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# Ecdysteroid Titters in Mated and Unmated *Drosophila melanogaster* Females

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## Abstract

Radioimmunoassay was used to determine ecdysteroid titers in mated or unmated *Drosophila melanogaster* females. Whole-body ecdysteroid titers increase after mating and this response is more pronounced after 12–24 hours than it is immediately after mating. In one experiment, females were mated to transgenic males deficient in accessory gland proteins to test whether these peptides mediate the observed increase in female whole-body ecdysteroid titers. Females mated to such transgenic males do not show a pronounced increase in whole-body ecdysteroid titers. The effect of mating on female hemolymph ecdysteroid titers was also investigated. Hemolymph ecdysteroid titers decrease after mating. The ecdysteroid titer change in the hemolymph may result from yolk protein uptake of ecdysteroids into developing vitellogenic oocytes as a consequence of male accessory gland protein stimulation of female oocyte maturation and yolk protein synthesis following mating.

**Keywords:** ecdysteroid titers, Ecdysone, 20-Hydroxyecdysone, *Drosophila melanogaster*

## 1. Introduction

One of the foundations of insect physiology is the correspondence between developmental events, or changes in reproductive state, and changes in hormone titers. Such associations play an important role in investigations of the biological function of hormones. For example, molting peaks of ecdysteroids are found in all insects (Sehnal, 1989) and in this context the epidermis is a focal system for the study of ecdysteroid function in relationship to well-defined biological events (Riddiford, 1989). Similarly, the role of ecdysteroids in embryogenesis or oocyte maturation is partially inferred from a correspondence between hormone

titers and biological events (Hagedorn, 1983; Lanot et al., 1989). Moreover, titer data are useful for investigating processes at the molecular level including hormonal control of expression of *Manduca sexta* ecdysone receptor genes (Fujiwara et al., 1995; Jindra et al., 1996). In general, extensive hormone titer data partially defines insect models for physiological studies (Gilbert, 1989). In contrast, although *Drosophila melanogaster* is an important model for genetics, developmental biology and other aspects of insect biology, there is a modest amount known about the correspondence between hormone titers and biological events in this species. Furthermore, an acute lacuna rises from the absence of information on hemolymph hormone titers in *D. melanogaster*.

Whole body ecdysteroid titers have been determined throughout the life cycle of *D. melanogaster*. This includes the embryonic, larval, and pupal stages (Handler, 1982; Hodgetts et al., 1977; Maroy et al., 1988; Bainbridge and Bownes, 1981; Pak and Gilbert, 1987; Richards, 1981). At pre-adult stages, an ecdysteroid peak is present at the time of germ band shortening in embryos and relatively small peaks are present at larval molts followed by a larger peak during the larval-pupal molt. Whole-body ecdysteroid titers in adult females have been investigated in relationship to ovarian maturation. For example, Hodgetts et al. (1977) found an increase in whole-body ecdysteroid levels after eclosion which apparently corresponds to the progression of ovary maturation. However, Handler (1982) observed a decrease in ecdysteroid titers during the first 36 hours after female eclosion and then observed an increase through the fourth day after eclosion which roughly corresponds to the age at which ovarian maturation is complete. Handler did not find ecdysteroids in the ovary. However, Schwartz et al. (1985) and Bownes et al. (1984) documented a substantial amount of ecdysteroids in the ovary. Clearly, there are differences in results among the relevant studies. It is noteworthy that the reproductive status of flies in these studies is usually not specified.

Reproductive status could be an important factor underlying differences in adult hormone titers. As a possible salient consideration, the male accessory glands of *D. melanogaster* produce approximately 100 peptides that are transferred to the female at the time of mating (Chen, 1984; Chen, 1991). It is known that one of these peptides, the sex-peptide, stimulates egg laying, causes females to be refractory to remating and has been shown to stimulate juvenile hormone biosynthesis (Kubli, 1996; Moshitzky et al., 1996).

The purpose of the present study was to investigate the effect of mating on ecdysteroid titers in female *D. melanogaster*. The first hypothesis tested was that mating changes whole body ecdysteroid titers in females. To test this hypothesis, ecdysteroid titers were determined in both virgin and mated females at intervals after mating using two *D. melanogaster* laboratory stocks. The second hypothesis tested was that male accessory gland secretions play a role in modulation of female whole body ecdysteroid titers after mating. This hypothesis was tested by mating females to males that produce accessory secretion compared to matings with transgenic line males that do not produce a significant level of accessory gland secretion. The third hypothesis tested was that mating changes hemolymph ecdysteroid titers. In order to test the third hypothesis, it was necessary to collect hemolymph from a large number of mated and unmated females. In general, the results of our work and other recent studies suggest an important role for mating and accessory gland secretion in modulating female hormone titers and subsequently oocyte maturation.

## 2. Materials and methods

### 2.1. *Drosophila* culture and experimental design

Flies were reared on a standard *Drosophila* medium. Per one liter of water, the following constituents formed the basis of the employed *Drosophila* medium: 7.5 g of agar, 58.3 ml of molasses, 100 g of cornmeal, 83.3 g of Torula yeast, 3.3 g of Tegosept (p-Hydrobenzoic acid methyl ester) in 16.7 ml of ethanol and 40 ml of propionic acid. Egg laying, larval growth, and maintenance of adults until use in experiments all took place under standard laboratory conditions. Standard laboratory conditions were 22°C and 12:12 (light:dark). For egg laying, 10 females and approximately the same number of males were held on medium in vials for two days. Adults emerging from these vials were isolated by sex as virgins using light ether anesthesia. Adults were four days old at the time of mating. The wild type laboratory stocks used in the study were Canton-S (CS) and Oregon-R (OR) (Lindsley and Zim, 1992). A transgenic line, DTA, was maintained as a source of sterile males that produce almost no male accessory gland secretion (Kalb et al., 1993).

Immediately after the beginning of photophase, single pairs of virgin males and virgin females were transferred by aspiration to a set of vials and observed for two hours at room lighting and temperature. The set of vials was visually inspected at regular intervals, approximately once every two minutes, such that the interval between inspections was much shorter than the duration of mating, which is typically 15–20 minutes. When mating was observed, the vial was marked and copulation was allowed to proceed to completion whereupon the male was removed. Mating was observed to ensure that females mated only once in the experiment. At defined intervals after the termination of mating, females were frozen at –20°C or hemolymph was withdrawn and then frozen.

Three different experiments were conducted corresponding to the three hypotheses described in the Introduction. The first experiment was a study of whole-body ecdysteroid titers in females at various times after mating. For this study, females from the Oregon-R lab stock were mated to males from the same stock. These females were frozen at intervals (1 h, 6 h, 12 h, 24 h) after the termination of copulation. For controls, females not given an opportunity to mate were handled and processed in an identical manner. This experiment was conducted three times except for the 24-hour time-point post-copulation when the experiment was conducted twice. For each replication, a different cohort of four-day-old virgin males and virgin females was used for mating and ecdysteroid extraction. Each extraction was used as the sample for one RIA determination of ecdysteroid titer. The second experiment was a comparison of Oregon-R (OR) and Canton-S (CS) female whole-body ecdysteroid titers after mating to transgenic males that produce almost no accessory gland secretion (DTA) and after mating to males with normal accessory gland function (*ry*) that were also derived from the transgenic stock. On each of three successive days, sufficient virgin males and virgin females for an experiment were collected from the stock sources. Correspondingly, sets of matings between four-day-old OR or CS virgin females and DTA or *ry* males of the same age were conducted on each of three successive days. The matings, and matching controls, on any one day were processed and assayed independently of the matings on other days to provide experimental replication. Each of the three mating sets generated an extraction sample for one RIA determination of ecdysteroid titer. The third

experiment was a determination of female Oregon-R hemolymph ecdysteroid titers in virgin females at 12 and 24 hours after mating. Matings were observed and hemolymph extracted from approximately 15 different sets of females which were pooled into two groups in order to accumulate sufficient ecdysteroid (volume of hemolymph) for analysis. Each group was extracted independently and each extract used as the sample for one RIA determination of ecdysteroid titer.

### ***2.2. Ecdysteroid extractions***

Ecdysteroids were extracted from whole bodies or hemolymph. Prior to whole-body ecdysteroid extractions, frozen females were lyophilized overnight in a vacuum centrifuge in eppendorf tubes. Each set of 25–90 dried flies was weighed using an analytical balance. These flies were returned to an eppendorf tube on dry ice and pulverized to a fine powder. A standard volume of 100% methanol was added to the powdered flies on a per weight basis. Specifically, 50  $\mu$ l of MeOH was added for each mg of weight of a set of flies. For example, if the lyophilized females weighed 20 mg, then 1 ml of methanol was added to the eppendorf tube. The suspension was vortexed and placed at 220°C for 5 days to allow ecdysteroids to diffuse from the powdered flies. During this 5-day period, the tubes were inverted and vortexed approximately once a day. At the end of this time and after pulse centrifugation at 13,000g, a standard aliquot of whole-body methanol extract was removed for RIA.

Ecdysteroids were extracted from hemolymph in a similar manner. Hemolymph was thawed on ice after removal from storage at 220°C. A standard volume of methanol (1 ml) was added to hemolymph from a set of flies. The hemolymph in methanol was mixed vigorously by vortexing and placed in a refrigerator at 4° for two days. After this period, the mixture was centrifuged for 10 minutes at 13,000g. For RIA, the supernatant containing extracted ecdysteroids was removed over a small pellet.

### ***2.3. Hemolymph removal***

Ecdysteroid titers were determined in hemolymph from females. For this purpose, a method was developed to efficiently obtain the requisite hemolymph volumes. Females were anaesthetized with light ether before injection with *Drosophila* Ringers solution. *Drosophila* Ringers is composed of 7.5 g NaCl, 0.35 g KCl, and 0.21 g CaCl<sub>2</sub> per liter of water. A fine pair of forceps was used to gently grasp an anaesthetized female along both sides of the thorax and head so that the dorsal portion of the thorax was pointing upward. The injection by glass needle was made into the pronotum ridge of the thorax. For this purpose, a needle puller was used to draw 20  $\mu$ l microcapillary pipettes to a fine tip. Using a plastic mouthpiece attached to latex tubing, a 0.5  $\mu$ l volume of *Drosophila* Ringers was gently injected into each female. Approximately 5 minutes after injection, the fly was reentered at the site of the injection and all of the available mixture of hemolymph and *Drosophila* Ringers was removed by very gentle aspiration and passive uptake. During this process, the needle tip did not penetrate the fly deeply and the initial withdrawal was by capillary action. The tip of the abdomen was gently compressed against the thorax to squeeze out and remove remaining hemolymph. Flies were injected in a series of 5 to 10 and thereafter

hemolymph from each fly was evacuated in the order of injection. The collected hemolymph was discharged from the microcapillary tube into an eppendorf tube on ice with PTU at a final concentration of approximately 0.1%. Similar to the procedure reported in Handler (1982), the hemolymph from each set of flies was extracted in an excess of methanol as described earlier.

#### **2.4. Radioimmunoassays**

The methods used for RIA are essentially as described in Warren and Gilbert (1988). In the RIA tube used for each assay, the solvent was vacuum evaporated from 200  $\mu$ l of whole-body extract or all of the extract from a pooled collection of hemolymph from 60–70 females. Each dried sample was dissolved by vigorous vortex mixing in 100  $\mu$ l of borate buffer (pH 8.4) with tritiated alpha-ecdysone (NEN, Dupont). To each RIA tube, except blank samples, 100  $\mu$ l of H22 antibody was added which is sufficient to precipitate approximately 1000 to 1100 cpm if no competing ecdysteroids are present. The H22 antibody tends to recognize the sidechain region of ecdysteroids, and it has been used for all of the previous studies of *D. melanogaster* hormone titers. In this study, the H22 antibody was used for whole-body ecdysteroid determinations. The SHO3 antibody recognizes the steroid nucleus of ecdysteroids. The SHO3 antibody has superior ecdysteroid recognition characteristics, but it is available in limited quantities. Consequently, it was used only for the hemolymph determinations. Standards consisted of a concentration series of unlabeled ecdysone. The final concentration of ecdysone standards was 2 ng, 1 ng, 0.5 ng, 250 pg, 125 pg, 62.5 pg, 31.3 pg, and 15.6 pg. To each tube, 20  $\mu$ l of a nonviable preparation of *Staphylococcus aureus* was added to precipitate the H22 antibody, or 40  $\mu$ l was added to precipitate the SHO3 antibody. The use of a nonviable preparation of the Cowan I strain of *Staphylococcus* for the ecdysteroid RIA is described in Warren et al. (1984). The unknowns and standards were incubated overnight at 4°C, precipitated at the same temperature by centrifugation at 5,000g, and resuspended prior to scintillation counting.

### **3. Results**

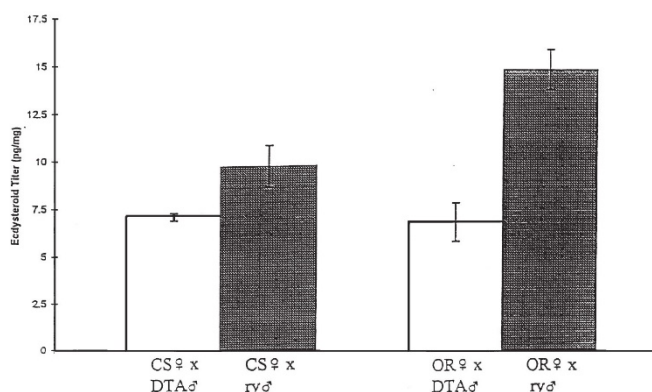
Table 1 presents means and standard deviations of whole-body RIA ecdysteroid titers in mated and unmated females from two laboratory stocks. The titers are expressed as ecdysone equivalents per mg of female dry weight. For Oregon-R females, ecdysteroid titers were determined at intervals after mating. A factorial analysis of variance of Oregon-R female titers indicates strong statistical support for an overall titer difference between mated and virgin females ( $P = 0.0012$ ). There also is statistical support for a time-dependent increase in ecdysteroid titer after mating ( $P = 0.0314$ ). A regression analysis of ecdysteroid titer as a function of time after mating indicates a better linear fit to the relationship between the two variables ( $P = 0.0062$ ) than a cubic fit between time and ecdysteroid titer ( $P = 0.3884$ ). There is no statistical support for a difference in lyophilized weight when virgins are compared with mated females or when female weight at different times post-copulation is compared (data not shown). At 12 hours after Canton-S females mate, analysis of ecdysteroid titers by *t* test indicates that there is no statistical support for a difference between mated and unmated females but the mean value differences are approximately

consistent with the Oregon-R 12-hour post-copulation data, which suggests that the results are not stock-specific.

**Table 1.** Mean (standard deviation) whole-body ecdysteroid titers in virgin and mated females from the Oregon-R laboratory stock. Ecdysteroid titers are expressed as pg ecdysone equivalents per mg dry weight of females.

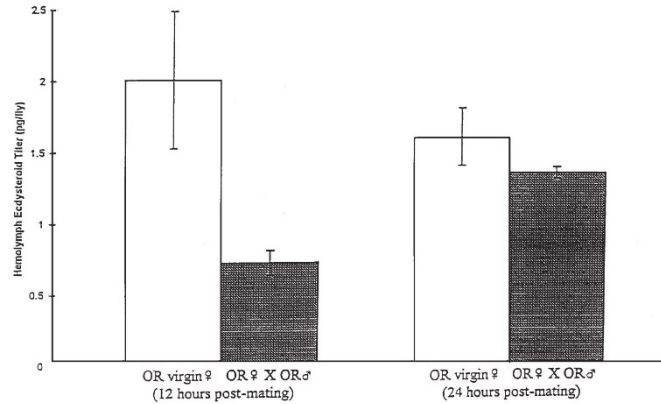
Time post-copulation	Oregon-R	
	Mated	Virgin
1 hour	11.6 (4.09)	9.5 (3.50)
6 hours	9.7 (1.08)	8.2 (1.38)
12 hours	19.6 (5.88)	8.4 (1.64)
24 hours	33.6 (17.89)	12.2 (3.40)
	Canton-S	
12 hours	16.1 (3.12)	9.8 (2.54)

Figure 1 presents whole-body H22 antibody RIA ecdysteroid titers corresponding to Oregon-R and Canton-S females 12 hours after mating to males without accessory gland secretion (DTA) or after mating to males (ry) from the same transgenic stock that have accessory gland secretions. Higher ecdysteroid titers are present in females mated to males with accessory gland secretions than found in females mated to males without accessory gland secretion. A factorial analysis of variance was conducted and the statistical support for a difference in ecdysteroid titers as a function of mating to a male with, or without, accessory gland secretion is  $P = 0.0004$ . There is no statistical support for a difference in female weight after mating to DTA males compared to mating with ry males (data not shown).



**Figure 1.** Mean and standard deviation of whole-body ecdysteroid titers in females 12 hours after mating to males having biologically ablated accessory glands (DTA) and males from the same line with normal accessory gland secretion (ry). Females are from either the Canton-S transgenic (CS) or Oregon-R (OR) laboratory stocks. Ecdysteroid titers are expressed as ecdysone equivalents per mg female dry weight.

Figure 2 presents hemolymph ecdysteroid titers corresponding to virgin and mated Oregon-R females. At 12 hours after virgin females mate, there is a substantial decrease in ecdysteroid titer. Prior to factorial analysis of variance, variates were subjected to a natural log transformation because in the untransformed data the variances were significantly correlated with means. The level of statistical support for a difference in hemolymph ecdysteroid titers between virgin and mated females is  $P = 0.0584$ . At 24 hours post-copulation, the ecdysteroid titer difference between mated and virgin females is markedly diminished.



**Figure 2.** Mean and standard deviation of hemolymph ecdysteroid titer (pg ecdysone equivalents per fly) in virgin and mated Oregon-R females at two times after the termination of copulation.

#### 4. Discussion

In the present study, it was determined that female whole-body ecdysteroid titers tended to increase after an interval of time following mating. The results of this work may be relevant to interpretation of earlier studies. Specifically, if in earlier studies females were held with males after eclosion, then mating may have caused whole-body ecdysteroid titers to increase. This may account for the results of Handler (1982) and Schwartz et al. (1989), who observed that Oregon-R female whole-body ecdysteroid titers declined over approximately the first day after eclosion then increased perhaps as a result of mating. The meconium contains about half of the ecdysteroid associated with females at the time of eclosion, and the loss of this material immediately after eclosion may account for the initial titer decline (Schwartz et al., 1989).

Previous studies of *D. melanogaster* using the H22 antibody produced a range of female whole-body ecdysteroid titer estimates within which our whole-body titer results apparently fall. Bownes et al. (1984) measured 5 pg of ecdysone equivalents per mg live weight in females that were 3 to 4 days post-eclosion. Schwartz et al. (1985) measured 34 pg of 20-hydroxyecdysone equivalents per female on day 3 post-eclosion and Schwartz et al. (1989) measured 4 pg of 20-hydroxyecdysone equivalents per female on day 4 post-eclosion. In our study, female whole-body ecdysteroid titers ranged from 7 pg to 22 pg of ecdysone



equivalents per mg of dry weight on day 4 post-eclosion (table 1). The variables associated with ecdysteroid titer change in our study were mating status and time post-mating, which suggests these factors may underlie some of the differences among earlier studies.

The only other analysis of *D. melanogaster* hemolymph ecdysteroid titers used the H22 antibody (Handler, 1982). In this study, approximately 2 pg of 20-hydroxyecdysone equivalents per female were measured on the first day post-eclosion (Handler, 1982). In the present study using the SHO3 antibody, 0.7–2 pg of ecdysone equivalents were detected per female. In the present study, mating status was observed to have an effect on hemolymph ecdysteroid titers. Specifically, mating was observed to result in lower female hemolymph ecdysteroid titer than found in virgin females of the same age (fig. 2). The change in ecdysteroid titer after mating may serve to regulate aspects of reproduction and adult physiology associated with mating. It will be difficult to evaluate this hypothesis until more is known about the role of ecdysteroid titers in controlling reproduction and adult physiology in *Drosophila*.

Yolk protein mediated oocyte uptake of hemolymph ecdysteroids, combined with continuing hormone secretion into the hemolymph by de novo synthesis, may underlie the increase in female whole-body ecdysteroid titers after mating that was observed in the present study. Ecdysteroids are frequently detected in insect ovaries (Ohnishi, 1990). Moreover, in various arthropods ecdysteroid conjugates are present in the egg presumably as a source of ecdysteroids for cuticulogenesis during embryonic development (Lanot et al., 1989). Handler (1982) reported an absence of ecdysteroids in the ovary of *D. melanogaster*. In contrast to Handler's result, it has generally been observed that *D. melanogaster* ovaries have ecdysteroids. In addition, ovarian synthesis of ecdysteroids has been documented in this species. For example, using the conditional mutation *ecd<sup>1</sup>*, Garen et al. (1977) suggested that the ovary is the primary site of ecdysteroid production in the female. Incorporation of tritiated precursors into ecdysteroids by larval ring glands and adult ovaries was investigated using the *ecd<sup>1</sup>* mutation and the results suggested similarities in the mechanism of ecdysteroid biosynthesis in different tissues (Warren et al., 1996). Bownes et al. (1984) described an increase in ovarian ecdysteroid content during the first three days post-eclosion. During a time course in vitro incubation of ovaries, Rubenstein et al. (1982) observed a constant level of ecdysteroids in ovaries accompanied by a substantial increase of hormone in the medium. In one study (Schwartz et al., 1989), 20-hydroxyecdysone, ecdysone and polar forms of ecdysone were found to be secreted by the ovary. Oocyte stages 8–9, early vitellogenic oocytes, produced more ecdysteroids than any other stage. There is evidence that yolk proteins bind ecdysteroids (Kraminsky et al., 1980; Bownes, 1992; Bownes et al., 1988). In general, active patency may result in increased adult whole-body ecdysteroid titers partially mediated by yolk protein steroidal uptake into the ovaries. As an extension of this hypothesis, relative ecdysteroid titer differences between mated and unmated females may vary temporally after mating as a joint function of egg-laying rate and vitellogenic activity which in turn may depend on endogenous factors, such as genetic constitution and environmental conditions.

Our study indicates that the effect of mating on female whole-body ecdysteroid titers is mediated by the action of male accessory gland proteins. In insects, modulation of various aspects of female reproduction and physiology by male accessory gland proteins has been

known for some time (Leopold, 1976; Chen, 1984). More recent biochemical, genetic, and molecular genetic studies have provided insight into the mechanism of this phenomenon in *D. melanogaster* (Kubli, 1996; Wolfner, 1997; Chen, 1991). For example, there is evidence that accessory gland peptides may have neurohormone-like characteristics (Monsma and Wolfner, 1988; Monsma et al., 1990). Two male accessory gland proteins, sex-peptide and the gene product of *Acp26Aa*, have been shown to act on oocyte maturation and egg laying. The sex-peptide also causes females to be refractory to remating. Sex-peptide remating and fecundity effects are present when the male transfers this peptide to the female at the time of mating or when it is expressed ectopically in transgenic females (Aigaki et al., 1991). There is evidence that the sex-peptide can stimulate yolk protein production and yolk protein uptake into developing oocytes (Soller et al., 1997). Sex-peptide has been shown to stimulate juvenile hormone biosynthesis (Moshitzky et al., 1996) and it is known that the progression from pre-vitellogenic oocytes to vitellogenic oocyte stages depends on juvenile hormone (Wilson, 1982). It may be relevant to note that a juvenile hormone analog, methoprene, was observed to stimulate ovarian ecdysteroid biosynthesis when applied to a mutation known to be deficient in juvenile hormone III titer (Schwartz et al., 1989). Stimulation of female ecdysteroid synthesis by mating is known from work on *Rhodnius prolixus* (Davey, 1993). *Acp26Aa* produces a peptide precursor product that has a region of amino acid similarity to *Aplysia* (sea slug) egg-laying hormone (Monsma and Wolfner, 1988). A null mutant of *Acp26Aa* has been used to show that the peptide stimulates egg laying on day one post-eclosion (Herndon and Wolfner, 1995) and there is evidence that the peptide product of *Acp26Aa* stimulates oocyte maturation. It may be hypothesized that male accessory gland proteins increase female whole-body ecdysteroid titers as a byproduct of stimulating juvenile hormone synthesis and subsequently stimulating vitellogenesis and oogenesis which results in increased yolk protein mediated uptake of ecdysteroids into vitellogenic oocytes. Presumably, active hormone biosynthesis partially compensates for ecdysteroid removal from the hemolymph. In general, the time dependent increase in whole-body ecdysteroid content parallels the temporal effect of sex-peptide on yolk protein synthesis and oocyte maturation (Soller et al., 1997).

An extension of the hypothesis to explain the effect of mating on female whole-body ecdysteroid titer could explain our seemingly contradictory observation of a decrease in hemolymph titer after mating. Specifically, male accessory gland proteins stimulate female reproduction and increased yolk protein mediated ecdysteroid uptake into maturing oocytes results in a net removal of ecdysteroids from the hemolymph. The results of our study, integrated with those of Handler and Postlethwait (1978), Moshitzky et al. (1996), and Soller et al. (1997, 1998), suggest a general scenario for some of the physiological effects of mating on female reproduction. As outlined immediately below, Soller et al. (1998) have described much of this scenario. Ecdysone administration is known to suppress vitellogenic oocyte stages (Handler and Postlethwait, 1978; Soller et al., 1998) and this may be physiologically analogous to the high ecdysteroid titer state in virgin females. Mating stimulates juvenile hormone biosynthesis, yolk protein synthesis, and yolk protein uptake into the developing oocytes. Yolk protein uptake into the developing oocytes may have the effect of decreasing hemolymph ecdysteroid titers which facilitates the progression of oocyte

maturation by removing the suppressive effect of high titers. Mating induces two prominent responses in female *D. melanogaster* (Chen et al., 1988; Soller et al., 1997): increased egg production and refractoriness to remating. The present study suggests that increased egg production after mating causes a decrease in female hemolymph ecdysteroid titer.

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