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Cephalic control of ovarian development in the black blowfly, Phormia regina (Meigen).

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CEPHALIC CONTROL OF OVARIAN DEVELOPMENT IN THE BLACK BLOWFLY, PHORMIA REGINA (MEIGEN)

A Thesis Presented

By

HONGYU DUAN

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DEDICATION

To my parents Song-Li Duan and

Feng-Xian Wang and my wife Jun-Lan Fan:

Their love makes all of this possible.

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This thesis results from the efforts of many people to whom I owe a great deal of thanks, but without the guidance and encouragement of my advisor, Dr. Chih-Ming Yin, this would have been impossible. I would also like to thank Dr. John G. Stoffolano, Jr. for his advice and support.

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ABSTRACT

CEPHALIC CONTROL OF OVARIAN DEVELOPMENT

IN THE BLACK BLOWFLY, PHORMIA REGINA (MEIGEN)

FEBRUARY, 1991

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The cephalic control of ovarian development of Phormia regina was investigated by using neck-ligation. It was found that the ovarian development of Phormia regina was influenced by cephalic factor or under cephalic control. The critical moment of this cephalic control was from 7 to 12 hr after the onset of liver feeding. Transections of the cardiac-recurrent nerve bundle (CRN) and the ventral nerve cord (VNC) indicated that this cephalic control was non-neural, since severing the CRN or VNC had no significant effect on the ovarian development once the flies had an adequate protein meal. The results of nerve transections before protein feeding also indicate that activation of the cephalic factor(s) by the protein meal was non-neural. The possible activation mechanisms were discussed.

Measurement of the hemolymph vitellogenin profile and hemolymph ecdysteroid titer showed that ecdysteroid biosynthesis and vitellogenin biosynthesis was significantly reduced in the neck-ligated flies. Radiochemical assay of corpus allatum activity showed that juvenile hormone biosynthesis was not fully activated in the neck-ligated

flies. It is suggested that the cephalic control of ovarian development in this anautogenous fly is via an allatotropic hormone and/or a hormone which stimulates the ovary to produce ecdysteroids..

Since an adequate protein meal is required for oogenesis in Phormia regina, protein ingestion and utilization of the female flies were studied. Data showed that crop rather than midgut was the major storage site of protein meal. The protein content of the crop was gradually transferred to the midgut during oogenesis. The transfer rate in the neck-ligated flies was slightly slower than that of the liver-fed control flies.

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CHAPTER I

LITERATURE REVIEW

A. Protein Meal and Ovarian Development In Anautogenous Insects

In anautogenous insects ovarian development does not proceed without a protein meal. Although there are differences among different groups of insects, the stages of oogenesis can be divided into previtellogenic and vitellogenic stages. When anautogenous insects are fed with a water and carbohydrate (non-reproductive) diet, female insects can be kept alive and healthy but their ovarian development stops at the so called "resting stage" (Hagedorn et al., 1977; Meola and Readio, 1988). Further growth of the oocytes depends on the ingestion of an adequate protein meal.

The nutritional requirements of oogenesis have been demonstrated in various insects. Dethier (1961) and Belzer (1978a, 1978b, 1978c, 1979) have demonstrated that a protein-specific hunger occurs in the female black blowfly, Phormia regina, prior to the period of ovarian development. A similar pattern of protein feeding behavior and protein hunger is observed in Calliphora erythrocephala (Strangways-Dixon, 1959, 1961), Chrysomya megacephala

(Spradbery and Schwelzer,1979) and Musca autumnalis (Van Geem and Broce, 1986).

In Calliphora erythrocephala, Fraenkel (1940) and Strangways-Dixon (1961) have also shown that ingestion of protein-containing substance is necessary for egg development. These authors also find that when the flies are fed with protein without carbohydrate they die. Therefore the nutritional function of the protein meal is for reproduction while the sugar meal is to provide an energy source for general metabolism.

In the fleshfly, Sarcophaga bullata, Wilkens (1968) find that incorporation of yolk into the oocytes requires dietary proteins or amino acids. Various other researchers have also shown that a protein meal is necessary for maturation of eggs in the stable fly, Stomoxys calcitrans (Moobola and Cupp, 1978; Venkatesh and Morrison, 1980a, 1980b).

The nutritional requirements of the mosquito, Aedes aegypti have been investigated by Macgregor (1931), Greenberg (1951), Larsen and Bodenstein (1959) and Spielman and Wong (1974). It has been confirmed that direct reception by the midgut of some essential food (blood or protein) is required for egg development. Without a proteinaceous meal, no eggs developed. Thus, protein is essential for egg development in this mosquito.

B. Protein Meal Triggers Egg Maturation Via Endocrine System

Further studies in oogenesis indicate that the ovarian development in anautogenous insects is under endocrine control. Evidence for the view that ovarian development is under hormonal control has been derived from experiments involving removal of the endocrine organs by decapitation, ligation, or extirpation. Thomsen (1948, 1952) has demonstrated that the median neurosecretory cells as well as the corpora allata are required for egg maturation in the blowfly, Calliphora erythrocephala. Wilkens (1968) reports that vitellogenesis in the fleshfly, Sarcophaga bullata, is controlled by the corpora allata and the neurosecretory cells of the pars intercerebralis. In aedine mosquitoes, it has also been shown that the corpora allata are essential for egg development and maturation (Lea, 1963). Gillett (1958) showed that the final vitellogenic stages of ovarian development in Aedes aegypti is under the control of a head-produced hormone. This is confirmed by Lea's results (1966) that median neurosecretory cells are required for egg maturation and furthermore, a blood meal triggers the release of egg development neurosecretory hormone (Lea, 1972). An ovarian hormone, the vitellogenin stimulating hormone, is found to be induced by a protein meal in Aedes aegypti (Hagedorn and

Fallon, 1973). This hormone is believed to control vitellogenesis.

It is now clear that in anautogenous insects, a protein meal is required not only to provide the building materials for egg development but also to trigger the endocrine system of the insect to produce, following a temporal pattern, a number of hormones which regulate the entire process of egg development.

In Phormia regina, requirement of an exogenous source of protein for egg development is first observed by Rasso and Fraenkel (1954). This has been confirmed by numerous other researchers (Orr 1964a, 1964b; Pappas and Fraenkel, 1977, 1978). The control of egg development has been studied by Orr (1964a, 1964b), Mjeni and Morrison (1976) and Yin et al. (1989a, 1989b, 1990). Although there are disagreements regarding the functions of different hormones in regulating ovarian development, they all agree that the protein diet serves a dual function of triggering the endocrine system and providing nutrients for vitellogenin biosynthesis.

C. Mechanism of Activating the Endocrine System

Communication between any two systems within the insects body can be conducted in several ways; neural, hormonal, humoral, or a combination of these methods. The mechanism of activating the endocrine organs by the protein

meal has been studied in anautogenous insects. In Aedes aegypti, Lea (1972) reports that the signal for releasing brain neurosecretory hormone is transported by the hemolymph. Several other studies confirmed this result (Bellamy and Bracken 1971; Chang and Judson, 1977). However, Gillett (1957), Gillett et al. (1975), Larsen and Bodenstein (1959) demonstrate that abdominal distention is involved in the initiation of this hormone releasing process. Using the methods of nerve transection and enema, Klowden (1987) shows that abdominal distention accelerates the release of the head factor and the message for its release travels through the ventral nerve cord. However, the ventral nerve cord is not essential. Thus, he shows that another non-neural factor is involved in brain activation.

In Phormia regina, Orr (1964a) has demonstrated that abdominal distention cannot initiate egg maturation. Thus, he suggests that some components of the protein meal or the digestion products of the protein meal may activate the endocrine system for egg development. However, Bennettova-Rezabova (1972) suggests that ovarian development is directly controlled by the brain through the ventral nerve cord, because severance of ventral nerve cord at the neck level inhibits egg development.

D. Control of Ovarian Development

1. Ecdysteroids

The presence of ecdysteroids in adult insects and their important roles in insect reproduction have been shown in many insects (Bollenbacher *et al.*,1978; Hagedorn, 1983; Adams *et al.*, 1988; Wilps and Zoller,1989). After the degeneration of the prothoracic glands in adult insects, the most important source of ecdysteroids has been traced back to ovaries (Hagedorn *et al.*, 1975; Lagueux *et al.*, 1977). Spielmen *et al.* (1971) find that mosquitoes fed 20-hydroxyecdysone produce eggs in the absence of a blood meal. Hagedorn and Fallon (1973) and Fallon *et al.* (1974) show that the ovary stimulates vitellogenin synthesis. It has been established that ecdysteroids stimulate vitellogenin synthesis by fat body (Hagedorn, 1985). Using an *in vitro* culture of fat body with 20-hydroxyecdysone, further studies by Ma *et al.* (1988) suggest that the initiation and control of vitellogenin synthesis in *Aedes aegypti* is a programmed response to 20-hydroxyecdysone. In *Phormia terraenovae*, Wilps and Zoller (1989) have demonstrated that during the first 10 days after eclosion each gonotropic cycle of the female fly is accompanied by a peak of hemolymph ecdysteriod titer. In *Phormia regina*, Yin *et al.* (1989b, 1990) show that a sharp increase of ecdysteroids in the hemolymph occurs several hours prior to

the appearance of a significant quantity of vitellogenin. They suggested that ecdysteroids in this species stimulate vitellogenin synthesis by the fat bodies. Thus, to date, research shows that ecdysteroids are important for the production of vitellogenin. However, juvenile hormone is also important (see discussion below).

2. Juvenile hormone

The involvement of corpora allata and juvenile hormone in ovarian development has been demonstrated in many insects (Engelmann, 1983; Kelly et al., 1987). In the majority of insect species studied, the corpora allata or their biosynthate, juvenile hormone, controls vitellogenin synthesis (Engelmann, 1979, 1983). In some other species, juvenile hormone is required for previtellogenic growth of the ovary (Gwadz and Spielman, 1973; Flanagan and Hagedorn, 1977; Hanaoka and Hagedorn, 1980; Tobe and Stay, 1977; Tobe and Chapman, 1979). It has also been shown that juvenile hormone is a prerequisite of the fat body for the development of competence to respond to 20-hydroxyecdysone in Aedes aegypti (Ma et al., 1988). In Calliphora erythrocephala, the corpora allata control the synthesis of neurosecretory materials by the median neurosecretory cells (Thomsen and Lea, 1968). In the cockroach Nauphoeta cinerea, Kindle et al. (1988) have found that juvenile hormone III can directly influence the uptake of vitellogenin.

In Phormia regina, Orr (1964a) shows that corpora allata must be present for two days after liver feeding for egg development. Mjeni and Morrison (1976) have demonstrated that the synthesis of female specific hemolymph proteins depends upon juvenile hormone. Pappas and Fraenkel (1978) suggest that corpora allata control vitellogenin synthesis. However, Yin et al. (1989a, 1989b) demonstrate that the role of corpus allatum or juvenile hormone in egg development is controlling the uptake of vitellogenin by the ovary instead of controlling the vitellogenin synthesis by the fat body, since the activation of juvenile hormone biosynthesis by the corpus allatum occurs a few hours later than the appearance of vitellogenin in the hemolymph.

3. Overall control of ovarian development: cephalic factors

The scheme of endocrine control of egg development in the anautogenous mosquito, Aedes aegypti, has been postulated (Hanaoka and Hagedorn 1980; Hagedorn, 1983). It is suggested that egg development in this species is an environmentally triggered and hormonally regulated process. In the previtellogenic stages, adult emergence causes the release of juvenile hormone by the corpora allata. Juvenile hormone then causes the undifferented ovary to develop to the resting stage. Another effect of JH is to stimulate or make the fat body competent to respond to 20-hydroxyecdysone. The vitellogenic stages of egg

development start when the blood meal triggers the brain to release the egg development neurosecretory hormone (EDNH). EDNH then activates the ovary to produce ecdysone. Shortly after being released from the ovary to the hemolymph, ecdysone is converted to 20-hydroxyecdysone. 20-hydroxyecdysone then activates the fat body to synthesize vitellogenin, which, after being released into hemolymph, is taken up by the developing oocytes.

In Phormia regina, Yin et al. (1989b, 1990) have shown that the protein meal causes an immediate increase in the hemolymph ecdysteroid titer, which is followed by an increase in the amount of vitellogenin in the hemolymph. Activation of the corpora allata follows the initiation of vitellogenin synthesis while the yolk deposition follows the appearance of a considerable amount of juvenile hormone released from the corpus allatum. Based on the above events, they suggest that ecdysteroids controls vitellogenin synthesis while juvenile hormone regulates the vitellogenin uptake.

However, mechanisms of how the protein meal activates the neuroendocrine system in Phormia regina is still unknown. Whether there is a similar involvement of the head (brain) in egg development of Phormia regina, as that occurs in Aedes aegypti and Calliphora erythrocephala, is not clear. The interactions among the brain, the nervous systems, the corpora allata, the ovary and the fat body, as well as the interactions among the cephalic factor(s),

ecdysteroids and juvenile hormone in regulating egg development needs to be further defined. The major objective of this thesis was to investigate the role of cephalic factors on oogenesis in Phormia regina.

CHAPTER II

MATERIALS AND METHODS

A. Insects

The stock colony of Phormia regina (Meigen) was reared in the rearing room at the University of Massachusetts. Larvae were raised on artificial medium, which has been described by Stoffolano (1974). Mature larvae were allowed to crawl out from the medium into sand to pupate. Pupae were collected daily and flies were allowed to emerge in experimental cages. Flies emerging within 12 hours were regarded as of same age (0 day old). Crystalline sucrose and water or 10% sucrose solution were provided to the flies during the first three days after emergence. During the experimental period, all flies were kept at 28 $\pm$ 2^{o}C, with a 16 hr light, 8 hr dark photoperiod regime.

B. Liver Feeding

1. Feeding of the untreated control flies

Beef liver was homogenized, depending on the experimental designs, either alone for the experiments to determine protein ingestion and utilization, or together with sucrose (10% of liver weight) for all the other experiments. The homogenization was done by using a mortar

and pestle. The homogenate was put into a Petri dish, which was then put into the experimental fly cage. Three-day old flies were allowed to feed on the homogenate for 4 hr. When flies were deprived of a protein source for the first three days of their adulthood, they became hungry for protein. The 4 hr feeding bout allowed almost 100% of the flies to feed to repletion. Flies were determined replete when their abdomens became distended and one could see intersegmental membrane between the abdominal segments.

2. Feeding of ventral nerve cord severed flies

When the ventral nerve cord was severed, Phormia regina was unable to stand still and the food detecting function of its foretarsi was lost. To assist these flies in feeding, wooden stick applicators (146 mm long x 3 mm diameter), with a tackiwax ball on the end of the stick, were prepared. Individual flies were secured on the applicator by sticking their wings to the tackiwax. The fastened fly was then put into a position so that the proboscis of the fly could touch the liver homogenate. The contact of the proboscis (i. e., especially the labellar chemosensilla) to the liver homogenate stimulated the fly to feed. These flies were also allowed to feed for 4 hr. However, unlike the untreated control flies, most of these ventral nerve cord severed flies ceased feeding before reaching full repletion within this 4 hr.

C. Ligation

Three-day old female flies were fed with liver-sucrose homogenate as described above. At various times (7 to 12 hr at 1 hr intervals) after the onset of liver feeding, the liver fed flies were divided into three groups: ligated, and sham-ligated and unligated controls.

Flies were placed into a glass vial equipped with a stopper and anesthetized by flushing the vial with CO_2. The vial was kept on ice while the flies waited for treatment. The ligation was performed on the surface of a wax-filled Petri dish. The animal was placed on the wax dorsal side down. A rubber band pinned down by two insect pins was stretched over the thorax to secure the fly. Neck ligation was performed by using a nylon thread with a diameter of 0.1-0.2 mm. The sham-ligated fly was first ligated at the neck with a human hair and immediately thereafter, the ligature was untied under a dissecting microscope. The blank control group was anesthetized with CO_2 and chilled similarly but no other treatment was made.

D. Staging of Follicles (Scoring of Ovaries)

The ovaries of the flies were dissected out under a dissecting microscope, 48 or 72 hr after feeding, depending

on the experimental design. The follicular developmental stage was recorded. The status of the follicles was categorized to three groups of previtellogenic (I. e., atages 1 to 3 of Adams and Reinecke, 1979), vitellogenic (I. e., stages 4 to 9) and mature (stage 10).

E. Measurement of Volume of Crop and Midgut

1. Protein Ingestion

Three-day old flies were allowed to feed on liver homogenate without sucrose for various time periods (5, 10, 30, 60 and 90 minutes). The flies were dissected immediately at the end of feeding. To facilitate the dissection, flies were submerged in a hemolymph isotonic solution, Phormia regina saline (Chen and Friedman, 1975). The crop and midgut were dissected out and transferred into separated depression wells on a ceramic plate, which was set on ice. The saline brought into the well by transferring the crop or midgut was absorbed with a strip of filter paper. Twenty ul of a carbonate buffer (0.05 M, pH 9.6) was then added to each well. The crop or the midgut was opened up by teasing the wall of the crop or midgut using a pair of fine pointed forceps. The contents of the crop or midgut were washed out into the buffer. Then the volume of buffer plus crop (or midgut) contents was measured by using a micropipette. The volume of the

crop or midgut was obtained by subtracting the buffer volume from the total volume.

2. Protein utilization

Three-day old female flies were allowed to feed on liver homogenate (without sucrose) for 2 hours. Only those fed to repletion were used. At various times (6, 9, 14, 26, 36 and 50 hours) after the onset of feeding, the crop and the midgut volumes were measured from a sample using the same method mentioned above.

F. Protein Determination

1. Method

Protein content of samples was measured using the method of Bradford (1976). A standard curve was done simultaneously with each set of protein determinations. Bovine serum albumin (BSA) was used to generate the standard curve and the data were read as BSA equivalents.

2. Feeding and crop emptying in normal flies

The crop and midgut were dissected out and the contents were washed out separately using the carbonate buffer. According to the contents of the crop or the midgut, various amounts of buffer were used to ensure complete dissolving of the protein as judged visually.

The time schedule of protein determinations followed that of crop and midgut volume determinations.

3. Crop emptying in ligated and sham-ligated flies

Three-day old flies were allowed to feed on liver for 2 hr. Ligated or sham-ligated treatments were given to these flies 7 hr after liver feeding began. At different times after the onset of liver feeding (12, 24, 48, 56 and 72 hours), the protein content of crop and midgut were measured using the same method described in section E, 1 of this chapter.

G. Severing the Ventral Nerve Cord

Flies at two times (4 and 8 hr) after liver feeding on day 3 or flies of same age after only sugar and water feeding were subjected to ventral nerve cord severing to test the possible function of the nerve cord during egg development. Flies were first anesthetized with CO_2 by using the same method described in ligation section (C). The anesthetized flies were fastened dorsal side down on the wax dish as described in the ligation method section (C). The head of the flies was pulled forward by a device possessing two hooks made of minuten pins so that the neck of the fly was well stretched to expose the ventral surface. An opening on the posterior end of the neck (the prothoracic side) was made by cutting the cervical

membrane. The fused ventral nerve cord can be seen clearly through this opening under the dissecting microscope (25 X). Then the ventral nerve cord was lifted up slightly by using a pair of fine-pointed forceps and severed with a blade, which was made up by a strip of shaving blade. The wound was sealed by a drop of melted wax. One hr after the ventral nerve cord transection, liver homogenate was fed to sugar-fed, ventral nerve cord severed flies using procedures described in B, 2 of this chapter. After the operation, the flies were kept on the surface of a moistened filter paper within a paper cup with a 15 cm diameter and a 10 cm height.

H. Severing the Cardiac-Recurrent Nerve Bundle (CRN)

Both liver-fed flies (operated at 4 hr after the onset of liver feeding) and three-day old sugar-fed flies (operated 1 hr prior to the liver meal) were used in this experiment. The fly was fastened ventral side down and the head of the fly was pulled forward using the same hooks described above. An opening was made at the dorsal side of the neck near the prothorax. The muscles at the open region were pushed aside. The aorta, corpus allatum and the "hypocerebral ganglion" became visible. A small hook made from a minuten pin was put under the bundle of aorta and the CRN. This bundle was lifted up slightly and cut with a blade at the region of the "hypocerebral ganglion"

and anterior to the corpus allatum. After this severing, the corpus allatum and corpora cardiaca complex was retracted into the thorax by the remaining part of aorta. The wound was sealed by using a drop of melted wax and the flies were kept under a high humidity condition within the paper cup described above.

I. Tracheal Severing

At 7 hr after the onset of liver feeding, the flies were anesthetized and fastened ventral side down on the wax dish using the same methods described in sections G and H. The neck of the fly was also stretched by pulling the head of the fly forward with the same hooks used in nerve transection. An opening was made at the dorsal side of the neck to expose the two cervical tracheal trunks. The tracheal trunks were then severed by using a fine-pointed hook made from a minuten pin. The sham control flies were treated similarly but the tracheal trunks were left intact.

J. Determination of Vitellogenin Level

1. Hemolymph sample preparation

Hemolymph samples were collected from three day old sugar-water fed flies, liver-fed control flies and ligated flies at various times depending on the experimental

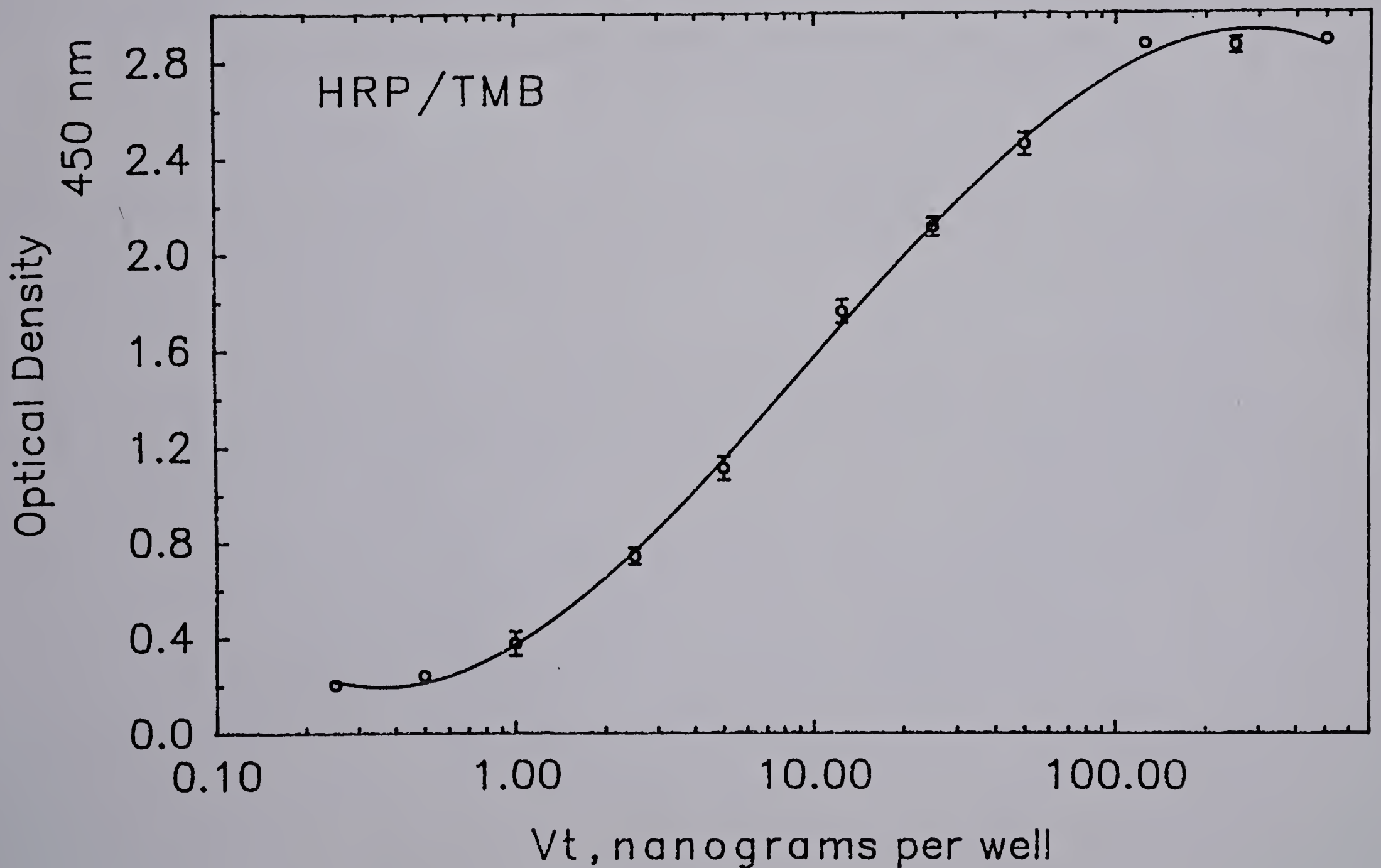
design. Collecting was done by pulling off one leg of the fly, pressing the fly body slightly and drawing the droplet of hemolymph, which came out of the opening, using a scaled micropipette. For each sample, 5 ul of hemolymph collected from 2 to 5 flies was mixed with 95 ul of the carbonate buffer mentioned above.

2. Vitellogenin titer determination

The hemolymph vitellogenin titer was measured by the enzyme-linked immunosorbent assay (ELISA). The ELISA procedure for Phormia regina vitellin/vitellogenin, virtually followed the methods described previously by Zou et al. (1988) except a few changes. Briefly, a 100 ul standard vitellin (Vt) carbonate buffer solution or hemolymph carbonate buffer solution (unknown sample) was added to each well of the polystyrene ELISA plate (CORNING, 25805-96) followed by incubating the plate at 37°C for 4 hr. After the incubation, the wells were emptied and washed three times with TBS-Tween 20 solution (20 mM Tris, 500 mM NaCl, 0.01% thimerosal, 0.05% Tween-20, pH 7.5). Two hundred ul of 1% BSA solution was added to each well and the plate was incubated at room temperature for 30 min.. The wells were emptied and washed again with TBS-Tween 20 three times. One hundred ul vitellin/vitellogenin antiserum diluted 1:1000 to 1:2000 in antibody buffer (TBS-Tween 20 buffer with 1% of BSA) was added to each well followed by incubating the plate at room temperature for 12

to 16 hr. After this incubation, the wells were emptied and washed three times with TBS (20 mM Tris, 500 mM NaCl, 0.01% thimerosal, pH 7.5). One hundred ul of horseradish peroxidase-conjugated secondary antibody in antibody buffer (1:2000 to 1:5000) was added to each well followed by incubating the plate for 2 hr. The wells were washed once more with TBS three times. Then, 100 ul substrate-chromogen solution, which was made up by mixing 0.1 ml 42 mM TMB (3,3',5,5'-tetramethylbenzidine) DMSO (dimethylsulfoxide) solution, 10 ml acetate/citric acid buffer (0.1 M, pH 6.0) and 1 ml 5 mM urea peroxide solution, was added to each well. The plate was then incubated at room temperature for 10 to 60 min depending on the rate of enzyme reaction. To terminate the reaction, 50 ul of 2 M H_2SO_4 was added to each well. The optical density (O.D.) of each well was read at 450 nm in a Vmax kinetic microplate reader. A standard curve was generated from the vitellin standard solution and used to determine the vitellogenin quantity in the unknown sample. By using urea peroxide-TMB system, the sensitivity of Phormia regina vitellin/vitellogenin ELISA was improved. Fig. 1 shows one of the standard curves used in this study. As indicated in Fig. 1, a range of 1 to 100 ng of vitellin or vitellogenin in an unknown sample could be measured using this method.

Figure 1. Standard curve for vitellogenin/vitellin ELISA using a horseradish peroxidase-conjugated secondary antibody and a urea peroxide-TMB substrate-chromogen system.


HRP/TMB
Optical Density
450 nm
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
0.10
1.00
10.00
100.00
Vt, nanograms per well

K. Radiochemical Assay of Corpus Allatum Activity

The corpus allatum activities of sugar-water fed, liver-fed and liver-fed, ligated flies were measured using the *in vitro* radiochemical assay of Zou *et al*. (1989). Since the corpus allatum activity in the normal flies reaches its highest point at about 50 hr after liver feeding, the corpus allatum activities of different treatment groups of flies were measured at 48 hr after liver feeding. One pair of corpus cardiacum-corpus allatum (CC-CA) complexes were incubated in 50 ul of tissue culture medium 199 lacking methionine (Gibco, special formulation) and supplemented with calcium chloride (5 mM) and Ficoll (20 mg/ml). L-[^{3}H]methyl-menthionine (specific activity 80 Ci/mmol; New England Nuclear, Boston MA) and cold methionine were added to the medium to make the total methionine concentration to 0.05 mM and 1 uCi L-[^{3}H]methyl-methionine in 50 ul of medium. Method of handling the corpus allatum followed that of Liu (1985). Incubation was carried out at 26^{o}C with 200 rpm shaking for 4 hr in the dark. The incubation was terminated and the culture medium, with the CC-CA complexes inside, was extracted with 250 ul of iso-octane (2,2,4-trimethlypentane). The extraction was done by emulsifying the medium and iso-octane using sonication and by centrifuging the emulsified mixture at 10,000 rpm for 10 minutes. The organic phase

(150 ul out of 250 ul) was carefully transferred to a 7 ml tube. To each tube with the 150 ul organic phase, 4 ml scintillation fluid (Aquasol-II) was added. The tube was vortexed and put into a 20 ml scintillation counting vial for radioactivity counting. Incubation without CC-CA complexes served as extraction controls for each set of measurements and the radioactivity of this extraction was used as background activity, which was subtracted from that of the experimental treatment samples. The extractions of all samples were quantified in a LKB 1209 Rackbeta liquid scintillation counter with counting efficiency of 48.55%. The incorporation rates of [^{3}H]methyl into JH were calculated based on the following formula modified from the formula of Liu (1985):

$$\frac{R - B}{(2.22 \times 10^{12}) \times E \times A \times S \times T} \times 10\% = \text{pmol/pair of gland/hr}$$

R = Radioactivity of sample (cpm)

B = Background radioactivity (cpm)

E = Counting efficiency

A = [^{3}H-methionine]/[cold methionine]

S = ^{3}H-methionine specific activity (80 Ci/10^{9} pmol)

T = Incubation duration (hr)

2.22×10^{12} dpm = 1 Ci

10% = Percentage of JH in total extractable radioactivity as determined by Liu et al., 1988 and Zou et al., 1989

L. Radioimmunoassy for Ecdysteroids

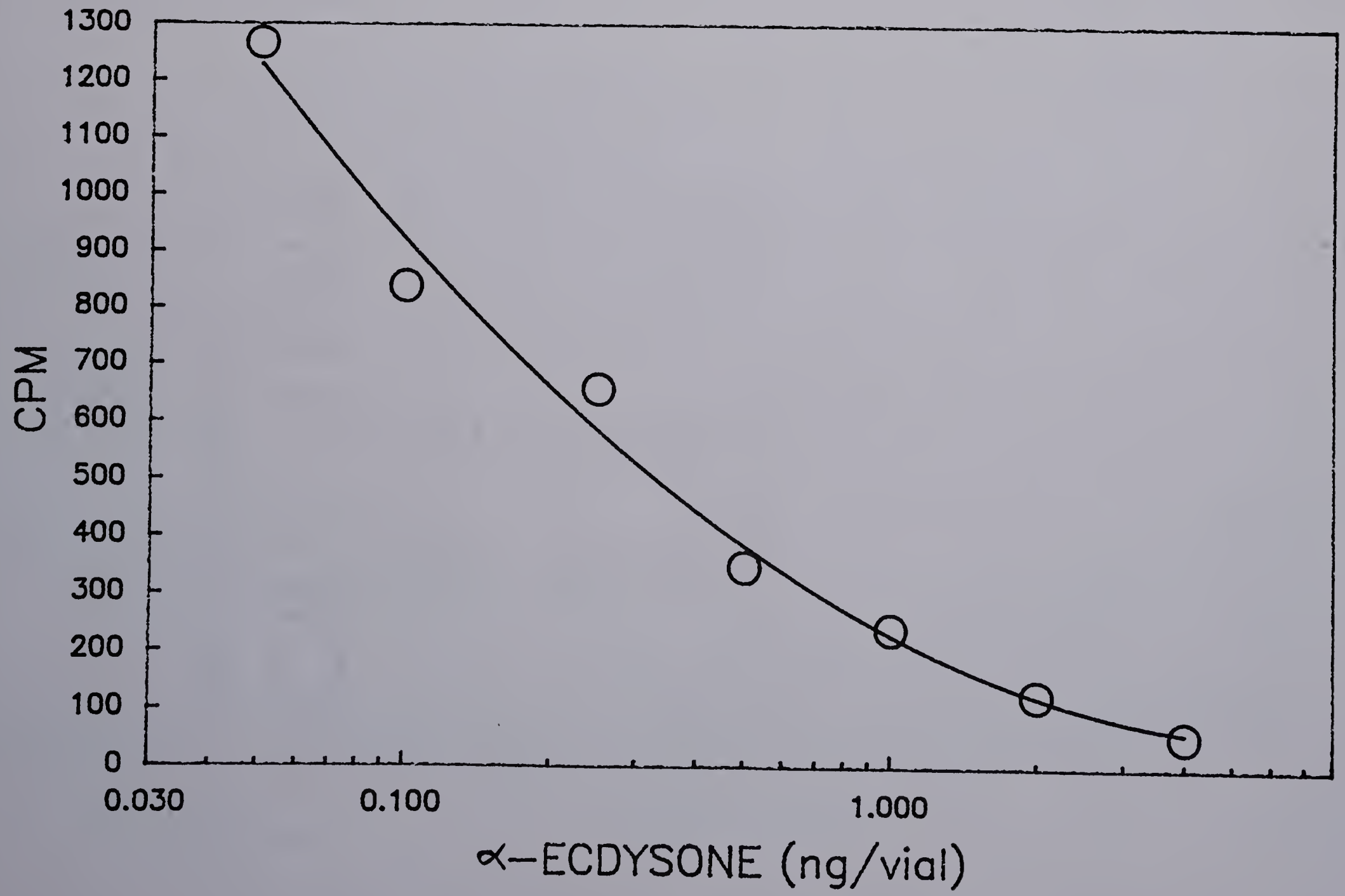
Hemolymph ecdysteriod titers were measured by using the radioimmunoassy (RIA) for ecdysteroids (Yin et al., 1990). The hemolymph was collected from ligated flies and liver-fed control flies 32 hr after liver feeding and from sugar-water fed flies with the same age using the hemolymph collecting method described in vitellogenin level determination section (J, 1 of this chapter). Five ul of hemolymph were collected for each sample from 2 to 5 flies. The hemolymph sample was then extracted three times with 100 ul 80% methanol by vortexing the mixture thoroughly and centrifuging it at 1000 rpm for 10 min.. The supernatant of the extraction was pooled together into a 6 x 50 mm tube for ecdysteroid measurement. In another set of 6 x 50 mm tubes, 0.05 ng to 4 ng of α-ecdysone dissolved in 100% ethanol was added to each tube for generating the standard curve. After the transfer, the sample and the standard solutions in the tubes were dried at room temperature with a gentle stream of air. To each of these tubes, 100 ul of H^3-ecdysone with about 6000 cpm was added. After vortexing, 100 ul of antiserum borate buffer solution were added to each tube. The tubes were vortexed again thoroughly and incubated for 12-16 hr at 4°C. After the incubation, 200 ul saturated ammonium sulfate solution were added to each tube to allow precipitation of the antibody-ecdysteroid complex. The tubes were vortexed and let stand

for 30 min at 4°C. Following the 30 min incubation, the tubes were centrifuged at 5000 rpm for 10 min and the supernatant was carefully aspirated off. The pellet in each tube was resuspended with 400 ul 50% saturated ammonium sulfate solution and the suspension was incubated for 30 min at 4°C. Each tube was centrifuged again at 5000 rpm for 10 min and the supernatant was aspirated off. To each tube, 20 ul of distilled water were added to solubilize the pellet. After vortexing each tube, 400 ul Aquasol-II were added. The sample tube was then put in a 7 ml tube which was placed in a 20 ml counting vial and counted for radioactivity using a LKB 1209 Rackbeta liquid scintillation counter. Equilibration of normal rabbit serum with H^3-ecdysone served as the counting background control in this experiment. The counted values of the samples and the standards were adjusted by subtracting the background value from each value. A standard curve was generated and the unknown samples were read from the curve. A range of 0.05 to 0.5 ng α-ecdysone in an unknown sample could be measured using the present RIA. Fig. 2 shows one of the standard curves used in this experiment. Results should be read as α-ecdysone equivalents.

M. Topical Application of Methoprene

Three-day old female flies were fed with liver for 4 hr. At 7 hr after the onset of liver feeding, the flie

Figure 2. Standard curve for ecdysteriod determination using ∝-ecdysone as standard.


1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
CPM
0.030
0.100
1.000
∝–ECDYSONE (ng/vial)

were ligated at the neck region. Fourty eight hr after the onset of liver feeding, these liver-fed, neck ligated flies were divided into three groups. The first group was treated with the juvenile hormone analogue, methoprene (ZR-515). The second group was treated with acetone and used as the solvent treated control. The third group did not receive further treatment and was used as the blank control. Another group of liver-fed flies which received no treatment was used as the non-ligated control. Methoprene was dissolved in acetone (5 ug/ul acetone) and topically applied to the lateral side of the thorax using a Hamilton syringe and a microapplicator (Instrumentation Specialties Co.). Each fly received 2 ul methoprene acetone solution with 10 ug methoprene. The solvent control flies were treated similarly but received 2 ul of acetone only. At 96 hr after the onset of liver feeding (i. e., 48 hr after neck ligation), the ovaries of the flies were dissected out and the ovarian developmental stage was recorded.

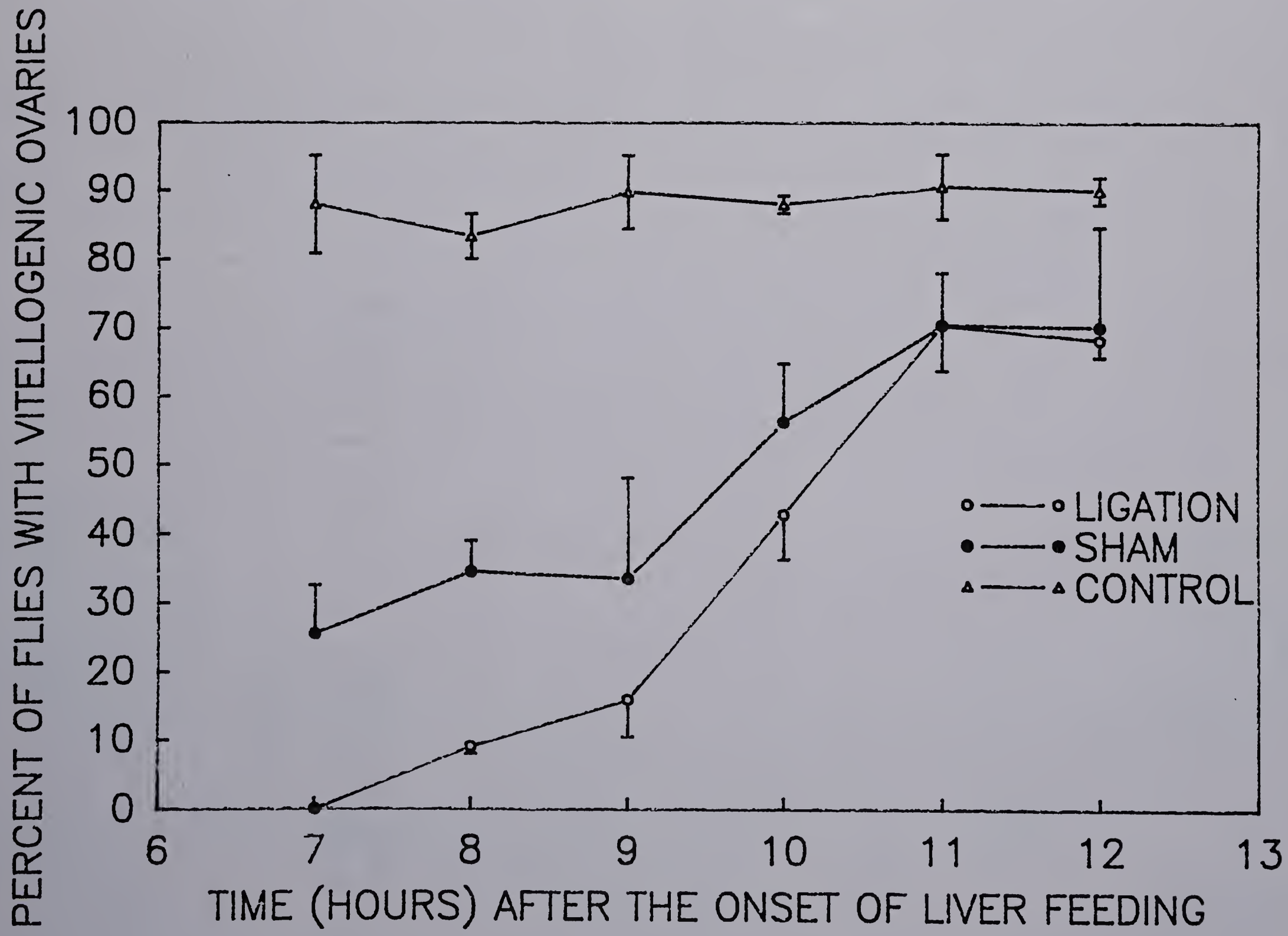
CHAPTER III

RESULTS

A. Effects of Neck Ligation on Ovarian Development

The effects of neck ligation, at different times after liver feeding, on the ovarian development of liver-fed Phormia regina are summarized in Fig. 3. When ligated at 7 hr after liver feeding started, ovaries of all ligated flies failed to develop beyond the previtellogenic stages when examined at 48 hours after liver feeding began. No difference in developmental status was observed between ovaries of the liver-fed, ligated flies, and the sugar-fed flies. However, in the liver-fed control flies, more than 85% of the flies showed vitellogenic ovaries and most of these ovaries contained fully matured eggs (stage 10). If ligated at 11 hr after liver feeding began, 70.4% of the ligated flies were able to develop their ovaries to vitellogenic stages when examined at 48 hr after liver feeding started. Thus, the reduction of ovarian development caused by ligation at 7 hr after the onset of liver feeding can be overcome by delaying the time of ligation. In the sham-ligated group, reduction in ovarian development also showed a time dependent reduction pattern, which is similar to that of the ligated flies.

Figure 3. Effect of neck ligation on ovarian development of Phormia regina at different times after liver feeding. Each datum point represents 3 replicates of 20 to 35 flies. Vertical bars = S. E. M.


PERCENT OF FLIES WITH VITELLOGENIC OVARIES
100
90
80
70
60
50
40
30
20
10
0
6
7
8
9
10
11
12
13
TIME (HOURS) AFTER THE ONSET OF LIVER FEEDING
LIGATION
SHAM
CONTROL

It is concluded from this experiment that ovarian development of Phormia regina is influenced by cephalic factor(s) or is under cephalic control. The critical period of this cephalic control is 7 to 12 hr after the onset of liver feeding. Since the sham-ligated flies showed a reduction in ovarian development, physical intactness of some tissue (or tissues) of the neck is very important for the proper function of this cephalic control.

B. Protein Meal Distribution in Crop and Midgut in Blank Control Flies

In the previous experiment it was noticed that the ligated and sham-ligated flies both had brown-colored food left in their crops at the time of dissection (48 hours after liver feeding began) while more that 90% of the blank control flies had empty crops. It is likely that the food left in the crop is the remaining part of the liver meal. This finding led me to examine the role of the crop in the protein meal and the role of the crop content in ovarian development. In this experiment the protein content of crop and midgut at various times during liver feeding (which may reflect food utilization) was investigated (Fig. 4).

The volumes of both crop and midgut during the same time span are showed in Fig. 5. In the beginning of liver feeding, the protein contents of crop and midgut were both

at undetectable levels. In this time, the volume of the crop was around 8 ul while the volume of midgut was too small to measure. Five minutes after liver feeding started, protein could be detected in both crop and midgut. This indicates that the crop and midgut were filled simultaneously during this period.

The rates of increases in volume and protein content for both the crop and midgut were higher at the first 10 min than those at the rest of the feeding time (from 10 min to 90 min). The volume and protein content of the crop peaked at 18.4 ul and 2338.7 ug 90 min after feeding started. At the same time, the midgut reached its peak volume of 2.2 ul and protein content of 512 ug. It is obvious from these results that the crop, rather than the midgut, is the major storage site of the protein meal.

Figs. 4 and 5 also showed that when the crop was full (i. e., reached the size of 18.7 ul 60 min after liver feeding started), the size increase stopped while the protein content continued to increase. It is apparent that the flies were able to concentrate their crop contents.

C. Utilization of Protein by Non-Ligated Control Flies

Since the crop has been proven to be the major storage site for the protein meal and significant digestion and absorption are unlikely to occur within the crop, I assumed that the contents of the crop is transferred to

Figure 4. Protein contents of the crop and midgut at various time intervals following liver feeding. Each datum point represents 5 to 10 replicates. vertical bars = S. E. M.

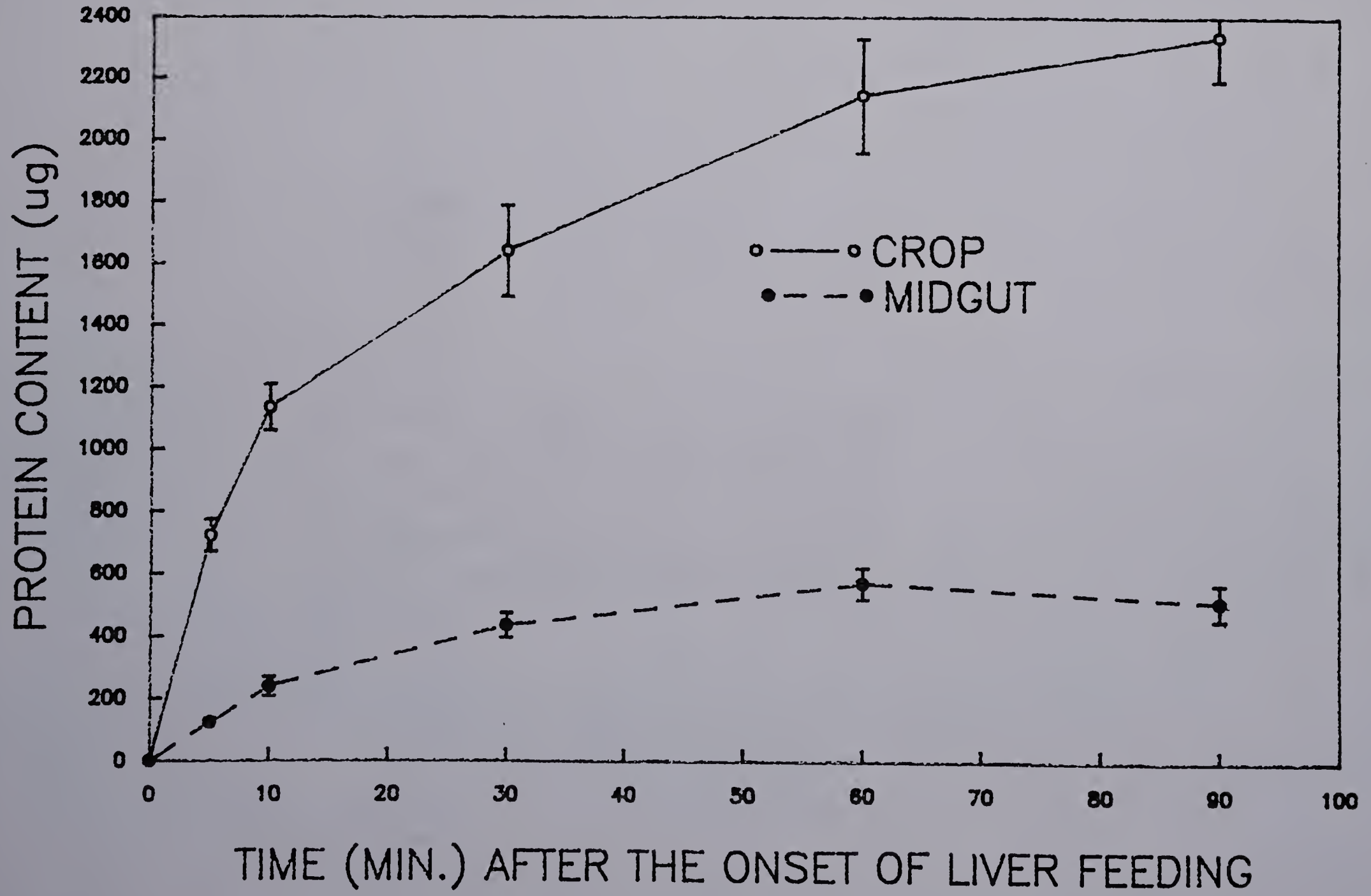
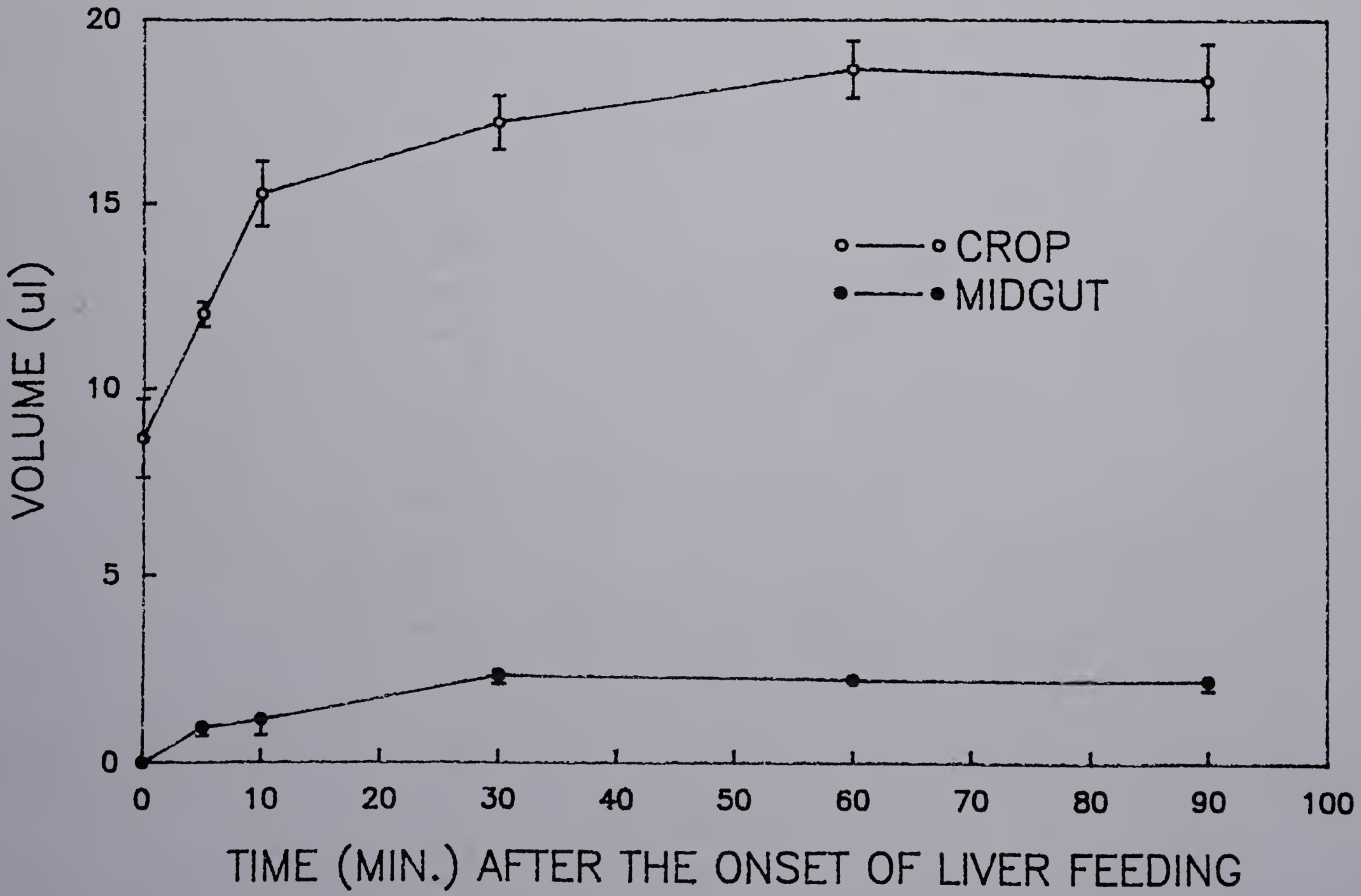

PROTEIN CONTENT (ug)
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
70
80
90
100
CROP
MIDGUT
TIME (MIN.) AFTER THE ONSET OF LIVER FEEDING

Figure 5. Volumes of crop and midgut at various time intervals following liver feeding. Each datum point represents 5 to 10 replicates. Vertical Bar = S. E. M.


20
15
10
5
0
VOLUME (ul)
CROP
MIDGUT
0
10
20
30
40
50
60
70
80
90
100
TIME (MIN.) AFTER THE ONSET OF LIVER FEEDING

the midgut for utilization. The volume and the protein content of the crop and midgut at various times after liver feeding were examined (Figs. 6 and 7). The contents of both crop and midgut showed a rapid decrease during the first 26 hours. At the end of the first day, about 75% of the proteinaceous materials ingested during feeding were emptied from the crop and 78% of the midgut content plus the transferred crop content were utilized. During the same time, the volumes of the crop and midgut dropped from 18.4 ul to 10.1 ul and 2.2 ul to 1.6 ul respectively. The internal volume and protein contents of both crop and midgut dropped to undetectable level at 50 hr after the onset of liver feeding.

D. Utilization of Protein by Neck-Ligated Flies and Sham-Ligated Flies

Crop emptying of Phormia regina depends on the operation of a series of valves and pumps located in the foregut near the neck region (Thomson and Holling, 1975; Thomson, 1975a, 1975b). This experiment was done to rule out the possibility that physical damage of the foregut (i. e., caused by the neck ligation) might block the transfer of the protein resource from crop to midgut, therefore suppressing indirectly ovarian development. The profiles of crop and midgut protein content of ligated flies (ligated at 7 hours after liver feeding started) and

sham-ligated control flies are depicted in Figs. 8 and 9. In the ligated flies, 48 hours after liver feeding, 67.9% of the crop-stored protein had been transferred to the midgut. In the sham-ligated control flies, 68.7% of the crop-stored protein was emptied at the same time. It is concluded from these results that the ligated and the sham-ligated control flies were able to transfer their crop contents to the midgut although the transferring rates were slower than those of the non-ligated flies (compare with Figs. 6 and 7).

The evidence thus suggests that suppression of ovarian development by the neck ligation and sham ligation is not caused by deficiencies in protein utilization. The suppression most likely results from a lack of a cephalic control factor (or factors).

E. Effects of Cardiac-Recurrent Nerve Bundle (CRN) Severing on Ovarian Development

Since ovarian development was suppressed by the sham-ligation treatment (see Fig. 3), and because this suppression was probably not caused by protein deficiency, some tissue(s) in the neck region other than foregut must have an important function in ovarian development. The possible role of CRN is therefore examined by severing the nerve bundle. The results of this treatment are shown in Fig. 10. When CRN was severed 4 hr after the onset of

liver feeding, there were no significant differences (single ANOVA $P \leq 0.05$) among the percentages of flies with vitellogenic ovaries for the nerve-severed flies, the sham controls and the unoperated controls. This indicates that the cephalic control of ovarian development is not via CRN although this nerve has some effect on feeding in Phormia regina (severing CRN causes hyperphagia of both sugar and protein as demonstrated by Dethier and Gelperin, 1967; Dethier, 1976). But the above experiment did not answer the question of whether CRN is required to convey the action of liver feeding to the brain so that the brain can execute its control over oogenesis. Results, summarized in Fig. 10, show, however, that severing the CRN 1 hr before liver feeding did not affect ovarian development. Thus, CRN is not essential in the activation of the brain control factor by the protein meal.

F. Role of Ventral Nerve Cord in Ovarian Development

Effects of ventral nerve cord severing at the neck region on oogenesis are shown in Fig. 11. No significant differences in ovarian development were detected in control flies, sham-operated flies and the flies with the ventral nerve cord served at 4 and 8 hr after the onset of liver feeding (single ANOVA, $P \leq 0.05$). These results suggest that once the protein meal requirement is fulfilled, ovarian development of the fly is independent of the ventral nerve

Figure 6. Volumes of the crop and midgut in liver-fed, untreated flies during egg development. Each datum point represents 5 to 8 replicates. Vertical bar = S. E. M.

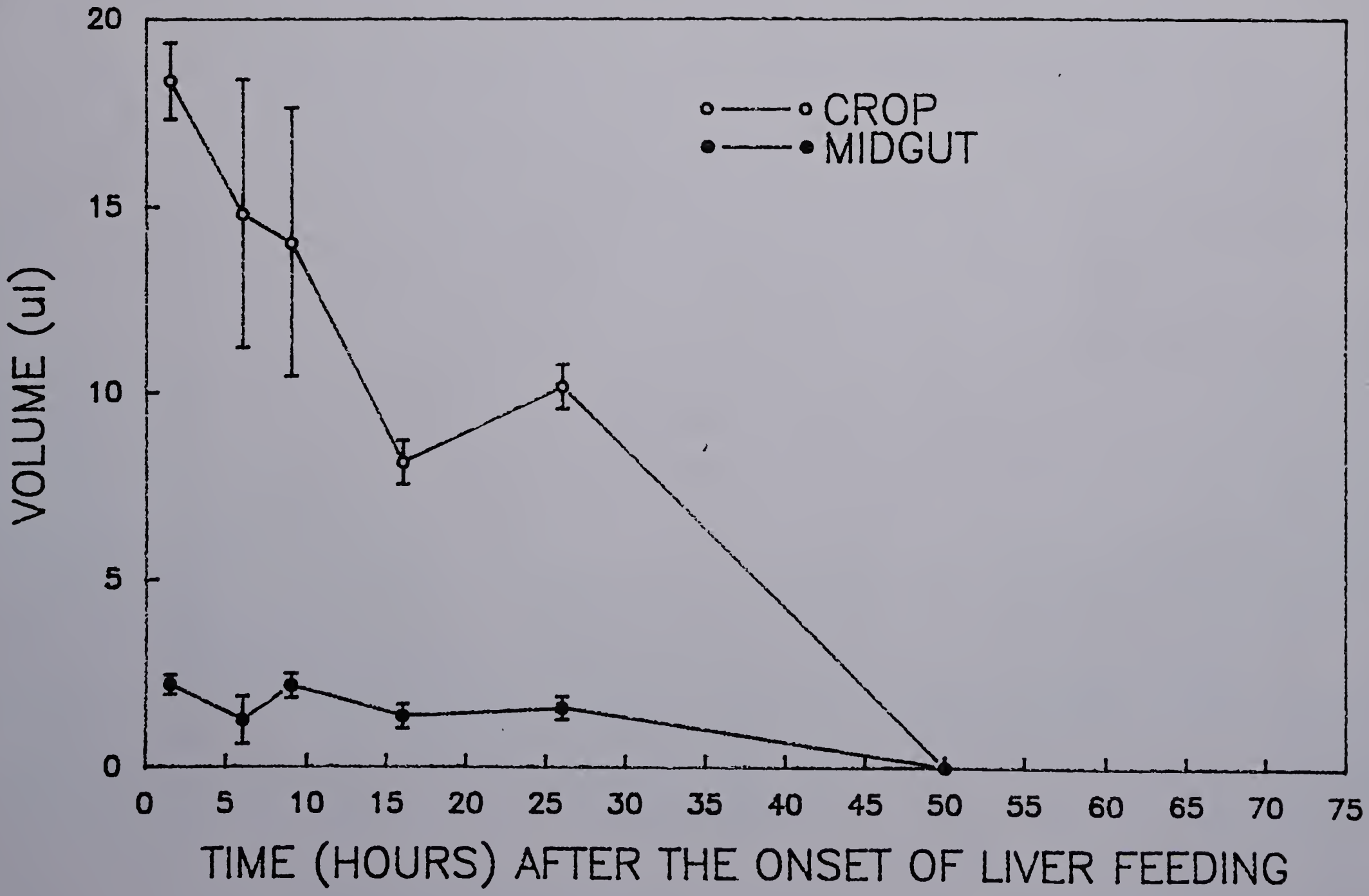

20
15
10
5
0
VOLUME (ul)
CROP
MIDGUT
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
TIME (HOURS) AFTER THE ONSET OF LIVER FEEDING

Figure 7. Movement of protein in the crop and midgut in liver-fed, untreated flies at various times following the commencement of liver feeding. Each datum point represents 5 to 8 replicates. Vertical bar = S. E. M.

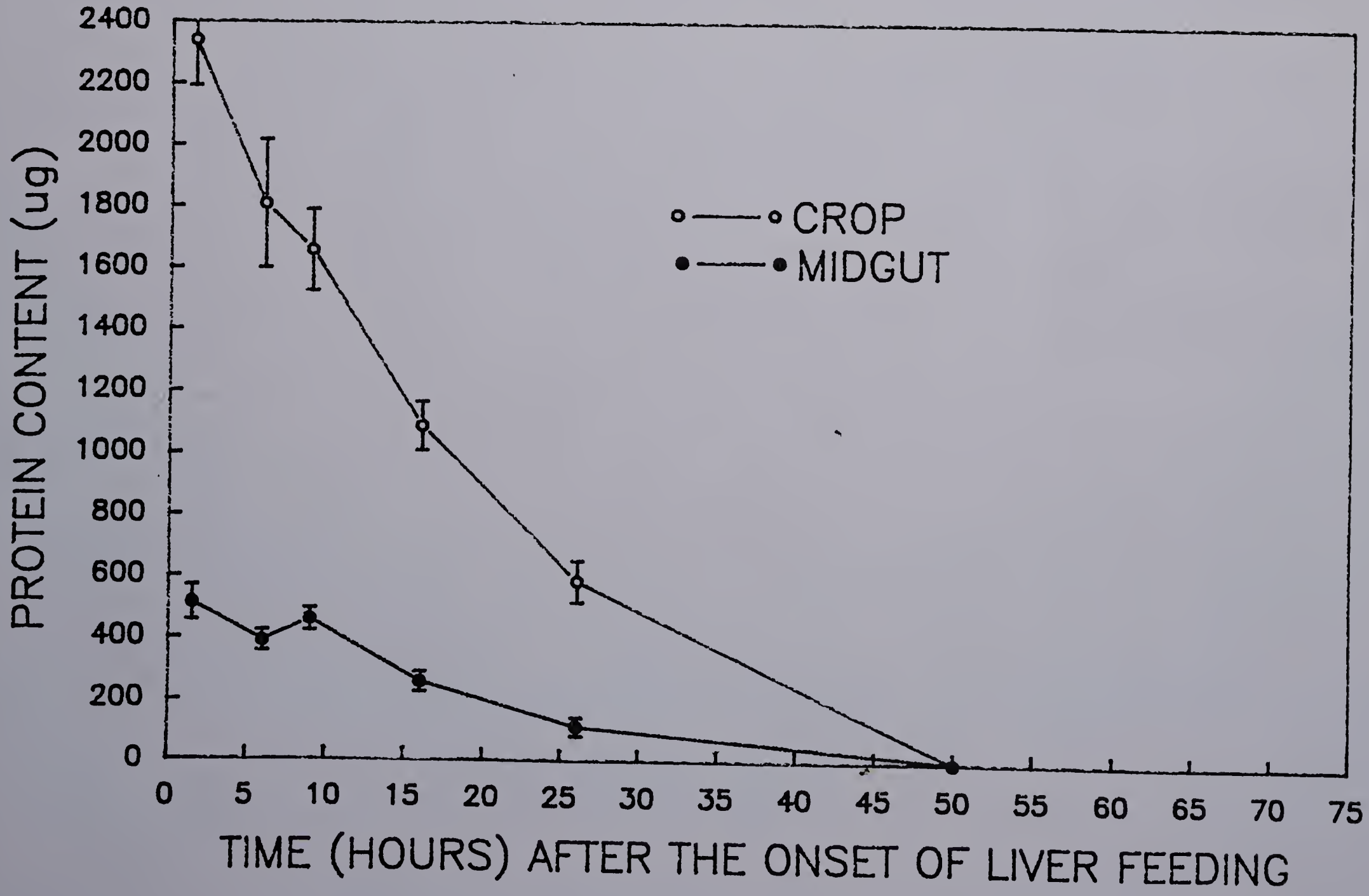

PROTEIN CONTENT (ug)
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
CROP
MIDGUT
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
TIME (HOURS) AFTER THE ONSET OF LIVER FEEDING

Figure 8. Movement of protein in the crop and midgut in neck-ligated female Phormia regina. Each datum point represents 5 to 10 replicate. Vertical bar = S. E. M.

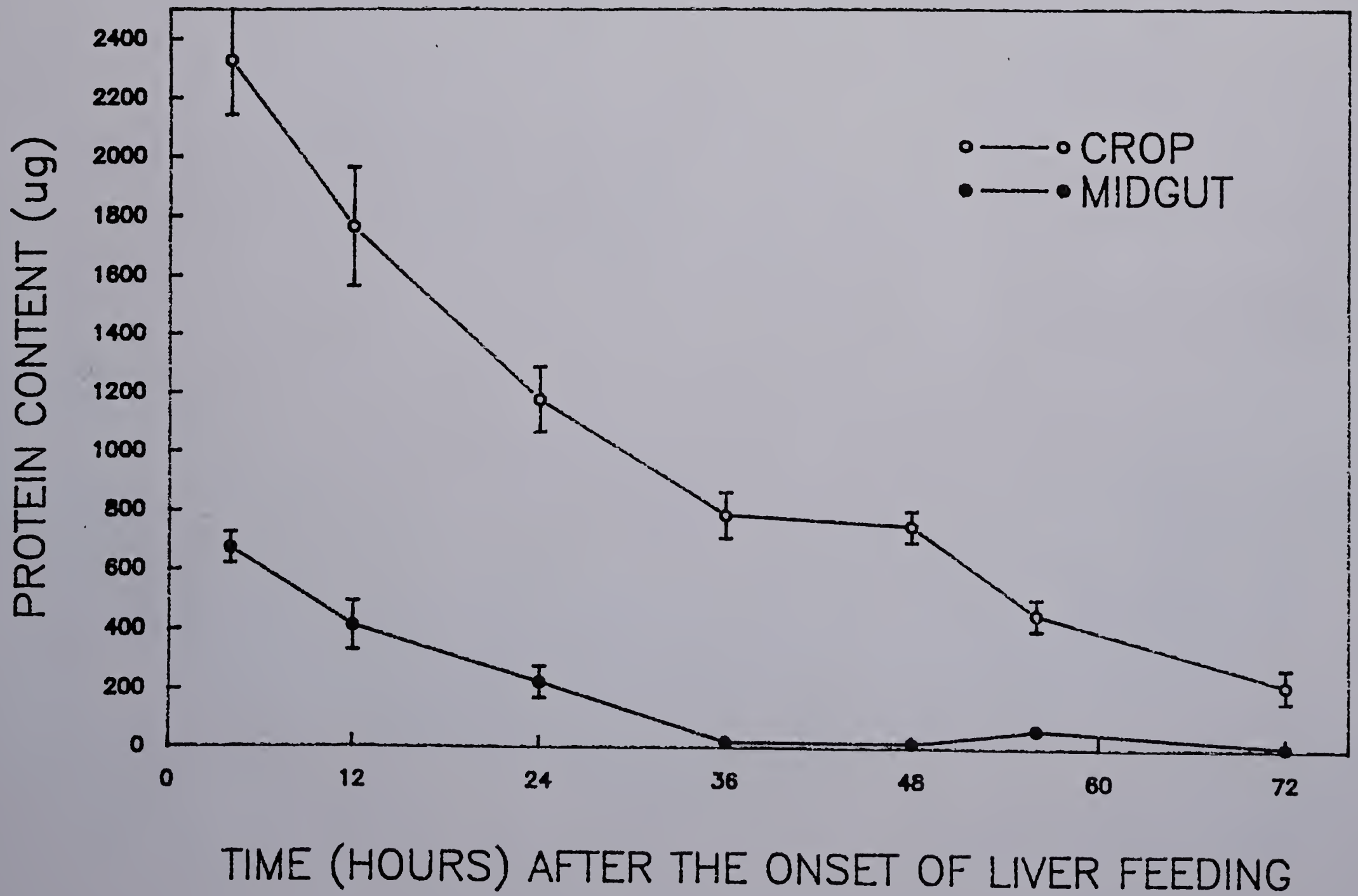

PROTEIN CONTENT (ug)
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
12
24
36
48
60
72
CROP
MIDGUT
TIME (HOURS) AFTER THE ONSET OF LIVER FEEDING

Figure 9. Movement of protein in the crop and midgut in sham-ligated female Phormia regina. Each datum point represents 5 to 10 replicates. Vertical bar = S. E. M.

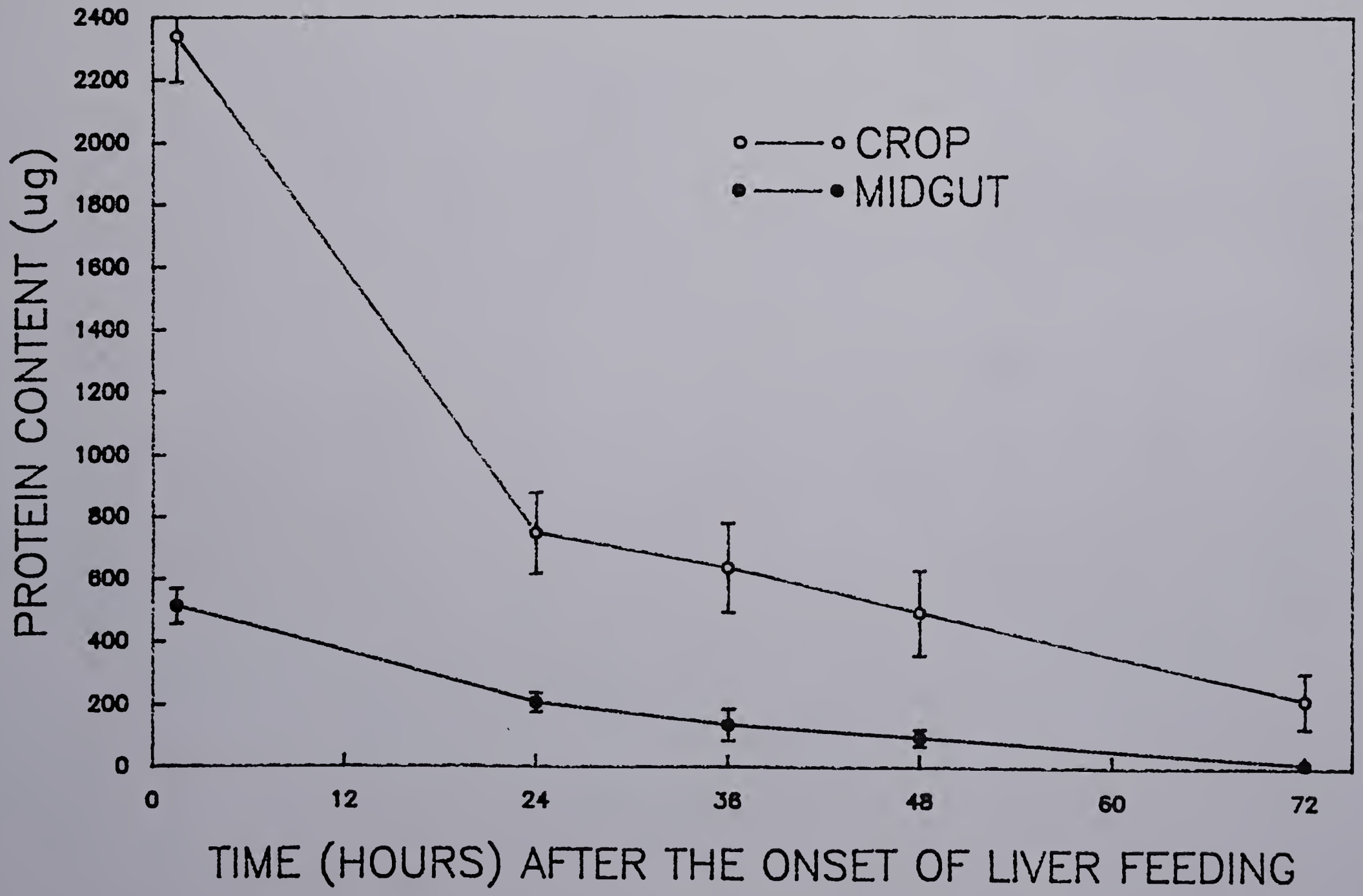

2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
PROTEIN CONTENT (ug)
CROP
MIDGUT
0
12
24
36
48
60
72
TIME (HOURS) AFTER THE ONSET OF LIVER FEEDING

Figure 10. Effect of CRN transection at various times on the ovarian development of Phormia regina. Each datum point represents 3 replicates of 16 to 33 flies. Vertical bar = S. E. M.

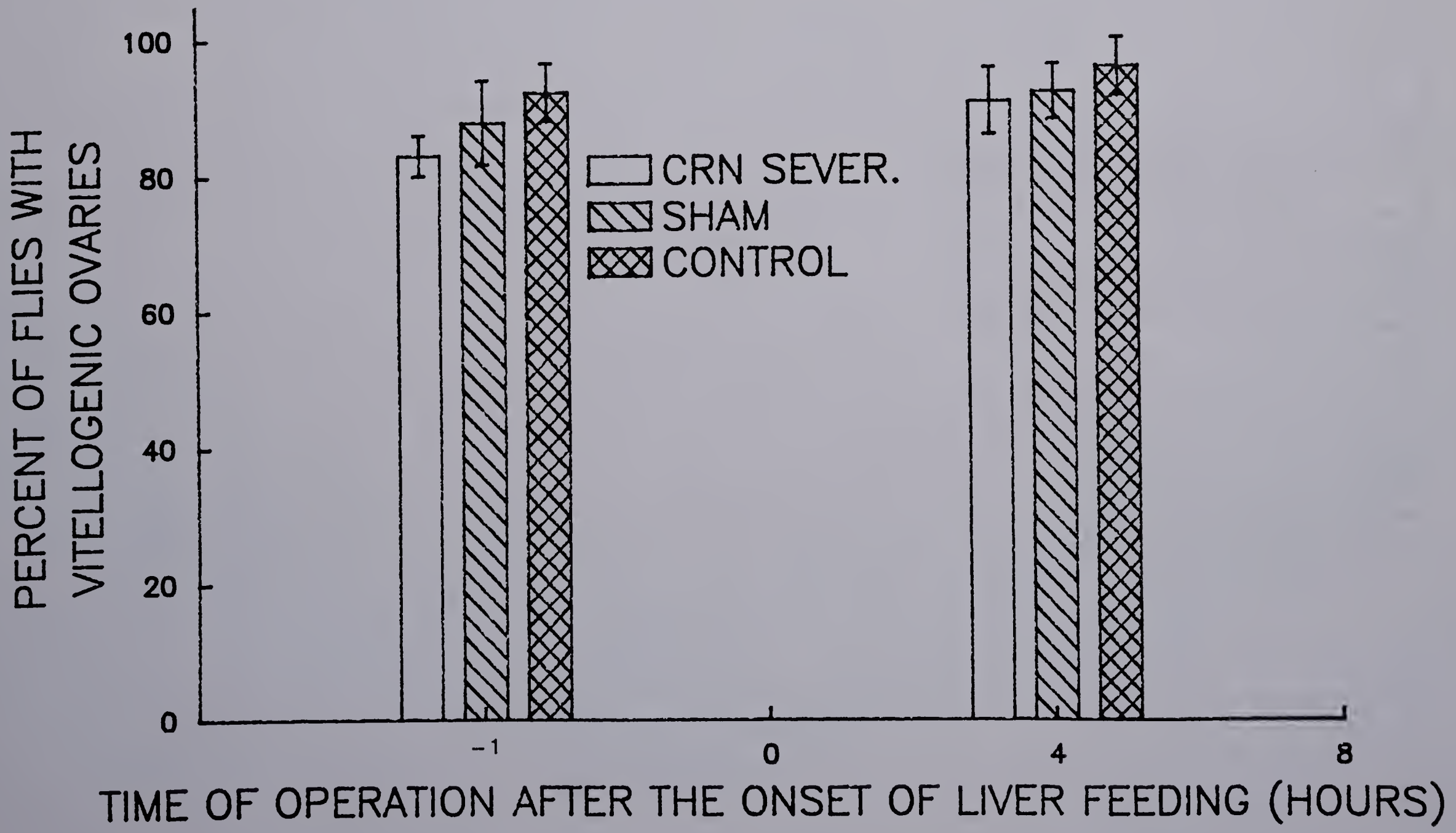
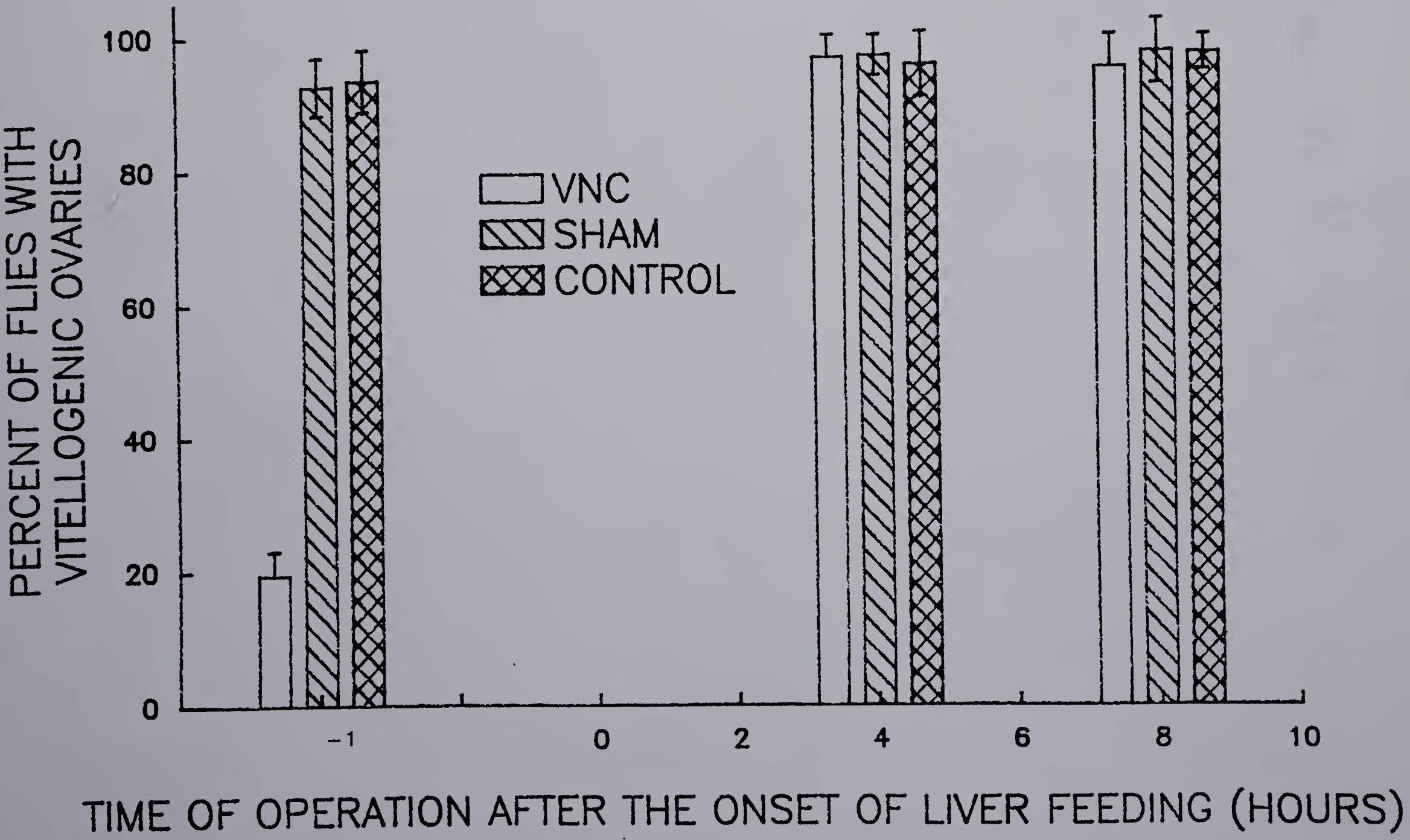

PERCENT OF FLIES WITH VITELLOGENIC OVARIES
100
80
60
40
20
0
CRN SEVER.
SHAM
CONTROL
-1
0
4
8
TIME OF OPERATION AFTER THE ONSET OF LIVER FEEDING (HOURS)

Figure 11. Effect of VNC transection on ovarian development of Phormia regina at various times after the onset of liver feeding. Each datum point represents 3 replicates of 16 to 33 flies. Vertical bar = S. E. M.


PERCENT OF FLIES WITH VITELLOGENIC OVARIES
100
80
60
40
20
0
VNC
SHAM
CONTROL
−1
0
2
4
6
8
10
TIME OF OPERATION AFTER THE ONSET OF LIVER FEEDING (HOURS)

cord. However, when the ventral nerve cord was sectioned 1 hr before liver feeding the operation prevented oogenesis in an average of 80.6% of the flies (72 hr after the commencement of liver feeding). Thus, the data suggest that the intactness of the ventral nerve cord before the liver meal is important for oogenesis. Since a repleted liver meal is essential for full egg maturation, it was assumed that feeding may be interferred by the transection of the ventral nerve cord.

G. Reduction of Liver Ingestion and Ovarian Development by Ventral Nerve Cord Severing

Table 1 shows that the sizes of liver meal and the corresponding ovarian status of the flies at 72 hr after liver feeding. Under the same category of ovarian status, the nerve severed flies and the sham-operated flies ingested similar amounts of proteinaceous food. In both nerve sectioned and sham-operated flies, which ingested less than 6 mg of liver, the ovaries failed to develop beyond stage 3. Therefore, a minimal amount of proteinaceous food is required to initiate and sustain the process of ovarian development. It is clear from these experiments that the minimal quantity necessary for developing vitellogenic ovaries is about 10 mg of fresh beef liver per fly.

Table 1. Effects of ventral nerve cord severing on liver feeding and ovarian developmenmt

	Ovarian Status		
	(‡) stage 10	(+) stage 4-9	(-) stage 1-3
Nerve Severed Flies			
liver ingested	9.6 mg	11.1±2.7 mg	5.7±1.2 mg
number of flies	1	7	17
% (n=25)	4%	28%	68%
Sham Control Flies			
liver ingested	13.2±1.5 mg	11.1±1.5 mg	6.8±2.5 mg
number of flies	13	11	8
% (n=32)	40.6%	34.4%	25%

(‡) and (+) = vitellogenic, (-) = non-vitellogenic

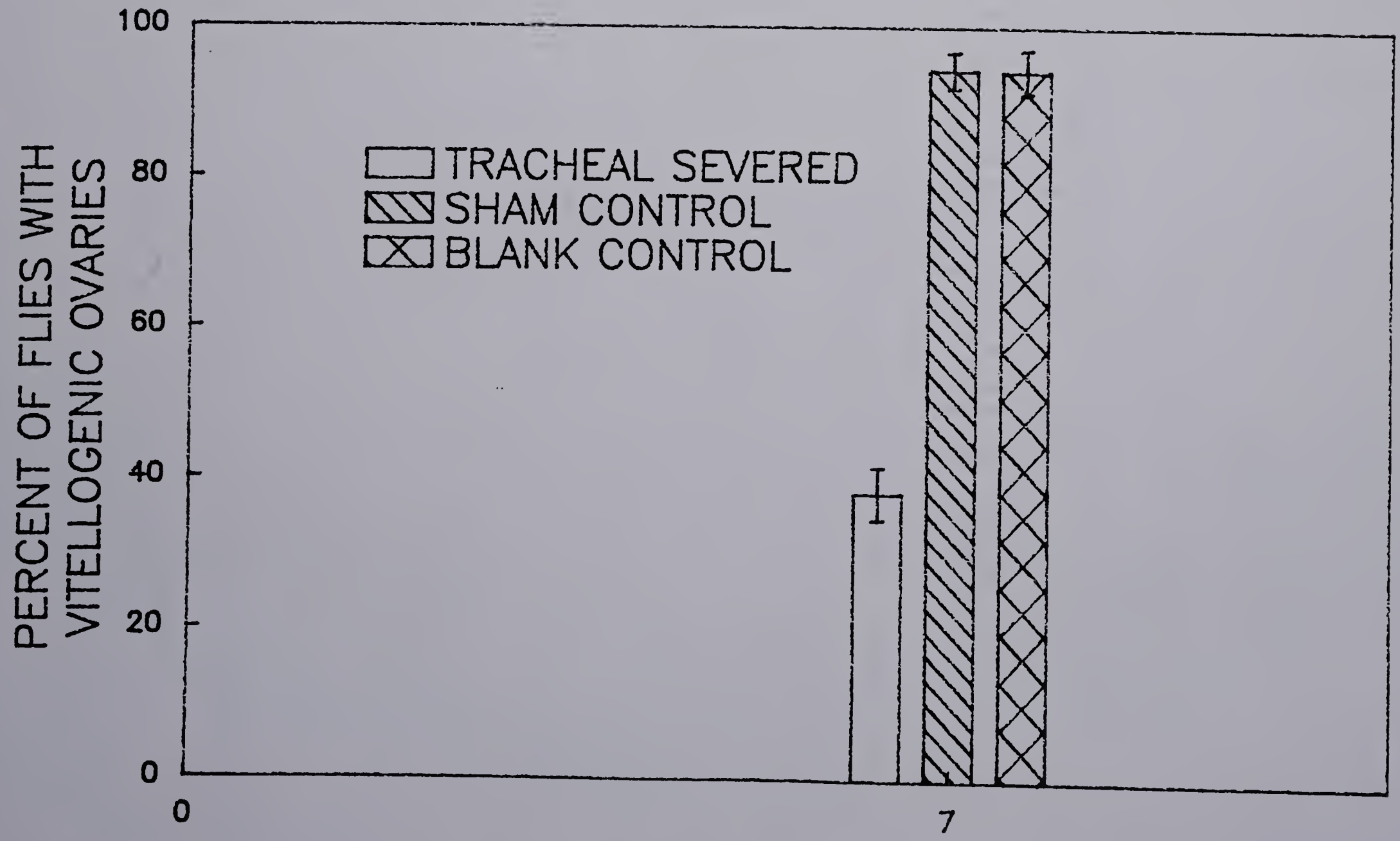
Liver ingested = body weight after liver feeding - body weight before liver feeding

The data show that indeed a reduction in protein ingestion occurred after the transection of the ventral nerve cord. It is conceivable that this reduction in protein ingestion caused the reduction in oogenesis as observed in the previous experiment.

H. Tracheal Severing and Ovarian Development

Previous experiments indicate that ovarian development in liver-fed, Phormia regina would proceed even after the severance of the ventral nerve cord and the cardiaca-recurrent nerve. The profiles of the protein movement from the crop to the midgut of ligated and sham-ligated flies also indicated that ligation and sham treatment did not block the movement of the protein diet. Therefore the results, that "sham-ligation" could prevent ovarian development, can not be explained simply by attributing the effect to the damage of nerve and food utilization caused by the sham-ligation. Since sham-ligation also damaged the tracheal trunks in the neck, the function of trachea on egg development were tested by severing the two cervical tracheal trunks. Fig. 12 shows the percentage of flies with vitellogenic ovaries 48 hr after liver feeding. Ovarian development was arrested in many of the tracheal severed flies. Only 38.1% of the tracheal severed flies contained vitellogenic ovaries while 94.3% of the sham control flies and 94.2% of the blank control flies

Figure 12. Effect of cervical tracheal trunk severing at 7 hr after the onset of liver feeding on the ovarian development. Each datum point represents 3 replicates of 15 to 32 flies. Vertical bar = S. E. M.

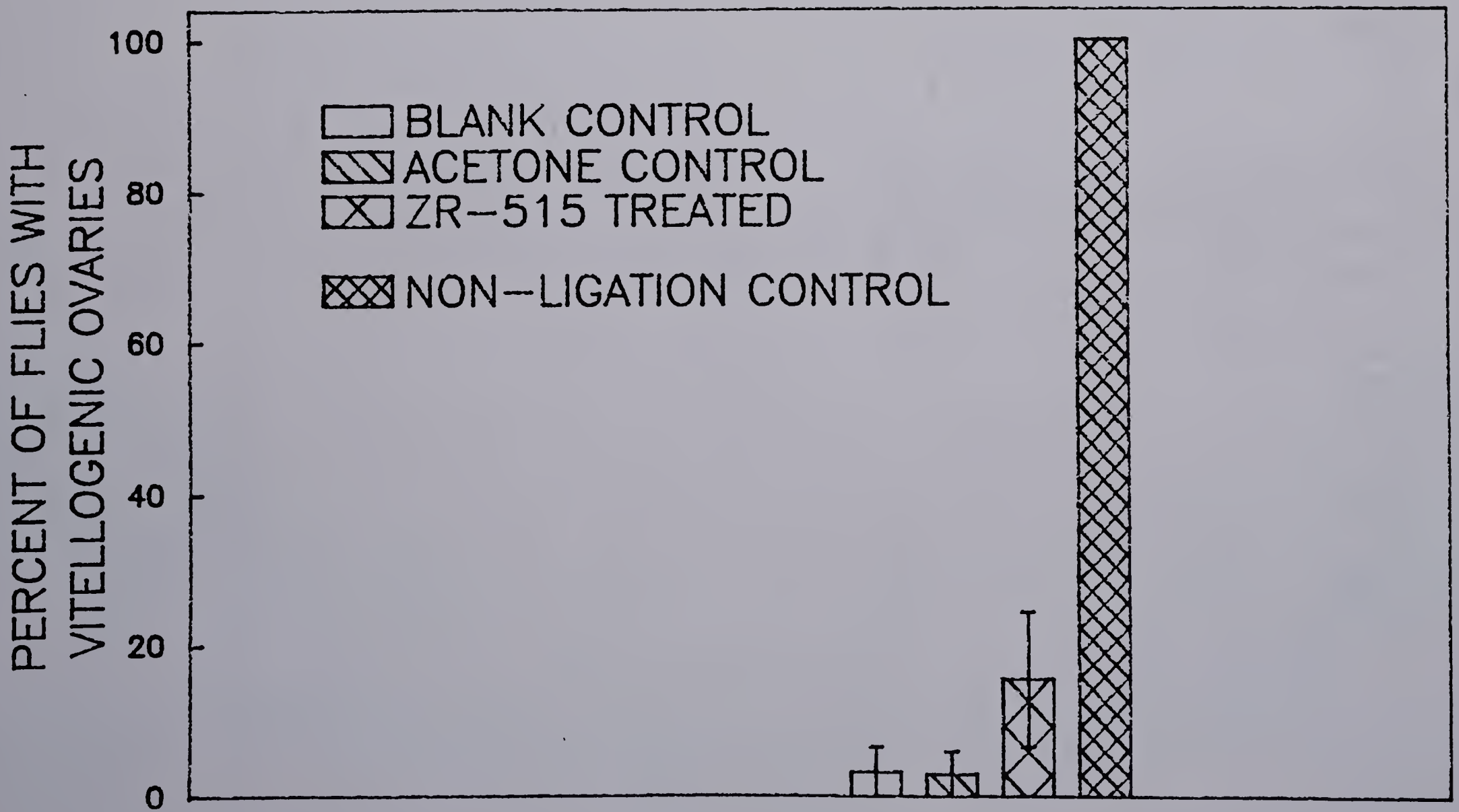

100
80
60
40
20
0
0
7
PERCENT OF FLIES WITH VITELLOGENIC OVARIES
TRACHEAL SEVERED
SHAM CONTROL
BLANK CONTROL
TIME OF OPERATION AFTER THE ONSET OF LIVER FEEDING (HOURS)

contained vitellogenic ovaries. These data clearly indicate that an adequate supply of air (oxygen) to the head is essential for normal ovarian development.

I. Effect of the Juvenile Hormone Analogue Methoprene (ZR-515) on Ligated Flies

Previous studies by Yin et al., (1989a, 1989b) have demonstrated that both ecdysteroids and juvenile hormone are involved in oogenesis. Ecdysteroid function at the level of vitellogenin synthesis while juvenile hormone functions at the level of vitellogenin uptake by the terminal oocyte (Yin et al., 1989a, 1989b). The mechanism of cephalic control of ovarian development could be accomplished through controlling either one of these processes or both. An adequate dose of JH analogue methoprene (10 ug/insect) was topically applied to the liver fed and ligated flies to test this hypothesis. Forty-eight hr after treatment (96 hr after liver-feeding) only 15.5 % of the methoprene treated flies showed vitellogenic ovaries and none of these ovaries reached maturation (stage 10) (Fig. 13). In the acetone control and the blank control (liver-fed, and ligated) flies, 3.3% and 3.0% individuals possessed vitellogenic ovaries. The result that methoprene failed to restore ovarian development in more than 84% of the ligated flies suggests that vitellogenin synthesis might be affected in the

Figure 13. Effect of ZR-515 treatment (10 ug/fly) on ovarian development of liver-fed flies neck-ligated at 7 hr after the onset of liver feeding. Each datum point represents 3 replicates of 18 to 32 flies. Vertical bar = S. E. M.

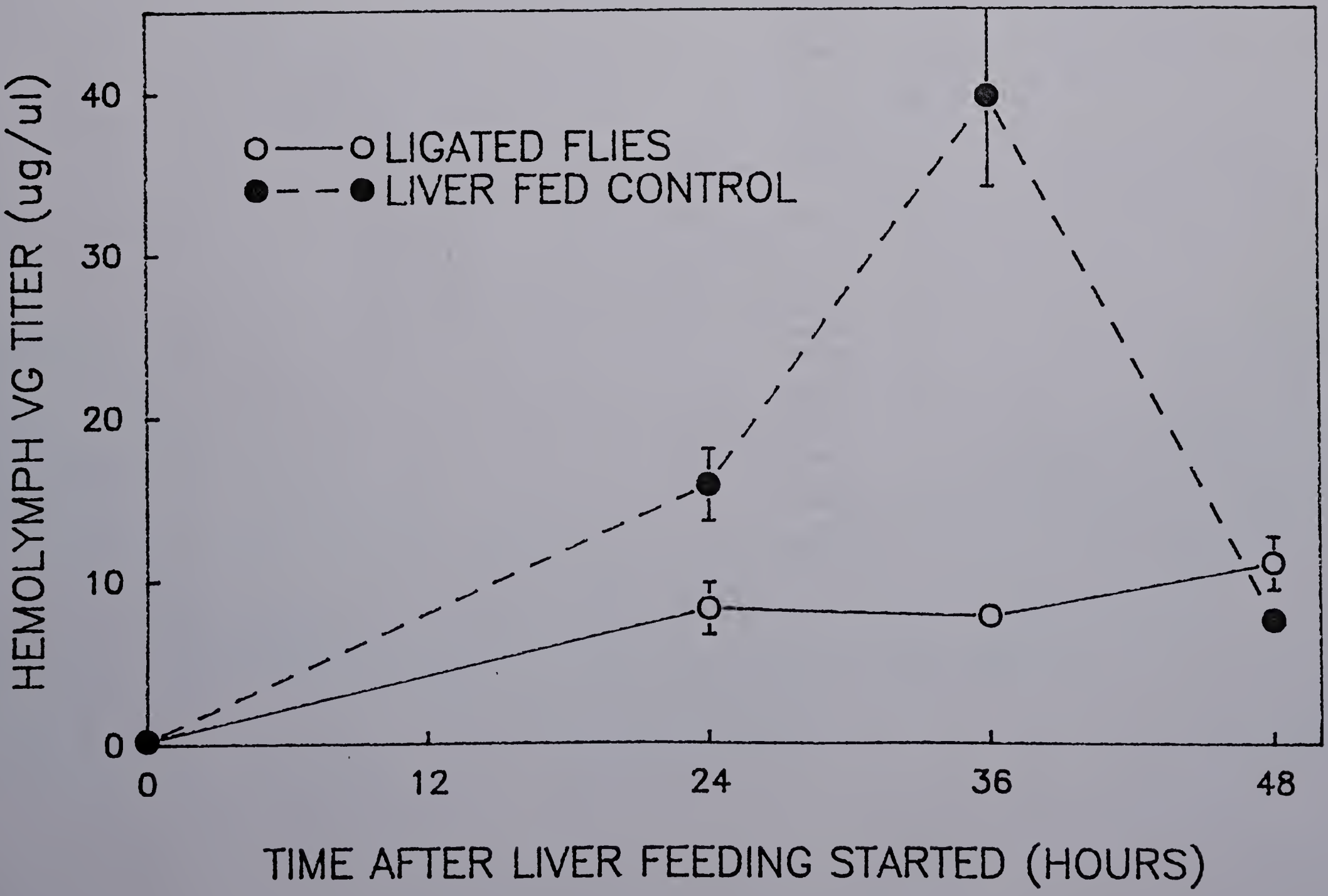

PERCENT OF FLIES WITH VITELLOGENIC OVARIES
100
80
60
40
20
0
BLANK CONTROL
ACETONE CONTROL
ZR-515 TREATED
NON-LIGATION CONTROL

ligated flies. It also suggests that the juvenile hormone analogue alone could not restore maturation of terminal oocytes in ligated flies even when protein resources were available.

J. Vitellogenin Profile in Ligated Flies

In order to determine whether neck ligation affected vitellogenin synthesis, the hemolymph vitellogenin titer of ligated flies was directly measured by using an ELISA specific for vitellin/vitellogenin. The vitellogenin profiles of both ligated, liver-fed and non-ligated, liver-fed flies are shown in Fig. 14. In the neck-ligated flies about 8 ug/ul of vitellogenin was found in the hemolymph 24 hr after the onset of liver feeding. This titer reached the highest point, 11 ug/ul, at 48 hr. Although this is a considerable amount of vitellogenin compared to that of the sugar-fed flies (about 0.25 ug/ul), this titer is much lower than the highest titer of 39.68 ug/ul in the liver-fed control flies. These data indicate that absence of cephalic factors (through ligation) reduced but did not eliminate vitellogenin synthesis. In the three-day old, sugar-fed flies (i.e., 0 hr in Fig. 14) a trace amount of vitellogenin (0.248 ug/ul) was also detected. Since 11 ug/ul of vitellogenin was present in ligated flies, two working hypothesis might be worth testing. One may assume that this lowered vitellogenin titer reflects a

Figure 14. Hemolymph vitellogenin profiles in neck-ligated and non-ligated Phormia regina at various times after the onset of liver feeding. Each datum point represents 3 to 5 replicates. Vertical bar = S. E. M.


LIGATED FLIES
LIVER FED CONTROL
HEMOLYMPH VG TITER (ug/ul)
0
10
20
30
40
0
12
24
36
48
TIME AFTER LIVER FEEDING STARTED (HOURS)

correspondingly lowered ecdysteroid titer. In addition, the lack of oocyte development in neck-ligated flies, which still produced some vitellogenin, might indicate that vitellogenin uptake was impaired. In that case, one might further postulate that juvenile hormone biosynthesis may be hampered in neck-ligated flies. Juvenile hormone biosynthesis and ecdysteroid titer were measured in the following experiments

K. Effect of Ligation on Corpus Allatum Activity

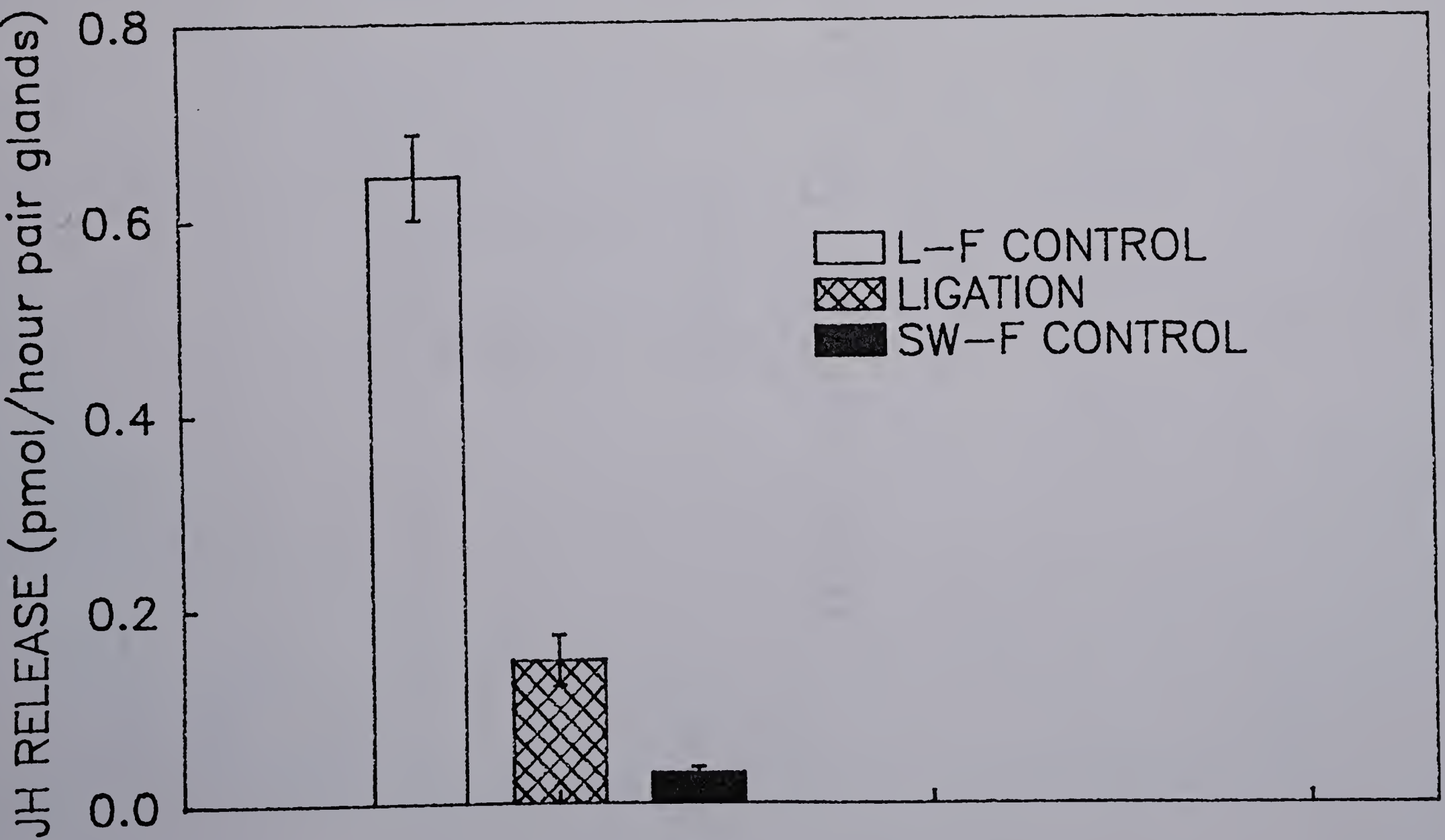
It has been shown previously (Liu et al., 1988; Zou et al., 1989) that a protein meal stimulated both juvenile hormone biosynthesis and release by the corpus allatum of Phormia regina. However, the activating (stimulating) mechanism of juvenile hormone biosynthesis by the dietary factor (liver meal) remains undefined. The possibility of the involvement of some cephalic factors in this process was investigated in this experiment. Three-day old flies were fed on liver for 4 hr and 3 hr later, these flies were divided into two groups. One group was neck-ligated and the other was left as liver-fed control. Another group of three-day old flies was deprived of liver and used as sugar-water fed control. At 48 hr from the onset of liver feeding, the rate of juvenile hormone release by the corpus allatum was estimated by a short-term, in vitro, radiochemical assay (Zou et al., 1989). Fig. 15

illustrates the rate of ^{3}H-methyl incorporation into juvenile hormone III by the corpus allatum from various groups of flies *in vitro*. Juvenile hormone III biosynthesis in ligated, liver-fed flies was 0.1472 ± 0.0255 pmol/pair gland/hr and that of the non-ligated, liver-fed and non-ligated sugar-fed flies were 0.6431 ± 0.0435 and 0.0316 ± 0.0060 pmol/pair gland/hr. The juvenile hormone III biosynthesis of non-ligated, liver-fed flies was significantly higher (single ANOVA, $P \leq 0.05$) than that of the ligated, liver-fed flies and the non-ligated, sugar-fed flies. There was also a significant difference (single ANOVA, $P \leq 0.05$) between the juvenile hormone III biosynthesis of ligated flies and non-ligated, sugar-fed flies.

L. Effect of Ligation on Ecdysterold Biosynthesis

It has been reported that the protein meal somehow stimulates ecdysterold biosynthesis in *Phormia regina*. The ecdysterold profiles of liver-fed flies and sugar-fed flies have been determined (Yin *et al.*, 1989b, 1990). In the present study, the role of a cephalic factor in regulating ecdysterold biosynthesis was investigated. At 32 hr after the onset of liver feeding, the hemolymph ecdysterold titer in the liver-fed, unligated flies reached 43.4 ± 4.75 pg/ul hemolymph. At the same time, results from 6 samples indicated that the ecdysterold titer of the liver-fed,

Figure 15. Effect of neck-ligation on the rate of *in vitro* incorporation of [^{3}H]methionine into JH by the corpus allatum. Each datum point represents 10 to 23 replicates. Vertical bar = S. E. M.


JH RELEASE (pmol/hour pair glands)
0.8
0.6
0.4
0.2
0.0
L–F CONTROL
LIGATION
SW–F CONTROL

ligated flies remained at undetectable level in 67% of the hemolymph samples and at very low but detectable level of about 10 pg/ul hemolymph in the remaining 33% of the samples. This is very similar to the hemolymph ecdysteroid titers found in the sugar-fed control flies. Similarly, the results from 8 samples indicated that in 75% of the sugar-fed flies, the ecdysteroid titer was too low to be measured by the present method and in the remaining 25% of the samples the titer was about 10 pg/ul hemolymph. It is obvious that the ecdysteroid titer was significantly reduced by the ligation treatment. Therefore, in the absence of a cephalic factor, the process of ecdysteroid biosynthesis was not fully activated. This also suggests that another important function of the cephalic factor is to stimulate ecdysteroid biosynthesis.

CHAPTER IV

DISCUSSION

A. Liver (Protein) Feeding and Crop Emptying

Due to the importance of an exogenous source of protein in the reproduction of anautogenous insects, investigating the process of protein ingestion and utilization will provide information for a better understanding of egg development. As indicated by the protein contents of the crop and midgut at various times after the onset of feeding, protein food was ingested into both crop and midgut simultaneously by the flies. This pattern of protein ingestion is similar but not identical to the pattern of sugar ingestion by the same fly reported previously by Dethier and Bodenstein (1958). They find that the sugar solution passes initially into both the midgut and the crop. After the midgut has been filled, the cardiac valve closes and the additional ingested sugar is passed into the crop. Although protein and sugar meals are different in their chemical nature and functions, the separation of their storage site from the digestion-absorption site may increase the efficiency of food utilization. Since the crop can store 4 times more protein (in protein content) than the midgut, it is likely that the function of crop is chiefly storage. However, it has been

reported that sugar digestion occurs in the crop of Calliphora erythrocephala (Bay, 1978a, 1978b). Thus, the crop of Phormia regina may also serve as a site of protein digestion. This latter idea needs to be verified.

Since deprivation of protein increases the protein hunger of the female flies (Dethier, 1961), the presently observed size of the protein meal and its distribution pattern in the crop and midgut might be a result of the protein deprivation designed into my experiments in which flies were kept away from protein for the first three days of their adulthood. Deprivation of protein food in nature might not be as dramatic as what was allowed to happen in the laboratory. Therefore, a slightly smaller size of protein meal and a different food distribution pattern may be expected in wild flies. It is interesting to notice that the distribution pattern of protein food in female Phormia regina is also different from blood feeding dipterans, including mosquitos (Trembley, 1952; Day, 1954). In Tabanus nigrovittatus (Stoffolano, 1983), the majority of the flies divert the blood to their midgut only. This difference between Phormia regina and blood feeding dipterans might be an adaptation to different food resources and textures.

It is very interesting to notice that during the period of protein feeding, the volume of the crop reached 18.7 ul and its protein content reached 2143.3 ug at 60 min after the onset of feeding (see Figs. 4 and 5). From this point

on the increase in crop volume stopped while the protein content continued to increase to 2338.7 ug at 90 min after the onset of liver feeding. This suggested that the flies were able to concentrate their protein meal. It has been observed that most of the flies regurgitated (i. e., the fly spits out a droplet of liquid and keeps the droplet on the tip of the proboscis and re-drinks the liquid a few min later) during the pauses between bouts of liver feeding. It is also common for regurgitation to occur when the flies feed on diluted sugar solution. Thus, regurgitation is likely to be a food concentration process.

B. Crop Emptying in Liver-fed Flies

Crop emptying in liver-fed flies occurred at a decreasing rate during protein digestion. The crop emptied more rapidly when the volume and content of the crop was large (see Figs. 6 and 7). A similar phenomenon has been reported for the crop emptying in sugar-fed flies. Green (1964) has demonstrated in sugar-fed male flies that 90% of the ingested sugar was emptied from the crop during the first 5 'periods' (mean period=3.12 hour) while the remaining 10% of sugar emptied during the rest of the 15 'periods'.

C. Nerve Transection and Liver Feeding

It is well known that transection of the ventral nerve cord or recurrent nerve causes hyperphagia in Phormia regina (Dethier and Gelperin, 1967; Dethier, 1976; Belzer, 1978b, 1978c) when sugar solution or protein solution is provided to the flies. Data presented here indicates that in the case of liver feeding, severance of the ventral nerve cord at the neck region reduced the amount of food ingested instead of introducing hyperphagia. This difference could be explained by the fact that ingestion of semi-solid food (i. e., liver) requires more neural coordination than the ingestion of sugar or protein solution. In fact, after ventral nerve cord sectioning at the cervical level the flies were unable to stand and keep their posture. Special care, as described in the Materials and Methods section, was used to assist flies in liver feeding. It is also observed that salivation would cause liver liquefaction when the proboscis touched the liver during liver feeding. Therefore, it is possible that the ventral nerve cord sectioning may reduce liver feeding by affecting salivary secretion and liver liquefaction.

D. Cephalic Control of Egg Development in Phormia Regina

The results presented in Fig. 3 indicated the presence of a cephalic control factor which regulates the

development of primary oocytes in Phormia regina to the vitellogenic stage. The data also showed that there is a specific period for the release of this cephalic control factor. This is not unexpected, since the brain, specially its neurosecretory cells, have been found to regulate many aspects of insect physiology. Brain neurosecretory cells have also been shown to regulate reproduction in various insect species. In Locusta migratoria, McCaffery (1976) has shown that electrocoagulation of the median neurosecretory cells of the brain prevents vitellogenesis but not pre-vitellogenic oocyte growth. It is suggested that the neurosecretory cells of the pars intercerebralis are needed to produce stimulatory allatotropic factors which activate and maintain the corpora allata. A similar result has been obtained by Goltzene and Porte (1978) in Locusta migratoria migratorioides. In the migratory grasshopper, Melanoplus sanguinipes (Elliott, 1978), cautery of the median neurosecretory cells or allatectomy prevents vitellogenesis. These reports also show that the total protein content, in females after the cauterization of their median neurosecretory cells, is less than that of the allatectomized females. These results suggest that the median neurosecretory cells have not only an allatotropic effect but also a direct effect on protein synthesis in this species (Elliott,1978). In the monarch butterfly, Danaus plexippus, Barker and Herman (1973) demonstrate, by using neck ligation, nerve disconnection, removal of the

neurosecretory and the retrocerebral complex, and injection of a juvenile hormone mimic, that both brain and corpora allata act to regulate ovarian growth. They also suggest that the brain regulates ovarian growth by stimulating postecloslon corpora allata activity. In Labidura riparia, Juberthie and Caussanel (1980) show that a significant release of brain neurosecretory product at the neurohemal part of the aorta coincided with the egg-laying process. In tsetse fly, Glossina austeni, Ejezie and Davey (1974), using a histological study, show that the cyclic changes of net synthesis and net release of stainable materials by the median neurosecretory cells are correlated with ovulation and larviposition. In the fleshfly, Sarcophaga bullata (Wilkens, 1968), removal of the brain neurosecretory cells prevents yolk deposition and reimplantation of neurosecretory cells to the neurosecretory cell deprived females restores vitellogenesis. In the blowfly, Calliphora erythrocephala, Thomsen (1952) demonstrates that median neurosecretory cells are essential for the maturation of the ovary. Further histological study of the median neurosecretory cells also shows a cytological change of these cells correlating with ovarian development (Thomsen, 1965). In Phormia regina, 6 groups of neurosecretory cells (Hsiao and Fraenkel, 1966) and two types of brain median neurosecretory cells have been identified (Dai et al., 1987). A diet-dependent size change of the median neurosecretory cells are observed

(Hsiao and Fraenkel, 1966). Since the size increase of the median neurosecretory cells results from liver feeding and liver feeding eventually leads to ovarian development, the cephalic control of ovarian development in this fly might involve the product(s) from the median neurosecretory cells.

In mosquitos, an egg development neurosecretory hormone produced in the brain median neurosecretory cells has been purified and characterized (Borovsky and Thomas,1985; Masler et al, 1983). According to Hagedorn et al. (1979), egg development neurosecretory hormone is secreted in response to the blood-feeding message, to stimulate the ovary to produce ecdysteroids, which in turn stimulate vitellogenin biosynthesis by the fat body.

Data from the results of neck ligation in this study clearly showed that ovarian development of Phormia regina was under cephalic control (see Fig. 3). Furthermore, the data from CRN and ventral nerve cord transection experiments (see Figs. 10 and 11) demonstrated that this cephalic control was not neural. Since the corpus allatum juvenile hormone biosynthesis and hemolymph ecdysteroid titer in liver-fed, ligated flies were significantly lower than those in the liver-fed control flies, the functions of this cephalic factor(s) were likely to stimulate both juvenile hormone and ecdysteroid biosynthesis. Taken together, these results suggest that an egg development neurosecretory hormone-type and an allatotropin-type of

neurohormone are involved in regulating egg development in Phormia regina.

E. Activation of Cephalic Control System

In anautogenous insects, it is well known that further growth of the terminal oocyte beyond the "resting stage" requires an adequate protein meal, which not only provides the raw materials for vitellogenesis but also activates the endocrine system (Dethier, 1976; Liu, 1985). Although it has been studied by various researchers, the mechanism, through which a protein meal activates the endocrine system, remains undefined. The hypothesis that abdominal stretch activates the brain through the ventral nerve cord was postulated in the anautogenous mosquitos Culex pipiens and Aedes aegypti by Larsen and Bodenstein (1959). However, Bellamy and Bracken (1971) suggest that only the nutrient of the blood is crucial. Spielman and Wong (1974) suggest that both distention of the midgut and the presence of a sufficient concentration of specific chemical moieties are important in stimulating oogenesis. More recently, Klowden (1987) demonstrates that one or more of the substances in the blood meal are necessary for the release of the head factor which stimulates oogenesis. However, in mosquito, abdominal distention also modulates this release through the nervous system (Klowden, 1987). Our results of nerve sectioning in Phormia regina clearly

demonstrated that neither the ventral nerve cord nor the cardiaca-recurrent nerve (CRN) are essential for oogenesis, although the ventral nerve cord may have some indirect function. These results are also consistent with Orr's result that abdominal distention, as introduced by sealing the anus while feeding the flies with 20 ul/fly of 1 M sucrose, can not stimulate egg development in Phormia regina (Orr, 1964a). It appears that there are some conflicts between the present study and the result of Bennettova-Rezabova (1972) concerning the role of the central nervous system in egg development. However, since we now know that the quantity and quality (Stoffolano et al., 1989b) of the protein meal are crucial for egg maturation and that nerve sectioning may interfere with protein feeding (see Table 1), the data and conclusion Bennettova-Rezabova (1972) obtained without monitoring protein ingestion are difficult to interpret at present.

Although it is clear now that a non-neural factor is involved in activating the brain, the biological nature of this factor is unknown. Lea (1982) has suggested that an egg development neurosecretory hormone-releasing factor from the ovary of the mosquitoes stimulates the release of egg development neurosecretory hormone from the corpora cardiaca. Orr (1964a) suggests that some components of the protein food or the digestion products of the protein meal could serve as the messenger to the head in Phormia regina. Whether there is an egg development neurosecretory hormone-

releasing factor or other humoral and/or hormonal factors, which serve as messengers between the digestive system and the brain in Phormia regina, needs further study.

It is also possible that the brain could obtain the liver feeding message through the chemoreceptors of the proboscis during the feeding process in Phormia regina. For this to operate, however, one would have to show some neural connections that could make such a hypothesis possible. If so, the nervous system within the head might be involved in activating the cephalic neurosecretory system.

F. Activation of Corpus Allatum and Ovary by the Cephalic Factors

Since ventral nerve cord and CRN sectioning did not affect egg development once the flies had an adequate protein meal, it is concluded that activation of the corpus allatum and ovary by the cephalic factor(s) is also non-neural.

An previous study by Tobe et al. (1977) shows that neural connection is important for CA activity in some species. Couillaud et al. (1984) also report that severance of nervi corporis allati in one day old female, Locusta migratoria, results in a significant decrease of juvenile hormone biosynthesis by the corpora allata. However, the present results indicate that the effect of

CRN transection on corpus allatum activity in Phormia regina was not significant as far as the hormonal requirements of ovarian development are concerned.

The data of CRN sectioning and the corpus allatum activity in ligated flies also indicated that the corpus allatum was not activated by nerve transection. In this regard, Phormia regina is different from the cockroach Diploptera punctata (Woodhead and Stay, 1989), whose corpora allata are inhibited by neural input in protein deprived adults.

G. Tracheal Section and Egg Maturation

The proper function of the central nerve system, especially the brain, requires an adequate supply of oxygen. Lack of oxygen in the brain causes immediate death of the brain and ultimately the organism in higher animals. Unlike the mammalian system, a different mechanism of gaseous exchange is used by insects and death of the brain dose not necessary mean the death of the insect. This allows the surgical approach of decapitation and ligation in the study of insect physiology. However, the proper function of the insect central nervous system and the brain still depends on a proper oxygen supply. In fact, the insect brain is heavily supplied with a tracheal network, which is connected to the accessory air sacs, within the head capsule. In the brain of Musca domestica, the total

volume of the tracheal system takes up between 4% to 8% of the neuropile (Strausfeld, 1976). Phormia regina is probably similar in this regard.

Since it was showed that ligation had little effect on protein utilization and that nerve sectioning did not affect ovarian development in protein-fed flies, ovarian development suppression caused by tissue damage in liver-fed, sham-ligated flies was likely due to the cervical tracheal trunk malfunction. The result of tracheal sectioning in this study indicated that proper functioning of the brain including the release of the cephalic factors, which most likely are produced by the brain neurosecretory cells, required oxygen. Similarly, in dissected preparations of the cecropia silkmoth, Hyalophora cecropia (Truman, 1978), the central nervous system requires an air supply through the tracheal system in order to produce behavioral responses to the eclosion hormone. In the honeybee, Apis mellifera, protein synthesis by the ovary and the fat body, in vitro, increases 15 and 17-fold when the tracheal system of the organs was left intact and opened to the air during incubation (Kaatz et al., 1985).

H. Effect of Ligation on Vitellogenin Biosynthesis

Hemolymph vitellogenin titers can be measured by using an ELISA. The hemolymph vitellogenin profile of the liver-fed control flies in this study (see Fig. 14) was very

similar to the vitellogenin profile reported by Zou et al. (1988). This confirms that an adequate protein meal stimulates vitellogenin biosynthesis, as indicated by the sharp increase of hemolymph vitellogenin titer after the protein meal. These results also provide evidence that vitellogenin biosynthesis, which is stimulated by a protein meal, was under cephalic control since in the absence of a cephalic control factor(s), the hemolymph vitellogenin titers of liver fed, ligated-flies were reduced significantly. This result also coincides with the idea that egg development neurosecretory hormone, which is produced by the brain, stimulates vitellogenin biosynthesis (Hanaoka and Hagedorn, 1980).

The fact that in flies ligated at 7 hr after the onset of liver feeding there was still about 10 ug/ul of vitellogenin in the hemolymph indicates that at the time of ligation a certain amount of a cephalic factor(s) may have already been released into the hemolymph. Ligation at a time earlier than 7 hr after the onset of protein meal may further lower the vitellogenin titer. It is also very interesting to note that a trace amount (0.2 ug/ul hemolymph) of vitellogenin was detected in the sugar-fed flies with the present ELISA. In contrast, previous researchers (Zou et al.,1988) report that vitellogenin is non-detectable in the sugar-fed female flies using a less sensitive ELISA. The present ELISA uses a rabbit antiserum and has a range of detection of 1 ng/well to 100 ng/well.

The earlier ELISA used by Zou et al. (1988) employed a mouse ascitic fluid and has a range of 0.5 ug/well to 25 ug/well. The present study showed that Phormia regina are capable of producing vitellogenin without a protein meal, although the titer is very low. If this is confirmed by further study, the role of the protein meal in egg development is not to trigger the endocrine cascade which maximizes vitellogenin biosynthesis. The slight difference of hemolymph vitellogenin concentration of liver-fed flies between this study and the previous study of Zou et al. (1988) may also be due to the difference between the methods.

1. Corpus Allatum Activity in Ligated Flies

Since CRN sectioning had no significant effects on oocyte development and since neck ligation reduced but did not stop juvenile hormone biosynthesis by corpus allatum, it is clear that neither neural activation nor neural inhibition regulates corpus allatum activity in adult female Phormia regina. Alternately, an allatotropic hormone from the head of the fly may be involved in the activation of corpus allatum. It may involve a hormone from the neurosecretory cells of the brain. This neurosecretory allatotropic hormone most likely is different from the so called egg development neurosecretory hormone which stimulates the ovary to produce ecdysone.

Recently, allatotropin has been identified for tobacco hornworm, Manduca sexta (Kataoka et al., 1989).

Although an increase of juvenile hormone biosynthesis does not occur until 24 hours after a liver-meal (Liu et al., 1988, Zou et al., 1989), the requirement of activation of the corpus allatum was fulfilled much earlier. The fact that the corpus allatum was partially activated in flies neck-ligated at 7 hours after liver feeding suggested that the release of the allatotropic hormone had already started by that time. Since the corpus allatum of sugar-fed flies showed only very low activity, juvenile hormone regulation of the previtellogenic oocyte growth is most likely different from that postulated in Aedes aegypti (Hagedorn et al., 1977). Because the juvenile hormone titer related to the previtellogenic oocyte development in mosquito is very high in relative terms.

J. Effect of Neck-Ligation on Ecdysterold Biosynthesis

Direct measurement of the hemolymph ecdysterold titer indicated that in the flies neck-ligated at 7 hr after the onset of liver feeding, the hemolymph ecdysterold titer was reduced to a level very close to that of the non-ligated, sugar-fed flies. Since the ecdysterold titer in liver-fed control flies was significantly higher (single ANOVA, $P \leq 0.05$) than that of the liver-fed, ligated flies, it is likely that the biosynthesis of ecdysterolds was impaired

by neck-ligation. Thus, the activation and /or maintenance of ecdysteroid biosynthesis were under cephalic control. The data suggested again that the cephalic factors, which control ovarian development, have not only an allatotropic function but also can stimulate ecdysteroid production by the ovary.

The reduction of ecdysteroid biosynthesis by a neck-ligation is also consistent with the relatively low hemolymph vitellogenin titer found in the neck-ligated flies. Since a low ecdysteroid titer was found to correspond with a low vitellogenin titer, the present result also supports the hypothesis that ecdysteroids control vitellogenin biosynthesis in Phormia regina (Yin, et al., 1989b, 1990)

K. Regulation of Egg Development

Based on this study and some other previous studies, some of the regulating mechanisms of primary oocyte development in Phormia regina are postulated in Fig. 16. An adequate protein meal starts the events of oocyte development (i. e., vitellogenesis) by stimulating the synthesis and release of neurosecretory hormones. This could be accomplished by several possible ways. Firstly, presence of protein food in the midgut stimulates the midgut endocrine cells to release a hormone which circulates in the hemolymph and serves as a messenger to

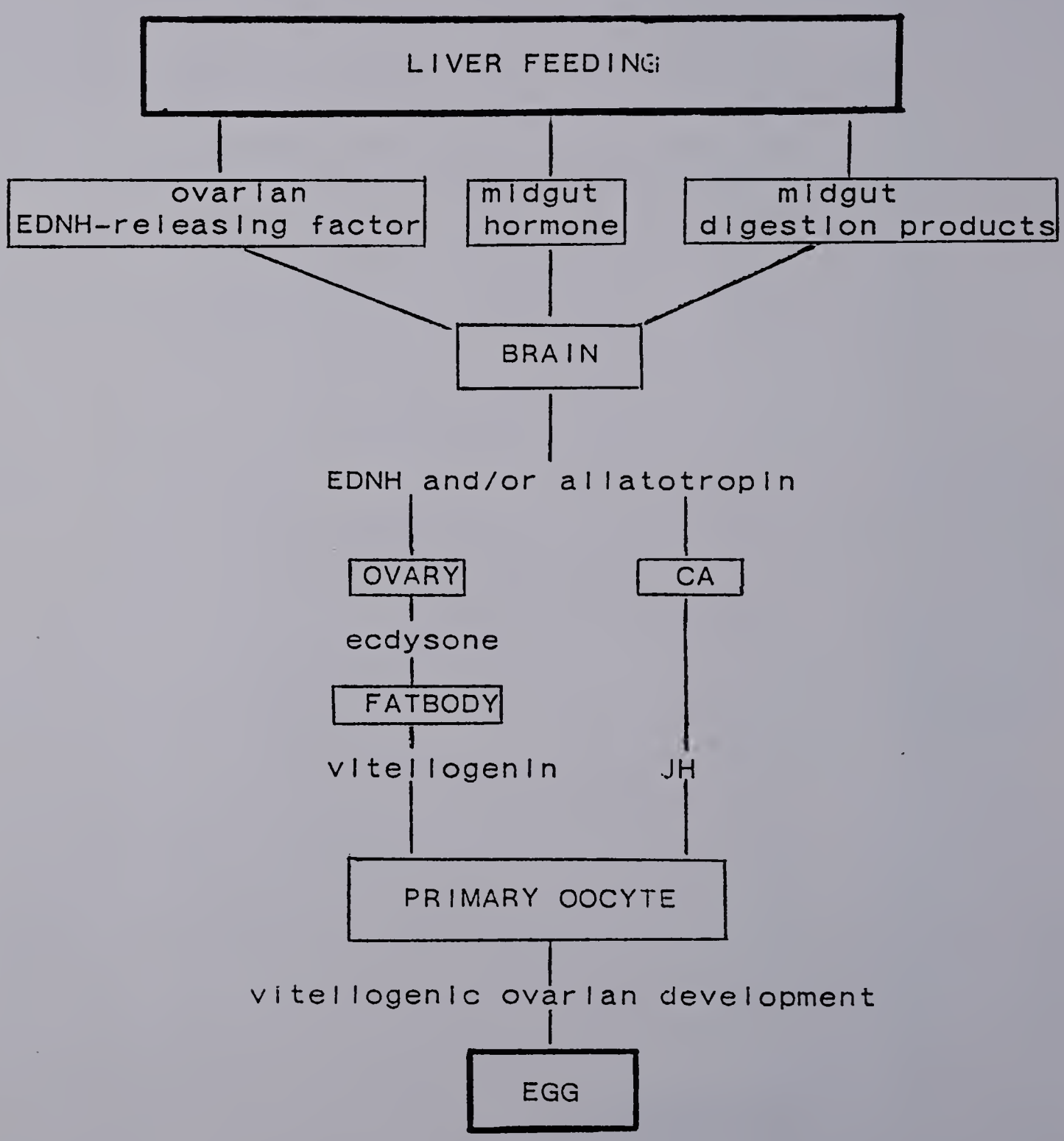
activate the brain neurosecretory cells. This possibility is supported by the evidence that a group of midgut endocrine cells has been found to secrete an unidentified material into the hemolymph after a protein meal (Stoffolano et al., 1989, Yin et al., 1990). Secondly, the digestion product of the protein food could serve as the messenger (Orr, 1964a). Thirdly, ingestion of protein food could somehow stimulate the ovary to produce a so called egg development neurosecretory hormone-releasing factor which circulates in the hemolymph and activates the brain neurosecretory cells (Lea, 1982). The activated brain neurosecretory cells then produce a gonadotropic hormone (e. g., egg development neurosecretory hormone) which stimulates the ovary to produce ecdysteroids and an allatotropic hormone which stimulates the corpus allatum to produce juvenile hormone. The whole process of brain activation and brain neurosecretory hormone(s) production can be completed within the period of 12 hr after the onset of liver feeding. Ecdysterolds then stimulate the fat body to synthesize vitellogenin. The highest ecdysone titer appeared at about 32 hr after the onset of the liver meal (Yin et al., 1989b; 1990). After the appearance of a high titer of vitellogenin in the hemolymph, an increase of juvenile hormone titer follows (Zou et al., 1989). The vitellogenin titer and the juvenile hormone titer reach their maximum at about 32 and 48 hr after the onset of liver feeding respectively. Responding to the increasing

juvenile hormone titer, the primary oocytes begin uptake of vitellogenin (Yin et al., 1989a, 1989b, 1990). By the time of 48 hr after the onset of liver feeding, the primary oocyte is fully mature.

As one can see in Fig. 16, the control of egg development is a very complex process and requires the coordination amongst numerous regulatory factors. Many of these regulatory factors in Phormia regina, including the cephalic factor for stimulating the ovary to produce and release ecdysone, the allatotropin and the "midgut messenger" need to be characterized. Other aspects of the egg development, including the physiological bases of "protein hunger" and the involvement of the so called oostatic hormone (Adams et al., 1968; Fraenkel and Hollowell, 1979; Borovsky, et al., 1985) in Phormia regina also need further study.

Figure 16. Control of egg maturation in Phormia regina.

CONTROL OF EGG MATURATION IN PHORMIA REGINA



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