

## Endocrine Regulation of Reproduction and Diapause in the Boll Weevil, *Anthonomus grandis* Boheman

Tina E. Taub-Montemayor,<sup>1\*</sup> James O. Palmer,<sup>2</sup> and Mary Ann Rankin<sup>1</sup>

<sup>1</sup>Department of Zoology, University of Texas, Austin, Texas

<sup>2</sup>Department of Biology, Allegheny College, Meadville, Pennsylvania

A review of the literature for the hormonal control of reproduction and diapause in Coleoptera is presented. The role of juvenile hormone and juvenile hormone esterase (JHE) in the control of the different life history strategies of the boll weevil are examined. Elevated levels of hemolymph JHE were found to be positively correlated with survival throughout the winter in South Texas population of weevils. Winter weevils were examined for hemolymph vitellogenin (Vg) and their subsequent survival was monitored. The majority of weevils surviving beyond ten weeks had no hemolymph Vg. We conclude that elevated hemolymph JHE and the absence of Vg are good predictors of survivors in the South Texas population. The hormonal basis of diapause termination was examined using hormone treatment and implant therapy. We were unsuccessful in our attempt to induce post-diapause reproductive development with all of our treatments. Only weevils given access to a food source were capable of reproductive development. Our recent experimental findings in the control of Vg synthesis and uptake in the boll weevil are reviewed. Arch. Insect Biochem. Physiol. 35:455–477, 1997. © 1997 Wiley-Liss, Inc.

**Key words:** Coleoptera; vitellogenin; juvenile hormone; juvenile hormone esterase; 20-hydroxyecdysone

**Acknowledgments:** We thank Greta Schuster for assistance in collecting and rearing weevils. Pete Krauter and K. Rod Summy provided many helpful suggestions throughout this study. A. Cavazos, J.M. Caballero, and K.R. Summy assisted our weevil collections in Weslaco, TX. K.H. Dahm, G. Bhaskaran, and K. Peck provided helpful guidance during preliminary studies.

Contract grant sponsor: USDA-CSRS, contract grant number 87-CRCR-2-301, contract grant sponsor: USDA Specific Agreement, contract grant number 58-6204-3-024; contract grant sponsor: Advanced Research Program from the Texas Coordinating Board of Higher Education, contract grant number #003658-424; contract grant sponsors: Natural Fibers and Food Protein Commission of Texas and Texas Agricultural Experiment Station.

\*Correspondence to: Tina E. Taub-Montemayor, Department of Zoology, University of Texas, Austin, TX 78712-1064. E-mail: ttaub@mail.utexas.edu

Received 15 August 1996; Accepted 14 February 1997.

## INTRODUCTION

This paper describes the hormonal control of reproduction and diapause in the boll weevil, and reviews the relevant literature for beetles. The Coleoptera are a large, diverse, and economically important insect group, yet there has been surprisingly little work investigating the endocrinology of reproduction and diapause\* in this order, with the exception of studies on the Colorado potato beetle, *Leptinotarsa decemlineata*. Even for *L. decemlineata* the endocrine control of reproduction is not entirely clear, and some coleopterans appear to differ in major ways from the *Leptinotarsa* model.

In the Colorado potato beetle, long days induce reproduction, short days induce onset of diapause 10–12 days after adult emergence (de Wilde et al., 1959). Adults feed actively during prediapause; however, no reproduction occurs and the onset of diapause is characterized by burrowing behavior. Extensive studies have been conducted to examine neuroendocrine regulatory factors involved in diapause control. Early work suggested that the absence of juvenile hormone (JH\*\*) was responsible for the diapause syndrome, in that when long-day beetles were allatectomized, their reproductive activity was arrested and they subsequently entered diapause (de Wilde and de Boer, 1961). Workers have consistently observed that allatectomy results in the appearance of diapause proteins in the hemolymph (Dortland, 1979).

JH titer determinations by various methods in several studies (de Kort et al., 1982; de Wilde et al., 1968; de Kort et al., 1985) revealed elevated levels of JH III in the hemolymph of long-day beetles and very low levels during prediapause in short-day animals. Both indirect criteria based on corpora allata (CA) volumes and direct measurement of CA activity *in vitro* confirmed these titer determinations (Schooneveld et al., 1977; Kramer, 1978; Khan et al., 1982a). Kramer and de Kort (1976a) and Kramer et al. (1977) suggested that JH esterases (JHE) play a major role in the breakdown of JH and the regulation of JH titers. JH-specific carrier proteins were identified; however, their role in the protection of JH and in the regulation of JH titer was considered minimal (Kramer and de Kort, 1978). CA activity seemed to be the most important regulator of JH titer, based on age-dependent changes in CA activity *in vitro* in beetles reared under short-day and long-day conditions (Kramer, 1978). Studies of CA regulatory mechanisms were pursued with the development of an improved assay for the measurement of CA activity *in vitro*, al-

\*In general, diapause is defined as a neurohormonally mediated state of physiological and behavioral changes that adapt insects for adverse conditions. Diapause is characterized by the cessation of reproductive development, reduced metabolic activity, movement to an appropriate hibernaculum, and enhanced fat body content in response to seasonal changes. Once the diapause program is initiated, metabolic activity is suppressed even if conditions conducive to development prevail, thus ensuring that the insect life cycle is kept in phase with changing seasons.

\*\*Abbreviations used: ANCOVA = analysis of covariance; ANOVA = analysis of variance; CA = corpora allata; CC = corpora cardiaca; DFP = *O,O*-diisopropyl phosphorofluoridate; EPPAT = *O*-ethyl-*S*-phenyl phosphoramidothiolate; Etoh = ethanol; 20-HE = 20-hydroxyecdysone; JH = juvenile hormone; JHA = juvenile hormone analogue; JHE = juvenile hormone esterase; JHM = juvenile hormone methoprene; LDHT = long-day, high-temperature; PBS = phosphate buffered saline; SDLT = short-day, low-temperature; TBS = tris-buffered saline; TTBS = tris-buffered saline with tween-20; OTFP = 3-octylthio-1,1,1-trifluoropropan-2-one; Vg = vitellogenin.

lowing determination of activity using individual gland pairs (Khan et al., 1982a). The significance of brain control of the CA via neural connections during starvation, short-day photoperiod, and following experimental elevation of JH titers was examined (Khan et al., 1982a, 1982b, 1983). Restraintment of gland activity due to starvation or JH III treatment occurred mainly via nervous pathways, while denervation studies suggest that during phases of elevated JH titer both neural and humoral factors regulate CA activity.

The role of JH in the control of vitellogenin synthesis is unclear. Dortland (1979) studied the effects of various endocrine manipulations on incorporation of  $^{14}\text{C}$ -lysine into vitellogenin (Vg) and diapause proteins under both long- and short-day conditions. He showed that removal of the corpora allata-corpora cardiaca (CA-CC) complex at ecdysis or later did not inhibit Vg production or oogenesis. However, topical application of JH did stimulate egg production in short-day allatocardiacectomized animals. Injections of 20-hydroxyecdysone (20-HE) had no effect on Vg synthesis in short-day animals. The implication of Dortland's results is that JH is not necessary for Vg synthesis or oviposition. This study seems to contradict that of de Loof and de Wilde (1970), who reported that both a neurosecretory hormone from the brain and JH are necessary for Vg synthesis. More recently, Koopmanschap et al. (1992) report that treatment with pyriproxyfen, a JH analog, prematurely induced the appearance of Vg in last instar larval hemolymph.

Investigating the endocrinology of diapause termination, Lefevre et al. (1989) measured CA activity, JH III titer, and JH metabolism to examine the effects of external factors (i.e., temperature, photoperiod, and food supply) during and after diapause in the female beetle. JH titer was found to remain at a constant low level throughout diapause, but afterwards the JH titer increased progressively. The titer determinations were well correlated with CA activity rates, and full reactivation of the CA occurred only after emergence, suggesting that CA activity is a result of the resumption of activity of the beetle rather than its cause, although both may be necessary for complete or sustained emergence activity. JHE activity in the hemolymph was determined to be low throughout and after diapause, regardless of temperature and photoperiod. These authors conclude that after diapause, JH titer is mainly affected by CA activity and that JHEs in the hemolymph play only a minor role, confirming the findings of Kramer and de Kort (1976a) and Kramer et al. (1977). Furthermore, Kramer and de Kort (1976a) found high levels of JHEs in adults prior to diapause and suggest the involvement of JHE in the reduction of the JH titer necessary for diapause induction. Endocrine control of adult diapause termination was further explored by Lefevre (1989). Single doses of JH III and 20-hydroxyecdysone (20-HE) were injected either alone or in combination into diapausing females at various times post-diapause initiation. 20-HE alone failed to stimulate diapause termination, whereas JH III alone resulted in a temporary termination. Only the combined injections of 20-HE and JH resulted in permanent emergence from diapause. 20-HE appears to switch off the diapause program in the brain, and JH to stimulate reproduction. Since Lefevre did not use allatocardiacectomized animals, his experiments do not exclude the involvement of a brain factor in post-diapause Vg synthesis or ovarian development.

The effect of JH or JH analogue (JHA) application on reproductive development has been explored in a few other Coleoptera. JHA treatment induced synthesis of Vg in the fat body and ovary of the lady beetle *Coccinella septempunctata* (Zhai et al., 1984; Zhai and Zhang, 1984; Zhang and Zhai, 1985; Guan and Chen, 1986) and induced Vg synthesis followed by oviposition in estivating females (Sakurai et al., 1987; Okuda and Chinzei, 1988). Vitellogenesis was also promoted by JHA treatment in two other lady beetles, *Propylea japonica* and *Harmonia axyridis* (Shen et al., 1992). Similarly, in the dermestid beetle *Trogoderma granarium* females, JHA treatment resulted in egg-laying within 12–24 hours (Chellayan and Karnavar, 1989). In some cases, JH treatment alone is insufficient to induce complete ovarian development. For example, Kono and Ozeki (1987) found that treatment with JH I / JHA (ZR-512) induced ovarian development in the twenty-eight-spotted ladybird beetle *Henosepilachna vigintioctopunctata* under diapause-inducing conditions, but the beetles failed to complete the last stage of yolk deposition and chorion formation. Treatment with pyrethroids stimulated ovarian development and partly induced oocyte maturation and oviposition. These authors suggest that two endocrine factors, JH and a neurosecretory factor, are involved in control of ovarian development and that pyrethroid application stimulates both factors.

JH has also been suggested to inhibit vitellogenesis in some Coleoptera. In the pupae and adults of the sweet potato weevil *Cylas formicarius*, application of JHA (hydroprene and methoprene) inhibited vitellogenesis (Ram et al., 1987a, 1987b, 1988); in the pulse beetle, *Callosobruchus maculatus*, JHA (1-4 methylphenyl-3,7-dichloro-3,7-dimethyl octane) caused necrosis in the ovary, inhibition of yolk formation, and prevention of egg maturation (Sareen et al., 1992). However, these results have not been confirmed with natural hormone treatment or gland removal and replacement therapy.

In the beetle *Pterostichus nigrata*, Ferenz (1981) examined the effect of photoperiod on CA activity in vitro and showed that short-day conditions were correlated with low levels of CA activity; long days, after short-day treatment, with high activity, while long days alone had no effect. A correlation between CA activity and oocyte growth was also observed for *Coccinella septempunctata* adults (Guan and Chen, 1986). However, in *T. molitor*, Weaver et al. (1978) found no obvious relationship between JH production and a particular stage in oocyte development. These authors did observe an increase in JH synthesis just before vitellogenesis. Indirect methods, such as the *Galleria* wax test and observations of volume or morphological changes of the CA, have been used in the lady beetle *Coccinella septempunctata* (Fu and Chen, 1984), scarab beetle *Canthon cyanellus* (Martinez and Caussanel, 1984), and the sweet potato weevil *Cylas formicarius* (Ram et al., 1986), which indicate a positive relationship between ovarian development and an increase in JH production or CA volume for these coleopterans. Thus, among the Coleoptera there seems to be some ambiguity as to the actual roles of JH and the brain in vitellogenesis and there is little information in species other than *L. decemlineata* regarding the endocrine control of diapause.

The boll weevil *Anthonomus grandis* is an insect of tremendous economic importance in this country because of its pest status and because of the heroic efforts that have been mounted to achieve its eradication (USDA/APHIS,

1987). Yet very little is known about its reproductive physiology. There is almost no published information regarding hormonal control of Vg synthesis, oogenesis, or adult diapause in this species. We have examined the hormonal control of reproduction in *A. grandis* in two papers recently submitted for publication elsewhere and in Taub (1994). Boll weevils that had been reared in short-day, low-temperature (SDLT) conditions were decapitated behind the pronotum the day after adult emergence, thereby removing the anterior endocrine glands. Various replacement treatments were given, including implants of active CA from reproductive females, reproductive brain, reproductive brain plus retrocerebral complex, application of methoprene in acetone or wax, or sham treatment/implants. The presence or absence of Vg in the hemolymph was determined ten days after treatment. Vg was never detected in untreated or sham-treated decapitated animals nor in animals receiving reproductive brain alone. Methoprene treatment or CA implants with or without brain stimulated appearance of Vg in the hemolymph, i.e., any treatment and only treatments that included a JH source or a JHA treatment stimulated Vg production, indicating that JH is necessary and sufficient to induce Vg synthesis in this species.

These results are supported by studies of the temporal relationship between production of JH by CA *in vitro* and Vg production or oviposition in the same two populations of weevils. CA activity was determined using a modification of the short-term radiochemical assay developed by Pratt and Tobe (1974). The major JH synthesized by *A. grandis* was identified as JH III by high-performance liquid chromatography in collaboration with Drs. Karl Dahm and Govindan Bhaskaran. The profiles of CA activity for females reared in long-day, high-temperature (LDHT) conditions supported our earlier conclusion that JH is necessary for Vg production. CA activity increases as Vg appears in the hemolymph of females reared in conditions inducing reproduction. Fifty percent of the LDHT females in this experiment had detectable Vg in their hemolymph beginning on day 3, and 100% tested positive by day 5 (Fig. 1); 16.7% of these females began ovipositing on day 4, and 100% had done so by day 7. The early peak of CA activity was also present in females reared in SDLT conditions, but Vg was not detectable in the hemolymph of SDLT females until day 6 (33%), rising to a peak of only 41.6% by day 9. Females from this group did not oviposit at all, despite an early peak in CA activity on day 4 and a second peak on day 7. The fact that none of these females oviposited, even though some of them made Vg, suggests that a second factor, lacking in animals reared under SDLT conditions, may be necessary for full ovarian development. Figure 1, based on Taub (1994), illustrates these relationships.

In a second decapitation/replacement experiment, the hormonal control of Vg uptake into oocytes was examined. Ovaries were dissected from females ten days following decapitation and treatment with JHM, brain plus retrocerebral complex, or sham implant. Only females receiving implants of brain plus retrocerebral complex showed uptake of Vg into the terminal oocyte. We conclude that although JH can stimulate the appearance of Vg in the hemolymph of pre-reproductive animals, JH alone is not sufficient to induce Vg uptake into the terminal oocyte; a brain factor is also required. Taub

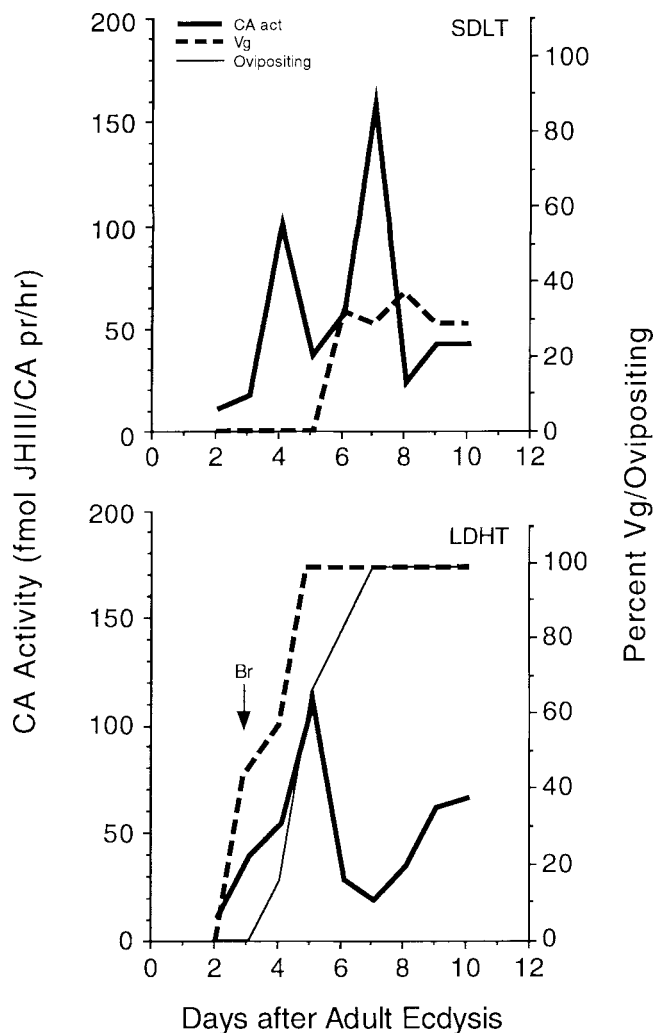


Fig. 1. Model of CA activity, Vg production, and oviposition in females reared in either LDHT or SDLT conditions. Data for this graph are adapted from Taub (1994). Dotted lines represent the percent of females at each age that show detectable levels of hemolymph Vg ( $n = 24$  for each age). Heavy solid lines represent CA activity ( $n = 12$  for each assay, with four replications). Light solid line represents percent of females ovipositing in LDHT conditions ( $n = 24$  for each age). No females oviposited in SDLT conditions. Br indicates proposed timing of brain factor involved in Vg uptake into the oocytes.

(1994) has also shown that CA taken from field-collected females that had been reared in diapause-inducing conditions showed little or no JH synthesis *in vitro*.

Building on this foundation, the present paper examines some of the endocrine correlates of adult reproductive diapause and overwinter survival in this species.

Some, but not all, strains of *A. grandis* enter adult diapause in response to short photoperiods experienced by larvae (Earle and Newsome, 1964). Feed-

ing on bolls usually increases the percent diapausing (Earle and Newsome, 1964; Brazzel and Hightower, 1960), but specific details of this effect are controversial. Phillips and Carter (1978) reported that abscissic acid, produced by maturing bolls, acts as a *deterrent* to diapause. Dietary studies (Earle and Newsome, 1964; Earle et al., 1966; Tingle et al., 1971; Hilliard, 1983) under laboratory conditions show that diet plays a role in the reproductive status of the female boll weevil, but it has not been possible to induce true diapause by manipulating nutrients in artificial diet. Temperature, as suggested by Lloyd et al. (1967) and demonstrated by Hilliard (1983), also affects reproductive status; however, it is not clear that this effect extends beyond simple reaction rate ( $Q_{10}$ ) effects. The diapausing boll weevil is difficult to identify, in that it does not display obvious behavior such as the burrowing behavior of the Colorado potato beetle. However, there is general agreement that diapausing boll weevils are characterized by atrophied gonads, arrested gametogenesis, and increased fat body content (Brazzel and Newsome, 1959).

In some parts of the species-range it may be important to distinguish between a diapause syndrome and the ability to survive the winter. There are numerous reports that only about 10% of the fall adults survive the winter (Hinds and Yothers, 1909; Price et al., 1985) and that those adults that emerge late in the fall are the most likely to survive (Hinds and Yothers, 1909; Wade and Rummel, 1978; Rummel and Carroll, 1983). Palmer and Cate (1992) monitored the effect of prior oviposition on winter survival and found that female boll weevils that had oviposited were much less likely to survive the winter than pre-reproductive animals, indicating that overwinter survival may be accomplished only by pre-reproductive animals, but the diapause syndrome can certainly be displayed by post-reproductive weevils.

## MATERIALS AND METHODS

### Insects and Rearing

Adult weevils for the JHE/behavior experiments were collected from infested bolls in central Texas (Brazos Valley, Burleson Co.) in mid-November, 1988. Bolls were kept in 0.33 m<sup>3</sup> emergence cages in an ambient environment in College Station, Texas. As adults emerged, they were collected and placed in fresh cages at a density of approximately 200 adults per cage with fresh, field-collected bolls (cotton fruit) for food and water wicks (cotton wicks in plastic cups). Food was replaced every few days and adults were allowed to feed for a total of 20 days. On day 21, adults were removed from food and placed in overwinter cages (200 unsexed adults/cage) filled half-full with wooden excelsior and a water wick. Samples of overwintering adults were removed from cages and bled on March 23, April 21, and May 26. Behavior was described as either active or dormant. Active weevils are defined as those actively crawling on top of excelsior or on the walls or lid of the cage, and dormant weevils were those weevils that were still inactive and buried in the center or bottom of excelsior.

Adult weevils for the JHE/survival experiments were collected from untreated fields from Brazos Valley on August 27 to 31 (n = 68), September 15 (n = 40), and October 25, 1989 (n = 40). On each collection date, individual

hemolymph samples were collected and immediately frozen. Weevils were individually marked and placed 10 per 250-ml clear plastic cup. Each cup contained a ball of poplar excelsior and a water vial, but no food. Cups were maintained in an environmental chamber at 20°C and a light regimen of 11L:13D. Cups were checked weekly thereafter and mortality recorded.

Weevils for the Vg/survival experiment were collected from infested cotton bolls during the last week in August, 1989, from untreated fields in Hunt Co., Burleson Co., and Hildago Co., Texas. In each collection, bolls were very green and punctures (oviposition plugs) were recent. Bolls were cleaned of any adult weevils, placed in separate, screened emergence cages, and kept under ambient field conditions at College Station, Texas. Emergence cages were sprinkled with water on a daily basis to prevent the bolls from drying out. Peak adult emergence occurred in the third and fourth week of September, 1989. On day 45 after adult emergence, weevils were removed from the food regimen and individual hemolymph samples were taken. Cages were opened once per week and inspected for dead weevils. The mortality of individually marked weevils was recorded for each of the three populations throughout the overwintering period until the experiment was terminated on April 30, 1990.

Winter field-collected females for the diapause maintenance and termination experiments were collected from USDA facility crops as well as commercial crops located in Weslaco, Texas and Lima, Mexico. Bolls and squares (cotton flowerbuds) were removed from the plant; pupae were harvested immediately and placed in petri dishes until emergence, while larvae were left in the plant material to feed until pupation. Plant materials containing larvae were surveyed daily for pupae, which were then removed from the plant material and placed in petri dishes. Dishes with pupae were checked daily for newly emerged adults, and adults were then placed into cohorts according to date of emergence (date of emergence = day 1). Each cohort was provided with water and fresh cotton bolls. Cohorts were maintained in environmental chambers under SDLT conditions at 12L:12D, [26°C L; 21°C D]. The diet source of fresh bolls was removed 20 days after ecdysis, following a modification of the starvation method of Mitchell and Taft (1966). Adults were segregated by sex for sample collection according to Burke (1959). Ovarian development was assayed following the physiological age-grading method of Grodowitz and Brewer (1987). This system divides oocyte maturation into three nulliparous stages (N1–N3) and four parous stages (P1–P4). Nulliparous stages are distinguished by the degree of follicle differentiation and vitellogenesis, while parous stages are separated by the quantity and appearance of follicular relic deposition in the ovariole base and the occurrence of degenerating follicular and germarial tissues.

#### Juvenile Hormone Esterase Assay

To determine whether we were working with a JH-specific esterase or a general esterase, we did a competition study of  $\alpha$ -naphthyl acetate with labeled JH. The concentration of JH substrate used was  $X = 1.32(10^{-3}) \mu\text{g JH}/\mu\text{l}$ . Three concentrations of  $\alpha$ -naphthyl acetate were examined (2X, 4X, and 10X) at a series of incubation times (5, 10, 30, 60, and 120 min). In collaboration with Drs. K. Dahm, M. Wozniak, and G. Bhaskaran at Texas A&M, we also

did a series of inhibitor studies following the methods of Sparks and Rose (1983) with 5th instar *Manduca sexta* hemolymph as a positive control. Inhibitors used were: DFP (*O,O*-diisopropyl phosphorofluoridate), EPPAT (*O*-ethyl-*S*-phenyl phosphoramidothiolate), OTFP (3-octylthio-1,1,1-trifluoropropan-2-one), and OTFP-sulfone. Hemolymph JHE activity was determined using the radiochemical partition assay of Hammock and Sparks (1977). Hemolymph samples were thawed and diluted 1:1000 in 0.1 M phosphate buffer (pH 7.4). One  $\mu\text{l}$  of substrate (final substrate concentration of  $5(10^{-6})$  tritiated JH III) was added to 100  $\mu\text{l}$  of dilute hemolymph in a 10 by 75 mm disposable culture tube and incubated at 25°C for 30 min. Following the incubation period reactions were terminated by adding 50  $\mu\text{l}$  MeOH/H<sub>2</sub>O/NH<sub>3</sub> (10:9:1). Undegraded juvenile hormone was extracted with 250  $\mu\text{l}$  of isooctane. For each sample, 100  $\mu\text{l}$  of the aqueous phase was counted in 3a70 liquid scintillation fluid (Research Products International). Calculation of JHE activity was performed according to Hammock and Roe (1985).

#### Vitellogenin Assay

Vitellogenin in individual hemolymph samples was assayed using immunoblotting with polyclonal antibodies that had been raised in rabbits injected with gel-purified boll weevil egg vitellin (Taub, 1994). Hemolymph samples were diluted 1/1000 in 0.1 M phosphate buffer (pH 7.4). Fifty  $\mu\text{l}$  of the dilute sample was applied to nitrocellulose in 350  $\mu\text{l}$  of tris-buffered saline (TBS: 20 mM Tris, 0.5M NaCl, pH 7.5). The nitrocellulose sheet was blocked for 30 min in 0.5% normal goat serum in TBS plus 0.05% tween-20 (TTBS), then washed in TTBS followed by TBS. The nitrocellulose was then transferred to a first antibody solution of the polyclonal anti-vitellin antibodies, incubated for 1 h with gentle shaking, and washed twice in TTBS followed by TBS. The nitrocellulose sheet was next incubated for 1 h in a second antibody solution of goat-anti-rabbit with a horseradish peroxidase conjugate, then washed in TBS. Finally, the nitrocellulose was transferred to a color development solution (30 mg of 4-chloro-1-naphthol in 10 ml ice-cold methanol, and 30  $\mu\text{l}$  ice-cold 30% hydrogen peroxide in 50 ml TBS) at room temperature for 10 min. Color development was stopped by washing the sheet in double-distilled water for 10 min. Individual hemolymph samples were scored as positive for Vg if any color developed and negative for Vg if no color developed. Sensitivity of the assay was at 22.5 ng purified protein.

#### CA Activity Assay

The rates of JH III release by CA were determined using the short-term *in vitro* radiochemical assay as developed by Pratt and Tobe (1974). Brains plus CA-CC from 12 individuals were incubated in 90 $\mu\text{l}$  Medium 199 (GIBCO, with Hank's salts, Hepes 25 mM) and 10 $\mu\text{l}$  L-[methyl-<sup>3</sup>H]methionine (70–85 Ci/nmol, New England Nuclear) with Ficoll (20 mg/ml) for 3 h at 30°C in the dark for each tissue culture dish. Following incubation, the reaction was terminated with 50 $\mu\text{l}$  ethanol with 15  $\mu\text{g}$  unlabeled JH III (Sigma Chemical), then JH was extracted from the medium using ethyl acetate. The organic phase was filtered through a Na<sub>2</sub>SO<sub>4</sub> column. Samples were then dried under nitrogen, redissolved in ether and spotted onto Sigma pre-coated silica gel plates

F<sub>254</sub> (20x20 cm) for TLC. Following separation by TLC using benzene/ethyl acetate/acetic acid solvent (84:15:1), plates were air-dried. Bands corresponding to JH-III standards were counted in a Beckman LS 1800 scintillation counter calibrated for a single label counting of tritium for 1 min for each sample.

### Hormone Treatments

The JH analogue, methoprene (JHM) [11-Methoxy-3,7,11-trimethyl-2,4-dodecadienoic acid 1-methylethyl ester] was applied topically in acetone to the abdomen at dosages of 0.5 $\mu$ g or 1.0 $\mu$ g. The methoprene-RS and -S forms were a gift from Thomas P. Bowman, Zoecon Co., Dallas, Texas. Application of JHM in wax was made at 10  $\mu$ g/treatment to the abdomen covering an incision site. The 20-HE (Sigma Chemical) was dissolved in ethanol, then aliquots were added to 10% ethanol/phosphate buffered saline (PBS, pH 7.5) for a final concentration of 0.5 $\mu$ g/0.2 $\mu$ l, and 0.2 $\mu$ l was injected into the clipped foreleg or the abdomen using a micropipette drawn into a needle. Streptomycin crystals were added to the PBS to reduce infection following injection. NU BASE PLATE wax was used for sealing incision sites.

Brain tissue for implantation was obtained from 4d laboratory-strain female weevils reared under LDHT conditions at 13L:11D, [31°C L; 26°C D] to induce immediate reproductive development. Implantations were made at an incision in the abdomen between the first and second sternite using fine forceps to insert the tissue into the incision site. The incision site was then sealed with wax.

## RESULTS

### JHE Assay

We have shown, in collaboration with K. Dahm, M. Wozniak, and G. Bhaskaran at Texas A&M, that the primary product of JH degradation after incubation of labeled JH with boll weevil hemolymph is JH acid, allowing the use of the Hammock and Sparks (1977) assay to determine JHE activity. We have found that *A. grandis* hemolymph esterases show high selectivity for JH as a substrate compared to  $\alpha$ -naphthyl acetate. Various concentrations of  $\alpha$ -naphthyl acetate in addition to labeled JH as a substrate for JHE had no effect on the rate of degradation of JH (Fig. 2). Furthermore, we have characterized *A. grandis* hemolymph esterases using a series of inhibitor studies in which 5th instar *Manduca sexta* hemolymph was used as a positive control. We used four inhibitors: DFP (*O,O*-diisopropyl phosphorofluoridate) a general esterase inhibitor that typically does not inhibit JH-specific esterases (for exceptions, see Yu and Terriere, 1978; Peter et al., 1979; Campbell et al., 1992), and three compounds that inhibit JH-specific esterases in other systems. As expected, DFP was not a potent inhibitor of JH-specific esterase in *M. sexta* nor did it inhibit *A. grandis* JHE. EPPAT (*O*-ethyl-*S*-phenyl phosphoramidothiolate) inhibited *A. grandis* JHE at higher concentrations than the JHE of *M. sexta*, while the two other JHE inhibitors, OTFP (3-octylthio-1,1,1-trifluoropropan-2-one) and OTFP-sulfone, inhibited at lower concentrations in *A. grandis* than in *M. sexta* (Table 1).

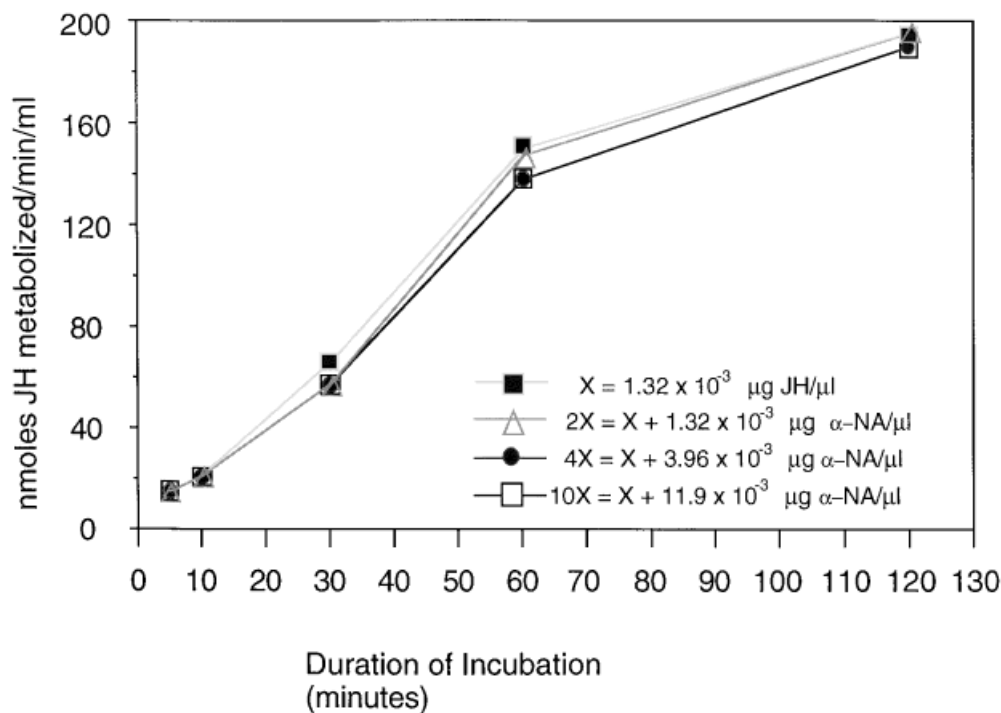


Fig. 2. Effect of  $\alpha$ -naphthyl acetate concentration on JHE activity plotted in nmol of JH metabolized against time of the reaction incubation. The concentration of JH substrate used was  $X = 1.32(10^{-3}) \mu\text{g JH}/\mu\text{l}$ . Three concentrations of  $\alpha$ -naphthyl acetate were examined (2X, 4X, and 10X) at a series of incubation times (5, 10, 30, 60, and 120 min).

Despite the presence of varying concentrations of an alternative esterase substrate,  $\alpha$ -naphthyl acetate, hydrolysis of JH remained unchanged. These results suggest that *A. grandis* hemolymph esterase is highly selective for JH as a substrate. Furthermore, this esterase activity is inhibited with relatively low concentration of specific JH-esterase inhibitors, and is unaffected by the general esterase inhibitor DFP below  $10^{-3}\text{M}$ , as seen in *L. decemlineata* (Kramer and de Kort, 1976b). Taken together, these results strongly suggest that the *A. grandis* hemolymph esterase is JH specific.

**Behavior and JHE Activity**

In field experiments with Dr. James Cate at Texas A&M University, weevils were collected from host plants in late summer and fall, fed for 20 days,

**TABLE 1. JHE Inhibitor Studies**

Inhibitor	Conc[M] at I <sub>50</sub>	Conc[M] at I <sub>50</sub>
	<i>A. grandis</i>	<i>M. sexta</i>
DFP	$>10^{-3}$	$>10^{-3}$
EPPAT	$9 \times 10^{-4}$	$7 \times 10^{-9}$
OTFP	$6 \times 10^{-10}$	$7 \times 10^{-7}$
OTFP-sulfone	$4 \times 10^{-7}$	$9 \times 10^{-4}$

then put into field cages with pine shavings, but no food, and monitored throughout the subsequent winter and spring. All populations showed high mortality in the first weeks after being put into field cages, and then a small percentage of the original population, usually 10% or less, survived the rest of the winter. A portion of the overwintering population remained quiescent throughout the diapause period, while at each census some individuals were out of the litter and active. Weevils that remained dormant had significantly higher levels of JHE activity than those weevils that were active on two of the three census dates (March 23 and May 26). On April 21, no active weevils were found but the dormant weevils had elevated levels of JHE activity (Fig. 3).

### JHE Activity and Survival

A series of experiments investigated the potential for JHE activity to predict subsequent survival of field-collected adults from the Brazos Valley population for which age and reproductive status were unknown. JHE activity varied widely among individuals for each of the three sample dates (Fig. 4). A bimodal distribution of JHE activity, among high and low levels, was clearly evident in the late-August sample, but was less evident in the September and October samples. Longevity data for each population were regrouped into two cohorts based upon whether the hemolymph converted  $<99$  nmoles of JH to JH-acid during the assay (low juvenile hormone esterase activity) or  $>100$  nmoles (high juvenile hormone esterase activity) and plotted against time (Fig. 5). In the August cohort, nearly 77% of the variation in survivorship was explained by the variation in the level of JHE in the hemolymph on day 20 (Table 2). In the September and October cohorts, differences in JHE explained 57% and 49% of the survival variance, respectively. Preliminary analysis of covariance (ANCOVA) with months as a discrete variable, natural log JHE as independent variable (covariate), and natural log of survival as dependent variable (covariate) showed no significant difference in slopes among the months (no significant interaction effect for month  $\cdot \ln JHE$ ;  $F_{(1,142)}$

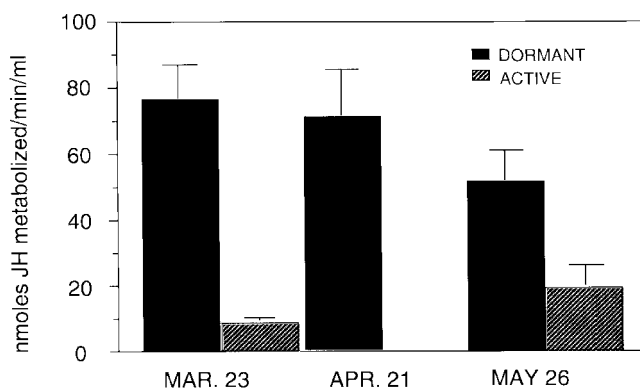


Fig. 3. JHE activity and behavior of overwintering weevils. Dormant weevils = weevils that were still inactive and buried in the center or bottom of excelsior. Active weevils = those actively crawling on top of excelsior or on walls or lid of cage. March 23: Dormant ( $n = 15$ ), Active ( $n = 13$ ); April 21: Dormant ( $n = 9$ ), Active: none; May 26: Dormant ( $n = 12$ ), Active ( $n = 11$ ). JHE activity is presented as nmoles of JH metabolized/min/ml during a two-hour incubation.

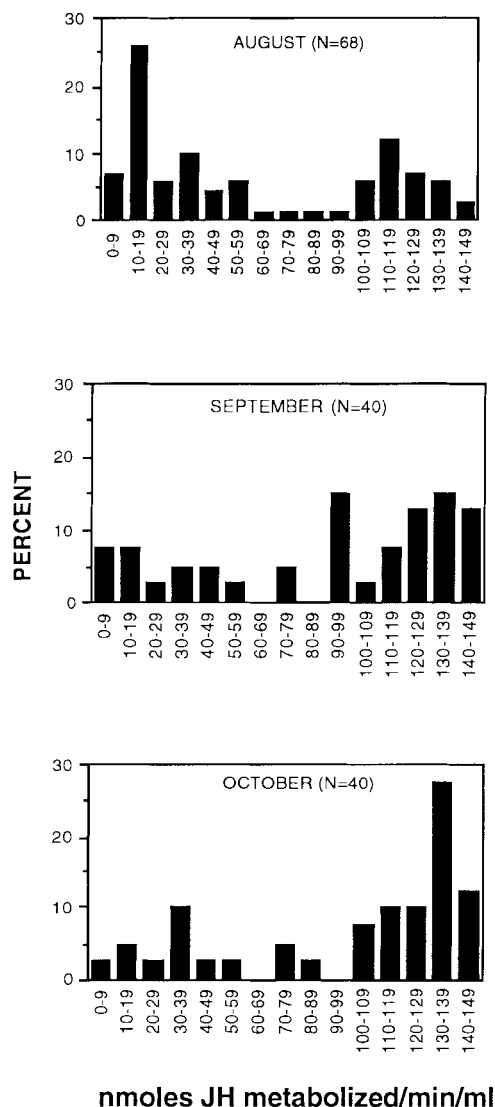


Fig. 4. Variability in hemolymph JHE activity among field-collected adults of the Brazos Valley population for each of three sample dates. Enzyme activity is expressed as nmol of JH metabolized/min/ml during the 30 min reaction incubation. Data include samples from males and females.

= 2.427,  $P = 0.092$ ) and the interaction was deleted in the final analysis. The final ANCOVA showed significant differences among months (Table 3). In August, there was a higher proportion of low-JHE animals in the population than in the later months. In all three cohorts, however, those animals that had high JHE levels prior to the overwintering period were, for the most part, those who survived the winter.

Females collected from Weslaco, Texas, during winter (December, 1990) were examined for JHE activity and hemolymph Vg on 10d, 20d, 30d, or 40d after

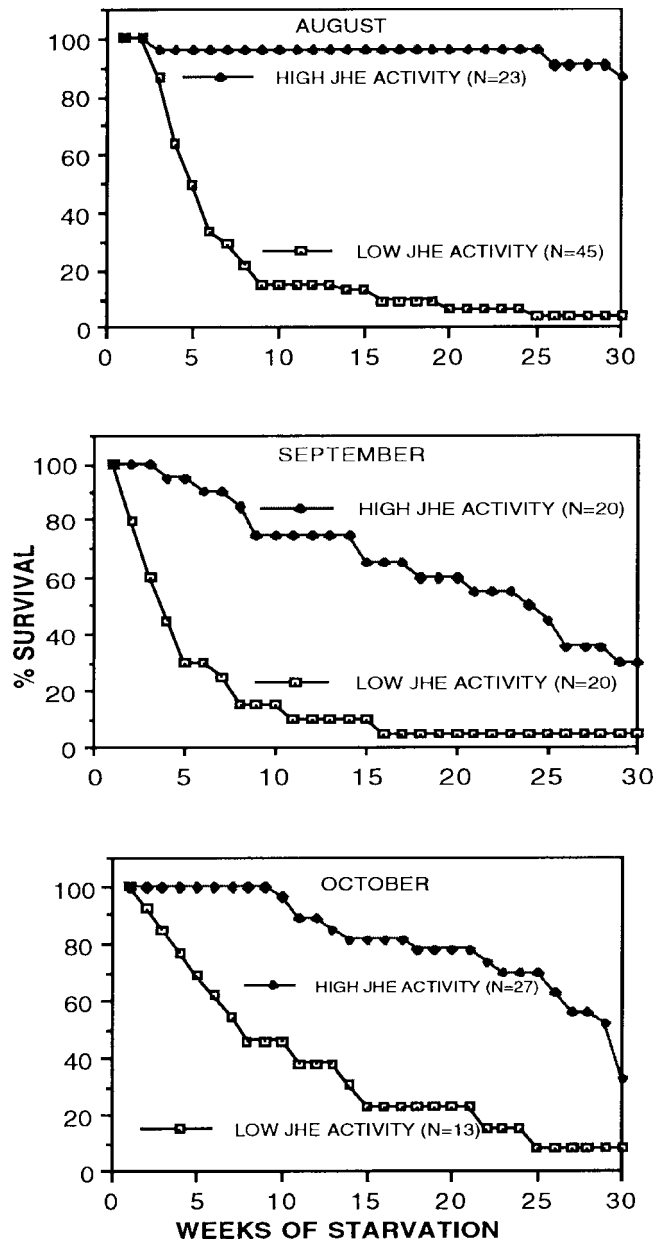


Fig. 5. Association between high or low hemolymph JHE activity and subsequent overwintering survival of field-collected adults from the Brazos Valley population at each of three sample dates. Data are grouped into cohorts of low JHE activity (solid circles) based on conversion of <99 nmoles of JH to JH-acid during the assay or high JHE activity (open circles) >100 nmoles and plotted against survival time.

TABLE 2. Regression Equation for Each Month for JHE/Survival Experiment

Month	Equation	R <sup>2</sup>
August	y = -1.241 + 0.958 (ln JHE)	0.768
September	y = -1.727 + 0.896 (ln JHE)	0.565
October	y = -0.131 + 0.661 (ln JHE)	0.490

adult emergence. These females showed elevated levels of JHE activity at all ages tested (Fig. 6); mean JHE activity of those tested at 20 days was significantly higher than for any other age group (one-factor ANOVA,  $P < .01$ ). Only 10% of 205 females tested positive for hemolymph Vg (Fig. 7).

**Relationship Between JHE Activity and CA Activity in Winter Weevils**

Rate of JH production by CA taken from overwintering females with either high or low JHE levels was determined. CA activity determinations were replicated and averaged for each group at each age with the exception of 3d females, for which insufficient numbers of females were available (n = 28). No significant differences were observed for CA activity between groups, within groups, as a function of age (ANOVA), or with age and JHE levels as categorical variables (two-way ANOVA), and all CA activity was very low relative to that observed in reproductive females (Taub, 1994).

**Vg and Survival**

To examine the correlation between winter survival and the presence of Vg in the hemolymph, field-collected weevils were bled on day 45 following emergence and hemolymph checked for the presence of Vg. The subsequent survival of each individual was monitored from November, 1989, to the end of April, 1990. Eighty-four percent of the 37 animals that survived more than ten weeks had no hemolymph Vg (Fig. 8). Furthermore, of the 40 ten-week survivors 93% had hemolymph Vg (data not shown).

**Diapause Termination**

To examine the endocrine control of post-diapause reproduction, we attempted to stimulate post-diapause Vg synthesis. Various hormone treatments were administered in single doses to late-fall, field-collected females that had been held for three to four months in diapause conditions following removal of food. Insects were tested for hemolymph Vg prior to treatment and at 24h, 48h, and 8d after treatments with JHM in acetone (0.5µg), JHM in acetone + 20-HE dissolved in ethanol then diluted with PBS (0.5µg/0.5µl acetone + 0.5µg/0.2µl ethanol + PBS), 20-HE (0.5µg/0.2µl ethanol + PBS), acetone, ethanol + PBS-injection, or no treatment (n = 20 each group). None of the treat-

TABLE 3. ANCOVA for JHE/Survival Experiment

	DF	Mean-square	F-ratio	P
LNJHE	1	108.799	261.358	0.000
Months	2	6.282	15.090	0.000
Error	144	0.416	—	—

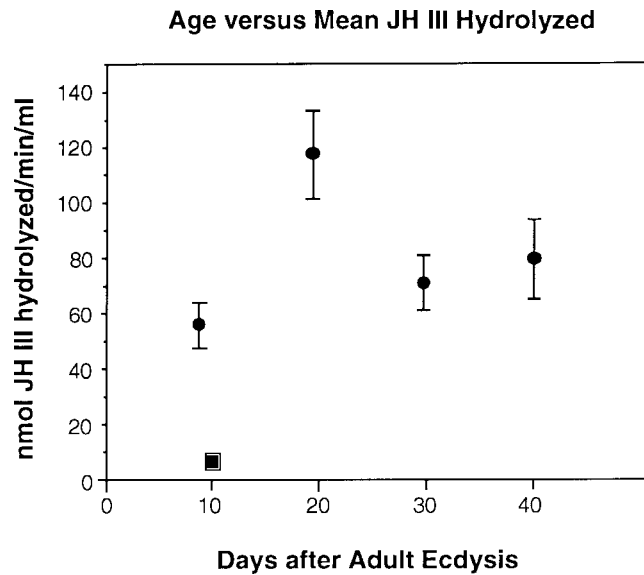


Fig. 6. Age versus mean JHE activity for diapause females from Texas. Age = days following adult ecdysis. JHE activity is presented as nmoles of JH hydrolyzed to JH acid/min/ml. Circles indicate diapause females, square indicates 10d reproductive females (n = 12). Number of diapause individuals in parenthesis were 10d (60), 20d (56), 30d (53), 40d (36). Bars show standard error of the mean. Females tested at 20 days are significantly higher than all other ages (one-factor ANOVA,  $P < .01$ ).

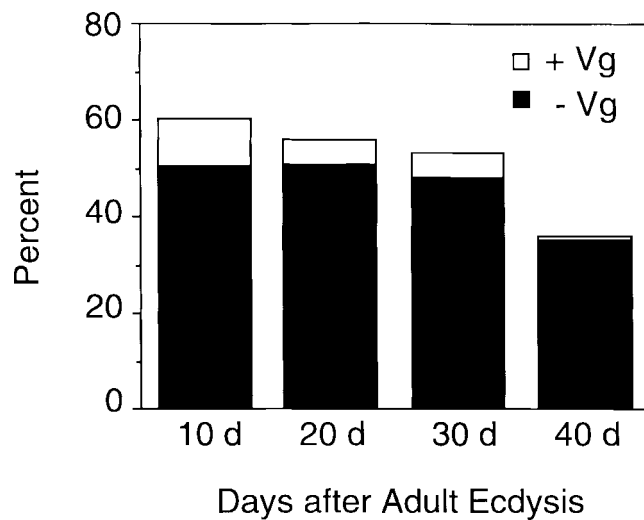


Fig. 7. Age versus Vg occurrence in diapause females from Texas. Age = days following adult ecdysis. Number of individuals in parenthesis: (+Vg, -Vg): 10d (10, 50), 20d (5, 51), 30d (5, 48), 40d (1, 35).

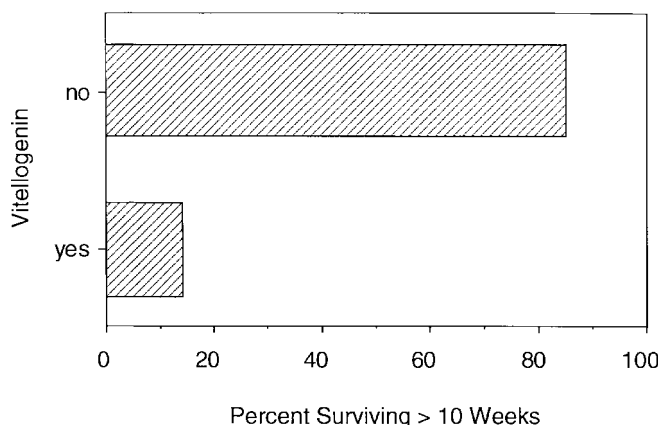


Fig. 8. Relationship of presence of hemolymph vitellogenin to overwinter survival in *A. grandis*. N = 37, 31/37 with no vitellogenin, 6/37 with vitellogenin.

ments used induced the appearance of Vg, although Vg production followed by oviposition was induced in diapausing females within one week among females that were placed into LDHT conditions with access to diet (Table 4).

In a second experiment, hormone treatments were administered in single doses at six or eight weeks following removal of food to field-collected females from Weslaco, Texas (December, 1993). This timing was chosen because we thought it possible that hormonal treatment during earlier stages of diapause might be more effective (Lefevre, 1989) than after three to four months holding time. In contrast to all our earlier experiments with South Texas populations, 41 of the 70 females tested positive for hemolymph Vg in the pre-treatment test. Thus, in this experiment females were monitored for hormonal effects on Vg uptake determined by dissection and confirmed by immunoblotting. Controls included untreated females and sham-implanted females given muscle tissue from females reared in LDHT conditions. Hormone treatment groups included: JHM in wax, reproductive brain plus JHM, and 20-HE plus JHM. In addition, three groups with ten females and ten males each were established to examine the effects of environmental conditions and exposure to diet on ovarian development. Two of these groups were given ac-

TABLE 4. Treatments and Results From Diapause Termination Experiment

Treatment	N	# With vitellogenin				# Oviposited
		0h	24h	48h	8d	8d
JHM	20	0	0	0	0	0
JHM + 20-HE	20	0	0	0	0	0
20-HE	20	0	0	0	0	0
LDHT + food	20	0	0	0	16	9
SDLT w/o food	20	0	0	0	0	0
Acetone	20	0	0	0	0	0
ETOH/PBS	20	0	0	0	0	0

Females were examined for hemolymph Vg prior to treatment (0h) and following treatment at 24h, 48h, and 8d.

cess to boll weevil diet, one was transferred to LDHT conditions, and the other remained in SDLT. The third group was placed in LDHT without access to diet. Among all treatment groups, only females with access to diet showed ovarian development, and both groups with food produced viable eggs regardless of photoperiod. Those in LDHT oviposited within seven days and those in SDLT oviposited within 11 days following access to food. All females were dissected within 15 days following treatment and none of the insects reared without food showed any ovarian development (Table 5).

## DISCUSSION

We observed a very high correlation between high JHE in the prediapause animals and overwinter survival. Thus, high JHE activity and absence of Vg appear to be diagnostic of a weevil's ability to survive the winter. Time-course studies have indicated that JHE peaks in prediapause animals at about 20 days post-ecdysis, drops to intermediate levels during diapause, and then declines rapidly to negligible levels as weevils emerge from diapause (Figs. 3 and 6; Hansen et al., unpublished data). In field cages, animals that had resumed activity during the winter had very low JHE activity, as did both lab- and field-reared reproductive weevils.

The correlation between the presence of Vg in the hemolymph of prediapause females and overwinter survival suggests that the onset of reproductive development virtually precludes winter survival, in agreement with Palmer and Cate (1992). This would indicate that successful overwintering is accomplished primarily by pre-reproductive animals. On the other hand, the results of the second diapause termination experiment, reported above, seem to indicate that during the diapause period survivors in some populations have initiated Vg synthesis.

The correlation of high JHE activity with overwintering success in our experimental regime suggested that the CA of diapausing boll weevils may be producing some JH. High JHE activity may be necessary during diapause to keep JH levels low enough to assure that no JH-induced, post-diapause de-

**TABLE 5. Results From Physiological Age-grading Analysis for Diapause Termination Experiment-2**

Treatment	Nulliparous			Parous			
	N1	N2	N3	P1	P2	P3	P4
JHM	10						
Brain + JHM	10						
JHM + 20-HE	10						
LDHT + food	7	—	1	2			
LDHT w/o food	10						
SDLT + food	3	1	2	4			
SDLT w/o food	5						
Sham	5						

Ovarian development was determined using the physiological age-grading system of Grodowitz and Brewer (1987).

velopment would begin until late spring. In experiments on diapausing females, we have shown that the CA of both low- and high-esterase animals synthesize only very low levels of JH, and there is no significant difference in CA activity between these two groups. Since both groups had an equal tendency to produce a small amount of JH, the critical difference between them would presumably be that the high-JHE animals would immediately metabolize the JH produced. Thus, it is possible that we have in the boll weevil population of South Texas a variety of endocrinological/diapause strategies. Some insects may be programmed to continue reproduction as long as they can (no JHE). Others may enter a brief or less intense diapause but emerge in a short time (low or only transiently high JHE), still others may have continuously high JHE levels that would metabolize any hormone produced and maintain dormancy until late spring. In our experimental regime, this final group were our "survivors." Those animals that became active during the overwinter period were nearly always dead at the subsequent census. Had they been free to leave the cage and search for food (pollen, feral cotton), however, they might have survived. In South Texas, where the winters are highly variable, but sometimes quite mild, early emergence may occasionally be highly advantageous.

Whether or not the function of JHE during diapause is as we propose, the correlation of high JHE activity with overwintering success may provide a tool for identification of winter survivors and may lead to a clarification of what physiological, environmental, and genetic factors induce the development of such animals.

Although JH seems to control Vg synthesis in the pre-reproductive female weevil (Taub, 1994) and high JHE levels are correlated with diapause onset and overwinter survival, simply supplying JH was not enough in these experiments to induce post-diapause Vg synthesis. Administration of various doses and combinations of JH and/or ecdysteroid did not induce the appearance of Vg in diapausing animals, although Vg production and oviposition could be induced in the same animals by giving them access to food. These results suggest that post-diapause Vg synthesis does *not* depend solely on the presence or absence of JH.

Lefevre (1989) found that reproduction in female *L. decemlineata* was triggered most effectively with treatments administered to animals after six or eight weeks in diapause, and following that lead we tried earlier treatment in the second diapause termination experiment, unfortunately without success. It is not clear that any of the animals in the second experiment were actually in diapause, since a large number displayed hemolymph Vg. It is possible that this group of weevils was an intermediate diapausing or non-diapausing strain, as described by Lloyd and Merkl (1961) and Lloyd et al. (1967), or already in the process of diapause emergence. The involvement of brain factor in the regulation of Vg uptake was described above and by Taub (1994). We suspect that this factor is probably important in the initiation of reproduction following diapause, and brain implants were done to test this possibility. This experiment was unsuccessful, but this potential control mechanism deserves further investigation.

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