH-ecdysone was detected by RIA in whole animal extracts than in extracts of sham-operated or unoperated controls (Table 3). Unfed animals contained barely detectable quantities of 20-OH-ecdysone. Topical treatment of CA-ablated animals with methoprene restored 20-OH-ecdysone content after blood feeding to 294 pg-eq, levels significantly higher than those in unoperated or sham-operated animals. However, mortality due to acetone treatment of operated animals reduced the sample size to two.

DISCUSSION

We have effectively modeled regulation of previtellogenic ovarian development and an early event of vitellogenic development,

TABLE 3
EFFECT OF CA ABLATION ON 20-OH-ECDYSONE
CONTENT OF FEMALES BLOOD FED 60–72 hr
AFTER OPERATION, ASSAYED 20 hr AFTER FEEDING

Operation	20-OH-ecdysone content (pg-eq/female)	
	Unfed	Fed
Unoperated	27 \pm 4 (6) ^a	215 \pm 13 (6)
Sham operated	36 \pm 4 (5)	224 \pm 29 (6)
CA ablated	33 \pm 2 (6)	72 \pm 3 (5)

^a Mean \pm SEM of number of individuals indicated.

ovarian ecdysone secretion, using *in situ* and *in vitro* systems. JH, in regulating previtellogenic growth of ovaries, also regulated competence of ovaries to secrete ecdysone in response to a brain hormone, EDNH, during vitellogenic development.

In vivo, the largest increase in responsiveness of ovaries to EDNH occurred between 24 and 48 hr after emergence, when 80% of total responsiveness was acquired; responsiveness and growth continued to increase through 60 hr. The period between 24 and 48 hr after emergence may thus include specific cytological events that account for development of ovarian responsiveness to EDNH. Assuming that the follicular epithelium in mosquitoes is responsible for ecdysone secretion, as in *Locusta migratoria* (Goltzene *et al.*, 1978; Glass *et al.*, 1978), one such event may be proliferation of follicular epithelial cells. Proliferation of these cells proceeds through 48 hr after emergence (Laurence and Simpson, 1974), and may account for development of responsiveness prior to 48 hr. Analogous development of ovarian follicles in immature rats is under the control of estrogen and follicle-stimulating hormone (FSH). Estrogen stimulates granulosa cell proliferation with a concomitant increase in receptors and response to FSH (Louvét and Vaitukaitis, 1976; Richards *et al.*, 1976). It is possible that proliferation of follicle cells with receptors for EDNH also occurs in the mosquito ovary.

Comparison between ecdysone secretion in saline and in extracts of heads shows that secretion above a basal level is due to a soluble factor in the extracts, which we presume to be EDNH (Table 1). Ovaries incubated in saline secreted a basal level that was low or undetectable, while secretion of ecdysone in extract was substantial, if ovaries had been previously exposed to JH. The increase in secretion by ovaries incubated in extract over incubations in saline was at least sixfold using ovaries from JH-I-treated abdomens or from intact animals.

At a dose of 500 ng/abdomen, JH-I stimulated growth and responsiveness equal to that found in intact animals, while ovaries from abdomens treated with methoprene secreted 43% more ecdysone in response to head extract than those from intact animals. Methoprene treatment also increased the basal level of ecdysone secretion. While basal levels of ecdysone are often detected before a blood meal *in vivo* and *in vitro* (Table 3) (Hanaoka and Hagedorn, 1980; Hagedorn *et al.*, 1975), they were not detectable in JH-treated or control preparations in this experiment. Methoprene was therefore more potent than the natural JH in stimulating basal levels of secretion, responsiveness, and growth of ovaries. This may be due to the analog's great physical (Henrick *et al.*, 1973) and biological (Weirich and Wren, 1973) stability. Unpublished results (Hagedorn and Wheelock) show JH-I to be the most potent of the natural juvenile hormones (JH-O, Bergot *et al.* (1980) was untested) in stimulating follicular growth.

The effects of increasing doses of JH-I (Table 2) demonstrated that follicle growth and responsiveness to EDNH are related yet do not directly correlate. A dose of 50 ng of JH-I induced follicles to undergo 75% of maximal growth, but induced responsiveness in ovaries to only 50% of maximum. The lack of strict correlation between growth and responsiveness was also seen during development *in vivo* (Fig. 1). Such

differences suggest that a specific developmental event may account for competence to respond to EDNH.

The model system effectively predicted results in the more complex *in vivo* situation, where CA ablation at emergence prevented the appearance of a high content of 20-OH-ecdysone 3 days later, after blood feeding (Table 3). Beckemeyer and Lea (1980) hypothesized that separation of secondary follicles from germaria is controlled by increases in 20-OH-ecdysone content. However, they found that CA ablation at emergence did not prevent separation of follicles upon blood feeding. Our findings may indicate that follicle separation requires only a small increase in 20-OH-ecdysone, rather than the increase of several hundred picograms that they found necessary.

Our results may help to explain some recent observations on egg development in *Aedes atropalpus*, an autogenous species, i.e., one which does not require a blood meal to develop the first batch of eggs. Masler *et al.* (1980) found that decapitation of *A. atropalpus* females at emergence prevented both egg development and an increase in 20-OH-ecdysone content; both were restored by applying JH-I (Kelly *et al.*, 1980). At least in *A. aegypti*, JH secretion has been found to be under hormonal or neural control of the brain (Gwadz and Spielman, 1973; Feinsod and Spielman, 1980). If so, decapitation of *A. atropalpus* may have prevented secretion of JH, and therefore prevented both ovarian growth and competence to secrete ecdysone. Since topically applied JH was sufficient to restore both ovarian development and high ecdysteroid content, ovarian development in *A. atropalpus* may require the brain to stimulate JH secretion by the CA, but not to stimulate ecdysone secretion by ovaries.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant AI 14771 and New York State Experiment Station Project No. 420 to H.H.H. We thank Dr.

Andrew Spielman, Dr. Philipp Rossignol, and Dr. Saul Moobola for demonstrating CA ablation techniques, Dr. Gerardus Staal and Zoecon Corp. for providing methoprene, and Dr. J. D. O'Connor for providing anti-ecdysone antiserum.

REFERENCES

- Beckemeyer, E. F., and Lea, A. O. (1980). Induction of follicle separation in the mosquito by physiological amounts of ecdysterone. *Science* **209**, 819-821.
- Bergot, B. J., Jamieson, G. C., Ratcliff, M. A., and Schooley, D. A. (1980). JH zero: New naturally occurring insect juvenile hormone from developing embryos of the tobacco hornworm. *Science* **210**, 336-338.
- Bohm, M. K., Behan, M., and Hagedorn, H. H. (1978). Termination of vitellogenin synthesis by mosquito fat body, a programmed response to ecdysterone. *Physiol. Entomol.* **3**, 17-25.
- Borst, D. W., and O'Connor, J. D. (1974). Trace analysis of ecdysones by gas-liquid chromatography, radioimmunoassay and bioassay. *Steroids* **24**, 637-656.
- Chang, E. S., and O'Connor, J. D. (1979). Arthropod molting hormones. In "Methods of Hormone Radioimmunoassay" (B. M. Jaffe and H. R. Behrman, eds.), 2nd Ed. Academic Press, New York.
- Fallon, A. M., Hagedorn, H. H., Wyatt, G. R., and Laufer, H. (1974). Activation of vitellogenin synthesis in the mosquito *Aedes aegypti* by ecdysone. *J. Insect Physiol.* **20**, 1815-1823.
- Feinsod, F. M., and Spielman, A. (1980). Independently regulated juvenile hormone activity and vitellogenesis in mosquitoes. *J. Insect Physiol.* **26**, 829-832.
- Flanagan, T. R., and Hagedorn, H. H. (1977). Vitellogenin synthesis in the mosquito: The role of juvenile hormone in the development of responsiveness to ecdysone. *Physiol. Entomol.* **2**, 173-178.
- Glass, H., Emmerich, H., and Spindler, K.-D. (1978). Immunohistochemical localisation of ecdysteroids in the follicular epithelium of locust oocytes. *Cell Tissue Res.* **194**, 237-244.
- Goltzene, F., Lagueux, M., Charlet, M., and Hoffman, J. A. (1978). The follicle cell epithelium of maturing ovaries of *Locusta migratoria*: A new biosynthetic tissue for ecdysone. *Hoppe Seyler Z. Physiol. Chem.* **359**, 1427-1434.
- Gwadz, R. W., and Spielman, A. (1973). Corpus allatum control of ovarian development in *Aedes aegypti*. *J. Insect Physiol.* **19**, 1441-1448.
- Hagedorn, H. H., and Fallon, A. M. (1973). Ovarian control of vitellogenin synthesis by the fat body in *Aedes aegypti*. *Nature (London)* **244**, 103-105.
- Hagedorn, H. H., O'Connor, J. D., Fuchs, M. S., Sage, B., Schlaefer, D. A., and Bohm, M. K. (1975). The ovary as a source of α -ecdysone in an adult mosquito. *Proc. Nat. Acad. Sci. USA* **72**, 3255-3259.
- Hagedorn, H. H., Shapiro, J. P., and Hanaoka, K. (1979). Ovarian ecdysone secretion is controlled by a brain hormone in an adult mosquito. *Nature (London)* **282**, 92-94.
- Hagedorn, H. H., Turner, S., Hagedorn, E. A., Pontecorvo, D., Greenbaum, P., Pfeiffer, D., Wheelock, G., and Flanagan, T. R. (1977). Post-emergence growth of the ovarian follicles of *Aedes aegypti*. *J. Insect Physiol.* **23**, 203-206.
- Hanaoka, K., and Hagedorn, H. H. (1980). Brain hormone control of ecdysone secretion by the ovary in a mosquito. In "Progress in Ecdysone Research" (J. A. Hoffman, ed.). Elsevier/North-Holland, Amsterdam.
- Henrick, C. A., Staal, G. B., and Siddall, J. B. (1973). Alkyl 3,7,11-trimethyl-2,4-dodecadienoates, a new class of potent insect growth regulators with juvenile hormone activity. *J. Agr. Food Chem.* **21**, 354-359.
- Horn, D. H. S., Wilkie, J. S., Sage, B. A., and O'Connor, J. D. (1976). A high affinity antiserum specific for the ecdysone nucleus. *J. Insect Physiol.* **22**, 901-905.
- Kelly, T. J., Whisenton, L. R., and Fuchs, M. S. (1980). Induction of ovarian maturation in *Aedes atropalpus* with JH-I and 20-hydroxyecdysone. *Amer. Zool.* **20**, 863.
- Laurence, B. R., and Simpson, M. G. (1974). Cell replication in the follicular epithelium of the adult mosquito. *J. Insect Physiol.* **20**, 679-701.
- Lea, A. O. (1967). The medial neurosecretory cells and egg maturation in mosquitoes. *J. Insect Physiol.* **13**, 419-429.
- Lea, A. O. (1972). Regulation of egg maturation in the mosquito by the neurosecretory system: The role of the corpus cardiacum. *Gen. Comp. Endocrinol. Suppl.* **3**, 602-608.
- Louvet, J.-P., and Vaitukaitis, J. L. (1976). Induction of follicle-stimulating hormone (FSH) receptors in rat ovaries by estrogen priming. *Endocrinology* **99**, 758-764.
- Masler, E. P., Fuchs, M. S., Sage, B., and O'Connor, J. D. (1980). Endocrine regulation of ovarian development in the autogenous mosquito, *Aedes atropalpus*. *Gen. Comp. Endocrinol.* **41**, 250-259.
- Richards, J. S., Ireland, J. J., Rao, M. C., Bernath, G. A., Midgley, A. R., and Reichert, L. E., Jr. (1976). Ovarian follicular development in the rat: Hormone receptor regulation by estradiol, follicle

- stimulating hormone and luteinizing hormone. *Endocrinology* **99**, 1562-1570.
- Roth, T. F., and K. R. Porter. (1964). Yolk protein uptake in the oocyte of the mosquito, *Aedes aegypti* L. *J. Cell Biol.* **20**, 313-332.
- Weirich, G., and Wren, J. (1973). The substrate specificity of juvenile hormone esterase from *Manduca sexta* hemolymph. *Life Sci.* **13**, 213-226.
- Yonge, C., and Hagedorn, H. H. (1977). Dynamics of vitellogenin uptake in *Aedes aegypti* as demonstrated by trypan blue. *J. Insect Physiol.* **23**, 1199-1203.