

UNIVERSITY HONORS PROGRAM

SENIOR PROJECT - APPROVAL

Name: Holly Avens

College: Arts & Sciences Department: BCMB

Faculty Mentor: Jac Park

PROJECT TITLE: Effect of Genetic Ablation of

lovazomn- Expressing Neurons on Ovarian

Diapause of Drosophila melanogaster

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: Jac Park . Faculty Mentor

Date: 5/2/05

Comments (Optional):

**Effect of Genetic Ablation of Corazonin-Expressing
Neurons on Ovarian Diapause of *Drosophila Melanogaster***

Holly C. Avens

University of Tennessee Honors Senior Thesis
Jae H. Park, Faculty Mentor

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Abstract

In normal *Drosophila melanogaster* reproduction, after the male and female copulate, the female stores the sperm in a seminal receptacle and then produces 40 to 50 eggs at one time that are fertilized in her uterus as they travel through her reproductive system to be laid in an appropriate medium. During winter, when conditions are not favorable for survival, reproduction is wasteful since it consumes energy that should otherwise be spent keeping the female fly alive and the offspring will most likely not survive. In order to stop reproduction, the ovaries of the female do not develop to the same extent as they do in favorable conditions. Instead, reproduction stops before deposition of vitellogen (yolk) around the egg. This condition is known as ovarian diapause. It has already been demonstrated that Corazonin, a neuropeptide thought to be involved in regulating *D. melanogaster* circadian rhythms, is involved in this phenomenon of halting development by mechanically killing neurons that express Corazonin and then subjecting blowflies, a larger fly species, to winter-like conditions (low temperatures and less light). Flies in which Corazonin function had been turned off continued to produce fully formed eggs, in stark contrast to their normal behavior. In fruit flies, however, it is impossible to mechanically terminate the effects of Corazonin. Therefore, genetic manipulation is utilized as an alternative approach. By linking the Corazonin promoter to an apoptotic gene, neurons that contain the necessary factor to express the Corazonin gene will be killed. When these transgenic flies are subjected to winter-like conditions in specialized incubators, the females should continue to produce eggs, thus proving that Corazonin is involved in ovarian diapause of *Drosophila melanogaster*.

Introduction

The *Drosophila* female reproductive system occupies the posterior two-thirds of the abdomen and consists of two ovaries, an efferent duct system, three sperm-storing organs, and two accessory glands (Miller 1994). The ovaries are compact, pear-shaped, and lie bilaterally in the third, fourth, and fifth abdominal segments, surrounded by adipose tissue (Miller 1994). The size of the ovaries varies with the extent of the development of the eggs and with the nurture of the individual, ranging from 220 by 350 to 900 by 1100 μ (Miller 1994).

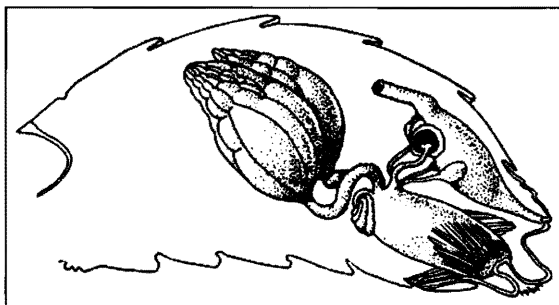


Fig. 1. Lateral Aspect of Adult Abdomen and Female Reproductive System at the Adult Stage

The reproductive system occupies the posterior two-thirds of the abdomen and the ovaries lie in the third, fourth, and fifth abdominal segments.

Image courtesy of;

Drysdale RA, Crosby MA, and The FlyBase Consortium (2005). FlyBase: genes and gene models. *Nucleic Acids Research* 33:D390-D395.

Each ovary consists of a compact group of parallel egg tubes or ovarioles, which ordinarily vary in number from 10 to 20 (Miller 1994). The ovariole consists of a series of four to six egg chambers that taper distally and are constricted at regular intervals so as to resemble a string of beads (Miller 1994). The end chamber is called the germarium and is the site where egg formation is initiated (Miller 1994). The germarium is 20 μ wide and 50-70 μ long and contains the oögonia, the cells that produce the primary oocytes, or eggs (Miller 1994). The basal chamber of the germarium merges into the first of the series of egg chamber, which constitutes the greater part of the ovariole (Miller 1994). The egg chambers, or follicles, are successively larger toward the base of the ovariole, increasing from spheres with an initial diameter of 25 μ to ovoids 450 by 170 μ , the size of the fully formed egg (Miller 1994). As yolk is deposited around the oocyte, the egg grows and the follicle

becomes greatly distended. At the base of the ovariole, the egg leaves the ovary and comes to rest in the uterus (Miller 1994).

During copulation, the sperm are deposited in the uterus and swim into the seminal receptacle and the spermathecae (Miller 1994). After an egg is in the uterus, sperm enter the space around its anterior end and penetrate through the micropyle, a differentiated area of surface in an egg intended for sperm entry (Miller 1994). Maturation and a variable degree of embryonic development may occur while the egg is still in the uterus (Miller 1994). Oviposition then pushes the egg out of the body (Miller 1994). Through the mechanism of storing sperm and producing many oocytes at once, a female may lay several hundred eggs in a short amount of time (Miller 1994).

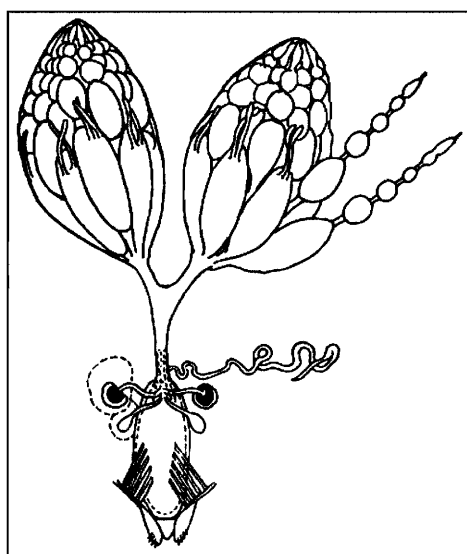


Fig. 2. Dorsal Aspect of Female Reproductive System

From top to bottom: 2 ovaries consisting of ovarioles, 2 of which have been separated to emphasize individual egg chambers. Ovaries are then joined by common oviduct, which empties into uterus and then the ovipositor. The coiled structure is the seminal receptacle and the lobes with dark centers are the spermatheca.

Image courtesy of;

Drysdale RA, Crosby MA, and The FlyBase Consortium (2005). FlyBase: genes and gene models. *Nucleic Acids Research* 33:D390-D395. <http://flybase.org/>

Most insects, and most living things in general, develop and reproduce during the summer months when conditions are favorable for survival (moderate temperatures, abundance of food, etc.), but then become dormant or enter diapause (a period of physiologically enforced dormancy between periods of activity) as winter approaches. Since reproduction is an energy costly biological mechanism, it would be wasteful for an organism to exert so much effort into producing offspring that would most likely not survive. Of the

18 species of *Drosophila* for which data are currently available, 16 overwinter in a reproductive or ovarian diapause in which short days (or long nights) elicit a block to vitellogenesis (Saunders 1989). When newly eclosed (within about 10 hours of eclosion) *Drosophila* females are transferred to fresh culture bottles and placed at 10 or 12°C, diapause incidence is highest in short days, with a maximum of about 10 hours of light, and lowest in long days, with a minimum of about 16-20 hours of light (Saunders 1990). This ovarian diapause is understood to be the cessation of oocyte development prior to yolk deposition around the egg (Saunders 1989). This change is easily evident since, when in diapause, the ovaries are small and transparent. In contrast, during reproduction, the ovaries are enlarged and opaque. This dormancy during winter may be a direct response to unfavorable conditions such as a lull in activity associated with cold temperatures or it involves programmed responses to seasonal changes in day length (photoperiod), resulting in interruptions or changes in endocrine regulation, which has many widespread effects of varying degrees (Saunders 1989). Thus, some insects, appear to have photoperiodic clocks that enable them to regulate their life cycles so that they develop and reproduce in favorable seasons and so that these events are arrested in diapause during unfavorable seasons (Beck, 1980; Saunders, 2002). The photoperiod is the principle cue by which insects are synchronized to both daily and seasonal cycles (Beck, 1980; Saunders, 2002). The physiological mechanisms underlying photoperiodism are thought to consist of: photoreceptors for the photoperiod, photoperiodic clocks that measure and sum successive light-dark cycles to a point at which a response is induced, and endocrine effectors that initiate appropriate physiological responses (Saunders 2002).

Corazonin (Crz) is a neuropeptide that has been implicated in many physiological mechanisms for several insects, including cardiac activity of the American cockroach, *Periplaneta americana* (Veenstra 1989), dark cuticular pigmentation exclusively in several orthopteran species, including the migratory locust, *Locusta migratoria* (Tawfik 1999; Hua 2000; Tanaka 2000), elongation of a larval spinning period prior to pupation in the silkworm, *Bombyx mori* (Tanaka 2002, 2003), induction of ecdysis behavior in the moth, *Manduca sexta* (Kim 2004), and promotion of pigment migration in the integument of a crustacean species (Porras 2003). Most importantly, Crz may be involved in the biological rhythms of *Drosophila* (Choi 2005). The somata of Crz-neurons in *Drosophila* adult brains could be in the vicinity of nerve terminals stemming from small ventrolateral neurons (s-LNV) (Choi 2005), which express the genes *per*, *tim*, and *Pdf* (reviewed by Stanewsky 2002). Furthermore, *Pdf* axon terminals are intimately associated with neurites originating from Crz-neurons, suggesting potential neuronal interactions between these two kinds of neurochemically differentiated cells (Choi 2005). Since *Pdf* has been implicated to be a principal circadian transmitter (Renn 1999), it is highly likely that Corazonin also plays role in circadian rhythms and could be involved in photoperiodism that would induce diapause. Additionally, it has already been shown that removal of nervous tissue containing Crz-expressing neurons in the Tobacco Hornworm, *Manduca sexta*, will impede induction of pupal diapause (Shiga 2003). Normally, the photoperiod controls induction of pupal diapause (short day lengths will prevent development into the adult form) (Rabb 1966; Bell et al. 1975), but after surgical ablation of the Crz-expressing neurons, a *Manduca sexta* pupae will still initiate adult development, even under diapause-inducing conditions (Shiga 2003).

However, since *D. melanogaster* is much too small for surgical ablation, an alternate approach must be used, i.e. genetic ablation. Therefore, we used transgenic lines of Crz-GAL4 and UAS-rpr. Crz-GAL4 contains the Corazonin promoter and the gal4 protein as the gene product so that in any cell, which normally transcribes Corazonin, GAL4 will be produced. The GAL4 protein is a potent transcription factor derived from yeast (Brand 1993). Since it is not native to *Drosophila*, the researcher can easily control its cellular expression (Brand 1993). UAS-rpr is a GAL4-responsive target gene in which reaper (rpr) has been subcloned into a vector behind a tandem array of five optimized GAL4-binding sites, referred to as upstream activator sequence (UAS) (Brand 1993). Reaper is an apoptotic gene that plays a central role in induction of cell death (White 1994) and is sufficient to kill only the cells that it is expressed in (White 1996). Therefore, cells that normally express Corazonin will produce GAL4, which will then bind to and activate UAS-rpr, producing the reaper apoptotic factor, those cells will be killed. Any neurons in flies that normally express Corazonin that now contain these two transgenic lines will therefore be killed (ablated) and their function turned off. If Corazonin regulates ovarian diapause, these flies will not undergo ovarian diapause even when subjected to diapause-inducing conditions (low temperature and short periods of light).

Materials and Methods

Canton-S was used as a wildtype strain of *Drosophila melanogaster*. Flies were raised at 25°C under 12:12-h light:dark cycles on food containing dextrose, cornmeal, agar, yeast, and methyl paraben as a preservative. First, in order to establish a scale to which *Drosophila* ovaries could be compared to, two female pupae were removed prior to eclosion.

One was raised under normal conditions described above. Another was placed in a vial containing only agarose to promote a starvation condition that would prevent ovary development. After 8 days, the ovaries were examined. Since there was no established laboratory protocol, I had to develop a method for this procedure on my own. I found best success by anesthetizing the flies by placing the vials on ice for 2 minutes, and then squeezing the ovipositor with forceps and pulling it out of the flies, bringing the reproductive system with it, including the ovaries. Reproductive organs not of interest and excess adipose tissue were excised using the forceps and the ovaries were placed in a glass well plate with phosphate-buffered solution, which mimics normal ionic concentrations found in organisms so as not to preserve the organs for the short duration of time needed. Images of the ovaries were acquired with an Olympus BX-61 microscope equipped with a CCD camera and processed by Adobe Photoshop.

In order to observe the effects of ovarian diapause-inducing conditions on wildtype flies, female pupae were removed from the Canton-S stock population prior to eclosion. Thirty were placed in a vial and subjected to non-diapause inducing conditions (25°C and 12:12-h light:dark cycles). Another 30 were placed in a vial stayed in an incubator that simulated diapause-inducing conditions (11°C and 8:16 light:dark cycles). After 8 days, the flies were anesthetized and their ovaries removed for observation.

Results

As expected, the ovaries acquired from the fly raised under normal conditions were enlarged and had a milky appearance. The ovaries from the fly raised on agarose were small and transparent.

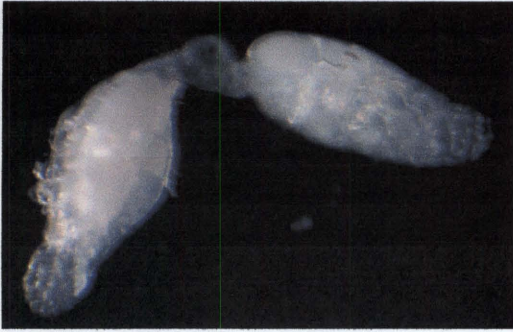


Fig. 3. Normal Ovary Development
The ovaries have a milky appearance due to the yolk deposition around eggs and become so enlarged that the female's abdomen becomes distended

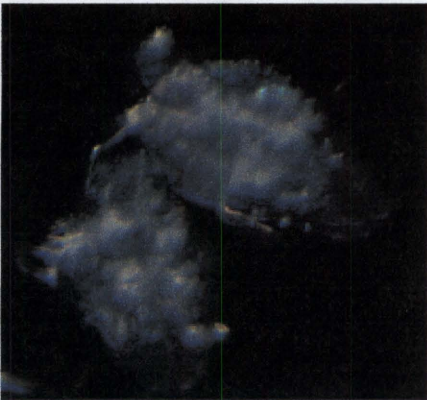


Fig. 4. Ovary Development under Diapause-Inducing Conditions
When development is arrested by diapause, the ovaries are smaller, more spherical and shape, and transparent.

Twenty-one flies placed into the vial meant for non-diapause conditions developed into adults. Upon extraction, all the ovaries appeared the same as the ovaries from the fly raised under normal conditions. Interestingly, of the 18 flies that developed into adults in diapause-inducing conditions, the ovaries were far less developed than from the fly raised under starvation condition. In fact, the ovaries were so miniscule that it was impossible to extract an intact pair from any of the flies using only the laboratory microscope, the forceps provided, and my skill level. Therefore, there are no images from these ovaries since the conditions were so favorable for creating ovarian diapause.

Problems Encountered

The next stage of the project was meant to cross two populations of flies using a protocol established by the *Carolina Drosophila Manual* (Flagg 1988): one with the transgenic line Crz promoter-GAL4 and another with the UAS-rpr transgenic line. This was attempted by collecting 10 female virgins from the Crz-gal4 line, taken early in the morning so that their lack of pigmentation makes them easily identifiable from the older, pigment females and placing them in a new vial with approximately the same number of males from the UAS-rpr line. Eight days after the cross was initiated, the parents were removed to preclude breeding between generations and to avoid confusion for future counts. Fidelity of the cross was ensured by using a Crz-gal4 line that had been cross-linked with a gene that produces yellow eye color and a lighter pigmentation in *D.melanogaster*, instead of the wildtype red eyes and darker pigmentation patterns. Therefore, the flies that contain both transgenic lines will have orange eyes and will be easily identifiable. However, a successful first filial population could never be obtained after several tries.

The next phase of the project would have been to obtain a viable population of flies carrying the Crz-GAL4/Uas-rpr transgenic lines. Upon subjecting them to different conditions, both groups should have ovaries that developed normally, even the group that was raised on diapause-inducing conditions. With the effects of Corazonin terminated, ovarian diapause would not have been allowed to proceed. This study would have proven Corazonin's role in ovarian diapause and provided clues to how it functions.

Discussion

The results obtained from the wildtype flies were as expected: when the flies were raised under normal conditions, all the ovaries developed normally, but when the flies were

raised under diapause-inducing conditions, the ovaries were severely underdeveloped. Unfortunately, we were not able to conduct the second phase of the project, due to unforeseen and unexplainable circumstances. However, had we been able to obtain a viable fly population on which to run the experimental conditions on, we expected that both groups of flies would have developed ovaries, despite whether they were raised under normal conditions or diapause-inducing conditions. This would have proven that Corazonin is involved in ovarian diapause, a function that could easily be associated with its role in Circadian rhythms, since the body is able to adjust its cycles to the daily amounts of light and dark. It is possible that Corazonin is responsible for *Drosophila melanogaster*'s reaction to light.

Further tests should also be conducted in which female and male flies are raised together in the same vial under the same conditions to see if the presence of the male has any effect on diapause.

Overall, the primary results of the experiment were hopeful, so perhaps with more time and expertise, this relationship can be proved.

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