ISOLATION AND IDENTIFICATION OF GENES EXPRESSED DURING DIAPAUSE IN HORN FLY, *HAEMATOBIA IRRITANS* (L.).

by

Lisa Kalischuk-Tymensen

B. Sc., University of Alberta 1992

Thesis Submitted to the School of Graduate Studies at the University of Lethbridge

in Partial Fulfilment of the Requirements for the Degree of

MASTER OF SCIENCE

LETHBRIDGE, ALBERTA

JUNE 2001

ABSTRACT

There is a discrepancy in the current literature concerning the stage of development in which horn flies arrest during pupal diapause. A study was therefore conducted to describe the morphologies of horn fly pupae and its central nervous system (CNS) throughout nondiapause pupal development and diapause.

Morphologies of diapausing pupae and CNS indicated that developmental arrest occurred early in pupal development during the interval between head eversion and pupal-adult apolysis. Morphological descriptions are necessary for defining comparable tissues between nondiapausing insects and diapausing insects. These tissues can then be used for molecular differential analysis to determine genes specific to either diapause or nondiapause. One such differential analysis technique, subtractive hybridization, was used to isolate putative diapause up-regulated genes from the horn fly. Seven different cDNAs were cloned and partially sequenced. Comparisons of the cDNA sequences with known DNA and protein sequences indicated homology with transferrin, cytochrome oxidase I, Kunitz family serine protease inhibitor, tyrosine hydroxylase (TH), and carboxylesterase. Two cDNAs did not have homology to entries in DNA and protein databases. Northern blot analyses were used to study expression of each gene by probing total RNA extracted from whole pupae throughout nondiapause pupal development and diapause. Expression of TH was also determined in total RNA extracted from CNS tissue of nondiapausing and diapausing pupae. Cytochrome oxidase was equally expressed in nondiapause and diapause destined pupae, and therefore not considered to be a diapause up-regulated gene. Expression patterns differed slightly for each of the

remaining clones; however, expression tended to be highest in diapause destined pupae during pupation compared to nondiapausing pupae. These genes and their products are involved in many aspects of insect physiology including metamorphosis, melanization and sclerotization of the puparium and cellular defense. The possible functions of these genes and products are discussed in the context of the diapause process.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Drs. Tim Lysyk, Brent Selinger, Kevin Smith and Jim Thomas for their guidance throughout my program. I would also like to thank the following people at the Agriculture and Agri-Food Canada Research Center, Lethbridge for their support in the various aspects of my research: Richard Lancaster, Blain Weisser, Dr. Danica Baines, Paul Coghlin, Dr. Ale Perotti, Dr. Larry Kawchuk, Frank Kulcsar, John Hachey, Dr. John Armstrong, Dr. Andre Laroche, Dr. Dennis Gaudet, Michele Frick, Therese Despins, Dr. Debbie Fujimoto, Lauri Lintott, Kimberly Irving, Dr. Bernie Benkel, Jon Davoren and Scott Richmond. I would also like to thank my sisters Andrea and Melanie, and my friends Jenny Gusse, Dr. Doug Inglis and Dr. Stewart Rood. Most of all, I am indebted to my partner, Wilco Tymensen.

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LIST OF ABBREVIATIONS

BPTI – bovine pancreatic trypsin inhibitor
cDNA – copy DNA
CNS – central nervous system
DA – dopamine
DDC – dopamine decarboxylase
DH – diapause hormone
DNA – deoxyribonucleic acid
EMBL – European molecular biology laboratory
G3PDH – glyceraldehyde 3-phosphate dehydrogenase
HPLC – high performance liquid chromatography
JH – juvenile hormone
mRNA – messenger RNA
MOPS – 3 [N-Morpholino]propanesulfonic acid
NCBI – National center for biotechnology information
PTTH – prothoracicotrophic hormone
RNA – ribonucleic acid
SDS – sodium dodecyl sulfate
SSC – sodium chloride sodium citrate
TH – tyrosine hydroxylase

CHAPTER 1

INTRODUCTION

The horn fly, *Haematobia irritans* (L.) is an economically significant dipteran pest of rangeland cattle. Survival and reproduction of both sexes are dependent upon blood meals obtained from cattle. Irritation and stress associated with blood feeding result in reduced weight gain of cattle leading to major economic losses.

Adult flies emerge in the spring and live exclusively on cattle; females leave only to lay eggs on freshly deposited manure. The eggs hatch, larvae feed on the manure and develop through three instars, pupate, undergo metamorphosis and emerge as adults. Several overlapping generations of flies are produced over the spring and summer. These reproductive life cycles continue until cooler fall temperatures are encountered. At this point, beginning usually in August or September and ending in October in Alberta, pupae enter a developmental arrest known as diapause (Lysyk and Moon, 2001). Pupae overwinter in a state of diapause under the manure pat, and adults emerge the following spring to start the next generation of flies. Diapause is an adaptation that ensures insect survival during periods of seasonal adversity, such as the winter.

Like all developmental processes, diapause is characterized by a unique pattern of protein and gene expression (Flannagan et al., 1998). During diapause, there is both a decrease in protein synthesis, and the expression of a number of unique proteins in the brain of the flesh fly, *Sarcophaga crassipalpis* Macquart

(Joplin et al., 1990). A number of unique proteins have also been noted in the gut of diapausing gypsy moth, *Lymantria dispar* L. (Lee and Denlinger, 1996). Protein work has not proven particularly informative, as few of these proteins have been identified or assigned a function. A molecular approach has also been used for the identification of gene expression differences in diapausing insects. Several diapause up-regulated and down-regulated genes from *S. crassipalpis* have been cloned, sequenced, and identified (Flannagan et al., 1998). Studies of the genes regulating diapause are more informative than studies of proteins, but relatively few have been undertaken.

There are a number of molecular techniques for isolating differentially regulated genes from two populations, such as diapausing and nondiapausing insects. Success of these techniques is dependent on having comparable tissues from each population. It is therefore important to morphologically define the stage of developmental arrest when analyzing gene expression differences between diapausing and nondiapausing insects.

The objectives of this study were to 1) morphologically define the stage of pupal-adult metamorphosis in which developmental arrest associated with diapause occurs in the horn fly, 2) identify up-regulated genes from diapause destined horn fly pupae, and 3) determine temporal and spatial patterns of up-regulated gene expression during diapause with the overall result of increasing knowledge about the genetics and molecular biology of the horn fly.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Diapause.

2.1.1 **Induction.** Diapause is an adaptation that ensures insect survival during periods of seasonal environmental adversity such as winter and drought. Insects respond to cues in advance of impending seasonal adversity. Photoperiod is considered the primary cue for the induction of diapause, followed by temperature. Other less common cues include food supply, density (crowding), and moisture (rainfall). Diapause may be induced by extremely variable regimes of photoperiod and/or temperature, depending on the insect species (reviewed by Tauber et al. 1986).

The sensitive period is the time where insects perceive these cues and stores the information for later translation into diapause induction. There is a large variability in the timing of the sensitive period and diapause. In some insects, both sensitive period and diapause may occur during the same developmental stage, and in other insects, the sensitive period may occur well ahead of the stage entering diapause. In some insects, photoperiod and temperature do not induce diapause in the insect itself, but affect its progeny. For example, in the blowfly, *Calliphora vicina* Robineau-Desvoidy, short days in the maternal generation result in larval diapause of progeny (Vinogradova, 1974; Saunders et al., 1986).

The events leading to diapause induction include perception of stimuli (photoperiod and temperature), measurement of stimuli length (photoperiod,

scotoperiod, thermoperiod, or cryoperiod), storage of the information, and finally translation into a hormonal regulatory signal (Saunders, 1982). The physiological mechanism for the measurement of stimuli length is the "clock". The "counter" is the mechanism for accumulating and storing the information throughout the sensitive period. A variety of models have been developed to explain the clock and counter mechanism. Vaz Nunes and Saunders (1999) reviewed these models.

The most recent model is the double circadian oscillator model (Vaz Nunes, 1998a, Vaz Nunes, 1998b). In this model, the clock mechanism is based on two circadian oscillators that measure stimuli length. During the sensitive period, the values from these oscillators are integrated into an "induction sum". Diapause is induced if the induction sum accumulated during the sensitive period is higher than a certain induction threshold. The threshold number is also termed the "required day number". This is the number of days under diapause-inducing conditions required to induce diapause in 50% of the insects (Saunders, 1981).

As representations of the regulatory mechanism, these models cannot describe what is happening on a biological level. It has been suggested that the circadian system may be the biological mechanism regulating diapause induction (Bunning, 1936; Pittendrigh, 1972; Lewis and Saunders, 1987). Circadian rhythms are governed by oscillators (or clocks) that function autonomously, but can be entrained by environmental cues such as photoperiod and temperature. Young (1998) and Dunlap (1998) offer reviews of the circadian system.

To date, only a single investigator has studied the regulation of diapause by the circadian system (Saunders et al., 1989; Saunders, 1990). *Period* (*per*) is one of

the core genes comprising the circadian oscillator. In *Drosophila melanogaster* Meigen, ovarian diapause is elicited in response to photoperiod. Flies with mutations of the *per* locus were capable of discriminating photoperiod length, and inducing diapause. It was concluded that mutations in *per* do not affect photoperiodic time measurement (Saunders et al., 1989; Saunders, 1990). The authors point out that although *per* is not causally involved in diapause induction of *D. melanogaster*, other genes in the circadian system besides *per* may be involved. Subsequent advances have been made in the molecular characterization of the circadian system, which may offer a new approach for the investigation of the clock and counter.

2.1.2 **Hormonal regulation.** Photoperiod and temperature are translated into hormonal signals that regulate diapause. Ecdysone and juvenile hormone (JH) are considered the primary hormonal regulators of diapause (reviewed by Tauber et al., 1986). Ecdysones function during molting, pupation, metamorphosis and gametogenesis (reviewed by Mordue et al., 1980). The synthesis of ecdysone occurs in the prothoracic gland and is under the control of a neurohormone, prothoracicotropic hormone (PTTH). JH functions during morphogenesis (embryogenesis, larval moulting, metamorphosis, polymorphism) and reproductive development (vitellogenin synthesis and ovarian development) (reviewed by Mordue et al., 1980). The site of JH synthesis is the corpus allatum.

JH and ecdysone work together during development (reviewed by Mordue et al., 1980). Ecdysone signals the progression of development (i.e. moulting and pupation) and JH determines the developmental program (i.e. larva or adult). JH as

its name implies maintains juvenile characteristics and modulates differentiation of the pupal and adult tissues in a complex fashion (Nijhout, 1994).

Depending on insect species and developmental state, diapause is induced and maintained by the presence or absence of either ecdysone or juvenile hormone (reviewed by Tauber et al., 1986). In general, diapause occurs when the hormonal status that normally characterizes that developmental stage is maintained. Diapause in the larval stage commonly, but not necessarily, involves the maintenance of high levels of JH, which maintain juvenile (larval) characteristics. Diapause in the pupal stage is often, but not necessarily, characterized by the lack of ecdysone. During the pupal stage, ecdysone must be present for adult development to begin. If ecdysone is absent, as it is in diapause, the insect remains in the pupal stage.

In some Lepidopteran insects, there are other hormones that regulate embryonic diapause. Diapause hormone (DH) is the only specific hormone known to elicit a diapause response in *Bombyx mori* L. (Ikeda et al., 1993). Several other hormones that share a similar structure with DH also induce diapause in these insects.

Recently, attention has focused on the role of neurotransmitters in diapause induction. In vertebrates, the biogenic amines dopamine, serotonin, and melatonin, are involved in the anticipation and adaptation to seasonal change (Reiter, 1991) resulting in hibernation and reproductive cessation (Weekley and Harlow, 1987; Nurnberger, 1995; Popova et al., 1993). Biogenic amines may also be involved in diapause induction and maintenance in insects. Distribution of dopamine in the brain of the European corn borer, *Ostrinia nubilalis* Häbner, is suggestive of involvement

in an endogenous time measuring system and/or diapause induction or termination (Houk and Beck, 1977). During diapause induction, serotonin may inhibit neurosecretory activity in the brain of *Pieris brassicae* (L.) (Puiroux et al., 1990). In addition, dopamine levels in haemolymph are significantly higher in *P. brassicae* pupae throughout diapause compared to continuously developing pupae (Isabel et al., 2001). Most recently, it was shown that dopamine is a factor for the induction of embryonic diapause in *B. mori* (Noguchi and Hayakawa, 2001). Dopamine content in the haemolymph and brain-subesophageal ganglia of *B. mori* increases in response to diapause inducing temperatures (Noguchi and Hayakawa, 2001). Increased dopamine in the maternal generation stimulates expression of diapause hormone and ultimately results in the production of diapausing eggs.

Involvement of specific amines in diapause varies between insect species. In diapause induction of the drosophilid fly, *Chymomyza costata* (Zetterstedt), serotonin is not involved and it remains unclear whether dopamine is involved (Kostal et al., 1999).

2.1.3 **Physiological, morphological, and behavioral changes during diapause.** Diapause induction ultimately results in physiological, morphological, and behavioural changes, that allow survival for extended periods of time in adverse environments. Some of the changes such as developmental and/or reproductive arrest, decreased metabolic rate, and resistance to environmental extremes (heat, cold, desiccation) are common to diapausing insects, while others such as diapause colour are species specific (reviewed by Tauber et al., 1986).

Diapause is characterized by arrested development and reproduction. In this way, diapause is an adaptation that serves to coordinate the insect's life cycle with seasonal environmental change. Arrest occurs at a specific stage of the insect's life cycle, these can be the embryonic (egg), larval, pupal, or adult stage. The specific stage of development in which diapause occurs is species specific.

Developmental arrest extends to the cellular level. The stage of cell cycle arrest is also species specific. In *B. mori*, cells are arrested at the G2 phase of the mitotic cell cycle (Nakagaki et al., 1991), whereas cells in brains of *S. crassipalpis*, are arrested at the G0/G1phase (Tammariello and Denlinger, 1998a).

Diapausing insects may undergo physiological and morphological adaptations that serve to provide physiological protection from freezing, desiccation or predators (reviewed by Tauber et al., 1986). Some insects form cryoprotectants such as glycerol or sugars, to lower freezing temperature and prevent tissue damage during freezing. In some insects, the cuticle and puparium may be modified. The puparium of the diapausing *S. crassipalpis* is lined with twice as much hydrocarbon as the nondiapause puparium (Yoder et al., 1992). The cuticle of the diapausing adult bean bug, *Riptortus clavatus* (Thunberg), is stiffer and contains more lipids (Morita et al., 1999). These modifications to the puparium and cuticle are likely associated with the ability to withstand desiccation during prolonged periods. Increased melanization of the cuticle of diapausing adult insects has been well documented for a number of species. Typically, overwintering colouration is an adaptation to camouflage these insects from predators.

2.1.4 **Maintenance and termination.** Diapause is terminated only after certain physiological processes (diapause development) have occurred. The persistence of the diapause state, even if conditions favorable for development are present, prevents premature termination of dormancy. In some insects, diapause termination may occur through a fast inductive process termed tachytelic termination (Hodek, 1982, 1983). In this type of termination, photoperiod and temperature serve as terminating stimuli. Termination may also occur through a gradual spontaneous process, termed horotelic termination (Hodek, 1982, 1983).

Throughout the process of diapause development, sensitivity to diapause maintaining stimuli decreases (reviewed by Tauber et al., 1986). There is also a decrease in the intensity of diapause (increase in the readiness to resume development). Changes in the intensity of diapause may occur, without simultaneous changes in characteristics such as cold hardiness. For example, diapause may be terminated, however an insect may still maintain the same glycerol content normally associated with diapause. Thus, physiological features cannot be used to study diapause development. Molecular techniques may therefore prove useful in the study of diapause development.

2.1.5 **Regulation of diapause at the molecular level.** During diapause, the resulting developmental arrest is not controlled by the simple down-regulation of gene expression and protein synthesis. Instead, diapause is a developmental process that is characterized by a unique pattern of gene and protein expression (Flannagan et al., 1998).

During diapause, there is both an overall decrease in protein synthesis and the synthesis of several unique proteins in the brain of *S. crassipalpis* (Joplin et al., 1990). In the gut of *L. dispar*, several proteins are up-regulated prior to and during early diapause (Lee and Denlinger, 1996). Unfortunately, these protein studies are not particularly informative as few of these proteins have been identified or been assigned a function.

Studies of gene expression are somewhat more informative. During diapause, several up-regulated and down-regulated cDNAs were identified from the brain of *S. crassipalpis* (Flannagan et al., 1998). One of the down-regulated genes was identified as a cell cycle regulator, proliferating cell nuclear antigen (Flannagan et al., 1998; Tammariello and Denlinger, 1998b). Another up-regulated gene was identified by a small heat shock protein transcript (Flannagan et al., 1998; Yocum et al., 1998). Although expression studies of differentially regulated genes are more informative (i.e. often provide sequence identities), they are uncommon.

2.2 Horn fly development.

Developmental times for the immature stages of horn flies are temperature dependent, tending to decrease with increasing temperatures. The developmental time on nondiapausing flies from egg to emergence of the adult is 41.6 days at 15° C and decreases to 8.4 days at 35° C (Lysyk, 1992a). The pupal stage requires about 50% of the total immature developmental period.

2.2.1 Metamorphosis.

During metamorphosis most adult tissues are formed in one of two ways. Adult structures such as eyes, antennae, and wings form from imaginal discs (Demerec, 1950). These discs are present in the embryo and

larva and remain undifferentiated until metamorphosis. The restructuring of larval tissues forms other structures such as the gut and central nervous system (CNS). Ecdysone induces simultaneous cell proliferation and programmed cell death during the restructuring of the central nervous systems of *D. melanogaster* (Awad and Truman, 1997) and *Sarcophaga peregrina* Robineau-Desvoidy (Fujii et al., 1999).

Metamorphosis in horn fly pupae has been described and categorized into 10 stages according to morphological features (Thomas, 1985). The first stage is pupariation in which the outer protective coat is formed. During the last instar, a larva contracts to become barrel shaped followed by tanning of the outside of the newly secreted cuticle. Stage 2, the pre-pupal stage, represents the interval between pupariation and larval-pupal apolysis. Stage 3 is the larval-pupal apolysis in which the larval hypodermis becomes separated from the wall of the puparium. This stage lasts from 9 to 18 hours after pupariation for horn flies undergoing metamorphosis at 23°C. Stage 4, the cryptocephalic pupal stage, represents the interval between larval-pupal apolysis and larval-pupal ecdysis. Stage 5 is the larval- pupal ecdysis, which is distinguished by head evagination. This stage is very short, and occurs during the interval from 24 to 26 hours after pupariation. Stage 6, the phanerocephalic pupal stage, represents the interval between larval- pupal ecdysis and pupal-adult apolysis. This stage lasts from 26 to 40 hours after pupariation. During stage 7, pupal-adult apolysis, the imaginal hypodermis separates from the pupal cuticle. This stage lasts from 40 to 65 hours after pupariation. Stage 8, the early pharate adult stage, lasts from 65 to 120 hours after pupariation. Stage 9 is the red-eyed pharate adult stage, and is distinguished by the red pigmentation of the pupae's eyes. This stage occurs

from 120-140 hours after pupariation. Stage 10 is the late pharate adult stage, and occurs from 140 hour after pupariation until eclosion, which occurs at approximately 160 hours after pupariation.

The specific stage of metamorphosis in which horn flies remain developmentally arrested throughout diapause is unclear. Horn flies are said to diapause either as yellow-eyed pharate adults (Depner, as cited in Kunz and Miller, 1985) or as red-eyed pharate adults (Thomas, 1985; Thomas and Kunz, 1986).

2.2.2 **Diapause induction.** The horn fly survives the winter by diapausing in the pupal stage. As temperature and photoperiod decline in the fall, an increasing proportion of pupae enter diapause. Depner (1961, 1962) carried out the initial studies on the factors governing diapause induction in the horn fly. It was suggested that a decreasing maternal photoperiod 'predisposes' the eggs to ultimately enter diapause during the pupal stage. Furthermore, the temperature that these predisposed larvae are exposed to determines the proportion that enters diapause. Field studies by Wright (1970) and later by Thomas et al. (1987) claimed agreement with Depner (1961, 1962). In these field studies, the effects of temperature and photoperiod were confounding, making it impossible to assess the individual contribution of each effect on diapause.

More recent laboratory studies indicated diapause induction was not influenced by maternal photoperiod (Lysyk, 1992b), length of photoperiod, or number of photoperiodic cycles experienced by larvae (Lysyk and Moon, 1994). Low temperatures experienced by larvae induced diapause (Lysyk, 1992b; Lysyk and Moon, 1994). The horn fly larval environment is manure, which is unlikely to

permit light penetration. Seasonal changes in photoperiod are likely negligible, hence the reliance on temperature to indicate the impending winter (Lysyk and Moon, 1994).

Horn fly pupae respond to temperature induction during a sensitive period corresponding to a physiological age of 0.10 to 0.82, where newly laid eggs are given the value 0, mean pupation occurs at 0.45 and mean eclosion occurs at an age of 1. In terms of active development, the period 0.10 to 0.82 is between the first-instar larval moult to just slightly before the first adult ecloses (Lysyk and Moon, 1994). Diapause induction is related to the number of days required for development during the sensitive period. Cooler temperatures result in longer developmental times, which increases the incidence of diapause. Temperatures below 15°C induce 100% diapause (Lysyk and Moon, 1994).

2.2.3 **Termination.** Termination of diapause in the horn fly is hastened by exposure to either high temperatures or chilling (Lysyk, 1999). Horn flies eclose within 6-8 days after exposure to 30°C. Chilling also terminates diapause, which is the more probable process under (natural) outdoor conditions. Diapausing horn fly pupae stored at – 5, 0.5, 5, and 10°C terminate diapause after 98, 84, 42, and 56 days respectively (Lysyk, 1999). Diapause termination is assumed to occur at some point throughout the winter. After diapause termination, insects are assumed to enter quiescence for the remainder of the winter, until environmental conditions are suitable for development (Tauber et al., 1986).

2.3 Method of differential analysis.

A variety of molecular techniques can be used to identify unique DNA sequences between two highly related DNA populations such as diapausing and non-diapausing horn fly pupae. These techniques include differential display, gene chip micro-array analysis, and suppression subtractive hybridization.

Differential display randomly amplifies cDNA fragments from a subpopulation of mRNAs from each population, and compares these fragments on sequencing gels (Liang and Pardee, 1992). Fragments expressed at different levels are isolated and sequenced. Enough subpopulations are amplified to represent all mRNAs. In gene chip micro-array analysis, DNA fragments corresponding to all genes of an organism are arrayed onto a solid support. Expression of all genes from the populations can be determined by hybridizing the arrayed fragments with labeled mRNA from each population. Although the most informative of the techniques, application of gene chip micro-array analysis is limited by expense and availability of equipment. In addition, application is usually limited to organisms whose genomes have been sequenced. Suppression subtractive hybridization eliminates cDNA fragments common to both populations, leaving only the unique cDNA fragments (Diatchenko et al., 1996; Gurskaya et al., 1996). This technique also normalizes the cDNAs within the target, resulting in the enrichment of rare messages.

Another differential technique, elimination hybridization, has been used to screen for the expression of diapause-specific genes in the brains of *S. crassipalpis* (Flannagan et al., 1998). A cDNA library was constructed from the brains of

diapausing *S. crassipalpis* pupae and screened with probes for nondiapausing brain RNA. Clones failing to hybridize with nondiapause probes were selected as putative diapause up-regulated genes. This resulted in the isolation of 19 cDNAs, of which 4 were diapause up-regulated, 7 were diapause down-regulated and 8 were expressed equally during nondiapause and diapause. The up-regulated cDNAs coded for proteins that shared homology with those involved in stress response and DNA repair, while the down-regulated cDNAs coded for proteins that shared homology with those involved in cell cycle repair.

CHAPTER 3

MATERIALS AND METHODS

3.1 Horn fly rearing.

Horn flies used in all experiments were obtained from a colony maintained on cattle housed at 25°C and a photoperiod of 16L:8D using methods described previously (Lysyk, 1991). Eggs were collected by placing adults in stoppered 500 mL Erlenmeyer flasks held at 30°C in total darkness for 30 - 60 min. For colony maintenance, immatures were reared at 25°C in fresh manure collected from alfalfa hay fed cattle. Pupae were extracted using floatation. The colony had been started with wild flies collected at Lethbridge Research Center in 1989 and was supplemented with wild flies in 1990 and 1991.

3.2 Diapausing and nondiapausing pupae.

Diapausing and nondiapausing horn flies were produced using standard methods (Lysyk and Moon, 1994). Eggs were placed on filter paper on top of fresh cattle manure and incubated at 25°C with a photoperiod of 16L:8D for 2 days. Diapausing pupae were produced by transferring the newly hatched immatures to 15°C and a photoperiod of 16L:8D for 30 - 40 d (Lysyk, 1992b; Lysyk and Moon, 1994). Nondiapausing insects were produced by rearing immatures at 25°C and a photoperiod of 16L:8D. All pupae were collected on the day of pupation (day 0). Nondiapausing pupae were held at 25°C, and collected daily (i.e. day 0, 1, 2, 3, 4 and 5). Diapausing pupae were held at 15°C and collected at assumed equivalent developmental times to nondiapausing pupae. Development is temperature

dependent such that one day at 25°C is the equivalent developmental time (for nondiapausing pupae) to 3 days at 15°C. Therefore, diapausing pupae were held at 15°C and collected every 3rd day (i.e. 0, 3, 6, 9, 12 and 15). Pupae were used immediately or stored at -80°C until required.

3.3 Pupal morphology during nondiapause development.

Morphology of horn fly pupae was determined daily during pupal-adult metamorphosis. Pupae could not be removed intact from the puparium on the day of pupation (day 0). Horn fly pupae were removed from their puparia 1, 2, 3, 4 and 5 days after pupation and photographed. Fixation in 70% ethanol for 24-48h was necessary for intact removal of pupae 1, 2 and 3 days after pupation. Observations of unfixed tissues were consistent with those of fixed tissues. A minimum of 10 insects were dissected and examined for each day. The pupae were viewed at 125X magnification and digitally photographed using a Nikon (Melville, NY) SMZ-10A microscope with a Pixera (Los Gatos, CA) VCS attachment and Pixera Studio Version 1.2 software for a Power Macintosh 7200/90 computer.

3.4 CNS morphology during nondiapause development.

Morphology of the horn fly pupal CNS was determined daily during pupal-adult metamorphosis. The CNS was dissected from pupae on the day of pupation (day 0) and 1, 2, 3 and 4 days after pupation. Fixation in 70% ethanol, for 24-48h was necessary for intact removal of the day 4 CNS. Observations of unfixed tissues were consistent with those of fixed tissues. A minimum of 10 CNSs were dissected and examined for each day. CNSs were viewed with transmitted lighting and the

day4 CNS was also viewed with incident lighting to show red pigment. CNSs were viewed at 250X magnification and digitally photographed as described in section 3.3.

3.5 Morphology of diapausing pupae and CNS.

Morphology of horn fly pupae and CNS tissues were determined for day 15 diapausing pupae. More than 60 diapausing (day 15) pupae were dissected from their puparium and examined for developmental stage. Fixation in 70% ethanol, for 24-48h was necessary for intact removal of diapausing pupae. Observations of unfixed tissues were consistent with those of fixed tissues. More than 60 brains from diapausing insects were dissected and examined. Pupae and CNSs were viewed and digitally photographed as described in section 3.3.

3.6 Morphology of pupae and CNS during long-term storage.

Day 15 diapausing pupae were stored at 5°C for 3 months. Pupae and CNS tissues were dissected every two weeks during the 3 months of storage. The developmental stage of pupae and CNS tissues were determined and recorded.

3.7 Isolation of diapause up-regulated cDNA from horn fly.

This study focused on comparing differences in gene expression between diapausing and nondiapausing horn flies. This was accomplished by extracting and purifying poly A(+) RNA from diapausing and nondiapausing horn flies and reverse transcribing the poly A(+) RNA to cDNA. Suppression subtractive hybridization was used to generate a diapause up-regulated cDNA library. Theoretically, this technique should eliminate cDNA fragments common to nondiapausing and diapausing populations, leaving only diapause up-regulated cDNAs. The first step in this technique was to digest the cDNA with a restriction enzyme which resulted in a

library consisting of small, partial cDNA fragments. A cDNA library was also constructed from diapausing horn flies that contained full length, non-fragmented cDNAs that represented transcripts from all cDNAs from diapausing pupae. The small fragments from the subtracted diapause up-regulated cDNAs were used as probes to screen the full-length diapause cDNA library. This resulted in the isolation of putative diapause up-regulated cDNAs. These cDNAs were sequenced and identified by comparing homologies to existing sequence databases. Northern blotting was used to determine relative abundance of the cDNAs in total RNA extracted from pupae throughout nondiapausing development and diapause. For one of the cDNAs, Northern blotting was used to determine relative abundance of the cDNA in total RNA extracted from CNS of nondiapausing and diapausing pupae. Throughout this study, standard recombinant DNA techniques were used (Sambrook et al., 1989).

3.7.1 **RNA purification from pupae.** RNA was extracted from nondiapausing and diapausing horn fly pupae. Whole pupae were homogenized in TRIzol reagent (50 mg pupae/mL reagent) (Life Technologies, Burlington, ON) and total RNA purified according to the manufacturer's protocol. Total RNA was dissolved in nuclease-free water and stored at -80°C until required. Poly A(+) RNA was isolated from total RNA using either the Quickprep Micro mRNA Purification Kit (Pharmacia Biotech, Baie d'Urfe, PQ) or the PolyATtract mRNA Isolation System (Promega Corp. Madison, WI). RNA was quantified by UV spectrophotometery.

3.7.2 **RNA purification from CNS.** RNA was extracted from CNS tissues dissected from nondiapausing and diapausing horn fly pupae. CNS tissues were homogenized in TRIzol reagent (30 CNSs/mL reagent) and total RNA purified. Total RNA was dissolved in nuclease-free water and stored at -80°C until required. For Northern Blotting, RNA extracted from the CNS of 30 pupae is required for an equivalent G3PDH (internal control) signal to RNA extracted from approximately 3 whole pupae.

3.7.3 **Subtraction library construction.** The PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) was used to perform suppression subtractive hybridization, and generate a subtraction library containing diapause up-regulated cDNA fragments. Poly A(+) RNA purified from diapausing horn flies was used as the tester, and poly A(+) RNA purified from nondiapausing flies was used as the driver.

3.7.4 **Full-length cDNA library from diapausing pupae.** A phage library containing full-length cDNAs from diapausing pupae was constructed with the ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol.

3.7.5 **Screening for diapause up-regulated cDNA's.** Plaques containing full-length cDNA were blotted onto Hybond-N+ nylon membrane (Amersham International, Buckingham, England). Blots were denatured in a solution containing 1.5 M NaCl and 0.5 M NaOH for 2 min, neutralized in a solution containing 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) for 5 minutes and then rinsed in a solution containing 0. M Tris-HCl (pH 7.5) and 2X SSC. DNA was bound to the membranes

by UV fixation. The subtraction library was labeled with the Gene Images random primer labeling kit (Amersham) and used as a probe to screen for plaques containing up-regulated genes. Blots were hybridized with labeled probe overnight at 60°C. Unbound probe was removed by a low stringency wash (0.1% SDS, 1X SSC, 60°C, 15 min) and a high stringency wash (0.1% SDS, 0.5X SSC, 60°C, 15 min). Positive clones were detected using the CDP-Star detection kit (Amersham). The membranes were wrapped in saran wrap and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, NY) for approximately 4 hours.

3.7.6 **Excision of phagemids and sequencing of cDNAs.** Bluescript phagemids containing putative diapause up-regulated cDNAs were excised from positive ZAP phage clones. Bluescript plasmid DNA was extracted using the Wizards™ minipreps DNA purification system (Promega Corp.). Nucleotide sequencing of the cDNAs was performed using the cycle sequencing method with an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Corporation, Mississauga, ON). Sequencing reactions were primed with the T3 primer (5'-AATTAACCCTCACTAAAGGG-3') in order to determine the nucleotide sequence of the 5' end of the cDNAs. Labeled reactions were resolved using an Applied Biosystems Model 373A DNA sequencing system (Perkin-Elmer Corporation). DNA and predicted protein sequences were analyzed using MacDNASIS DNA software (Hitachi Software Engineering Co., Ltd., San Bruno, CA). BLAST (Altschul et al., 1990) sequence analysis programs were used to compare sequences of putative diapause up-regulated sequences with sequence data from the GenBank, EMBL, and Berkeley Drosophila Genome Project databases. The

BLASTN function was used to compare the nucleotide sequences to a nucleotide sequence database. The BLASTX function was used to compare the nucleotide sequence translated in all six reading frames to a protein sequence database. For sequences with no known sequence homology, the PROPSEARCH (Hobohm and Sander, 1995) program found at the EMBL website was used for further analysis. This program ignores the sequence of the amino acids and instead compares properties of the amino acid sequence (i.e. hydrophobicity) to properties of 58 protein families. This program is useful for determining functional and/or structural homologues where sequence alignment has failed, as sequence homology is not always the most effective means of inferring protein function. Failure to find homology at the amino acid level may not mean that the proteins have dissimilar functions

3.7.7 **Northern blotting.** Northern blotting (Sambrook et al., 1989) was used to confirm that the isolated genes were preferentially expressed during diapause (Flannagan et al., 1998). Total RNA was electrophoresed on a 0.22 M formaldehyde denaturing gel, using MOPS running buffer that also contained 0.22 M formaldehyde. RNA was blotted onto Hybond-N+ nylon membrane (Amersham) by upward capillary action using 20X SSC as a transfer medium. RNA was bound to the membrane by UV irradiation. The sense strand of the diapause up-regulated genes was amplified by one-directional PCR, using only T3 primer. The sense strand was labeled with the Gene Images random prime labeling kit (Amersham) to form labeled anti-sense DNA probes. Blots were hybridized with labeled probe overnight at 60°C. Unbound probe was removed by a low stringency wash (0.1% SDS, 1X

SSC, 60°C, 15 min.) and a high stringency wash (0.1% SDS, 0.1X SSC, 60°C, 15 min.). The CDP-Star detection kit (Amersham) was used to detect probes binding to RNA extracted from diapausing and nondiapausing pupae. The membranes were wrapped in saran wrap and exposed to film overnight. Equal loading of the gel was ensured by quantifying RNA by UV spectrophotometery, staining the blotted RNA with 0.03% methylene blue in 0.3M sodium acetate pH 5.2, and probing the blot with a homologous G3PDH probe kindly provided by Dr. Felix Guerrero, USDA-ARS, Kerrville, TX. RNA was sized using 0.24-9.5 Kb RNA molecular size markers (Life Technologies).

Northern blotting was also used to compare temporal patterns of expression for each clone. Expression was examined for day 0, 1, 2, 3, 4 and 5 nondiapausing pupae and day 0, 3, 6, 9, 12 and 15 diapausing pupae. Spatial patterns of expression were compared for a single clone, HiD6, using Northern blotting. Expression of HiD6 was determined for RNA extracted from the whole body and CNS of day 0 and 2 nondiapausing pupae and day 0 and 15 diapausing pupae.

CHAPTER 4

RESULTS

4.1. Morphological characteristics of horn fly pupae during nondiapause development.

Horn fly pupae were removed from their puparium and photographed daily during the pupal-adult metamorphosis (Fig. 1). The stages and characteristics of horn fly pupae during development are summarized in Table 1. Within 24 hours of puparation (day 0), before head eversion, it is difficult to remove the insect intact from the puparium, therefore the puparium is shown in Figure 1A. During stage 1 (Fig. 1B), 24-48 hours after pupation, head eversion has occurred. The pupa is yellow in color. Body tagmatization has occurred, as separate head, thorax and abdomen are discernible. Adult structures including the wings and eyes are visible. During stage 2 (Fig. 1C), 48-72 hours after pupation, both the body and eyes are yellow, and respiratory horns are clearly visible. Antennae are visible from a frontal view (Fig. 2). During stage 3 (Fig. 1D), 72-96 hours after pupation, the body and eyes are yellow, and there is noticeable abdominal segmentation. Antennae appear enlarged, as a frontal view is not required for visibility. During stage 4 (Fig. 1E), 96-120 hours after pupation, the compound eyes are pigmented and appear red, and the rest of the body is yellow. During stage 5 (Fig. 1F),120-144 hours after pupation, sclerotization and melanization of the cuticle are obvious, and tanning of the bristles on the thorax and abdomen is evident. The adult fly emerges on day 6-7.

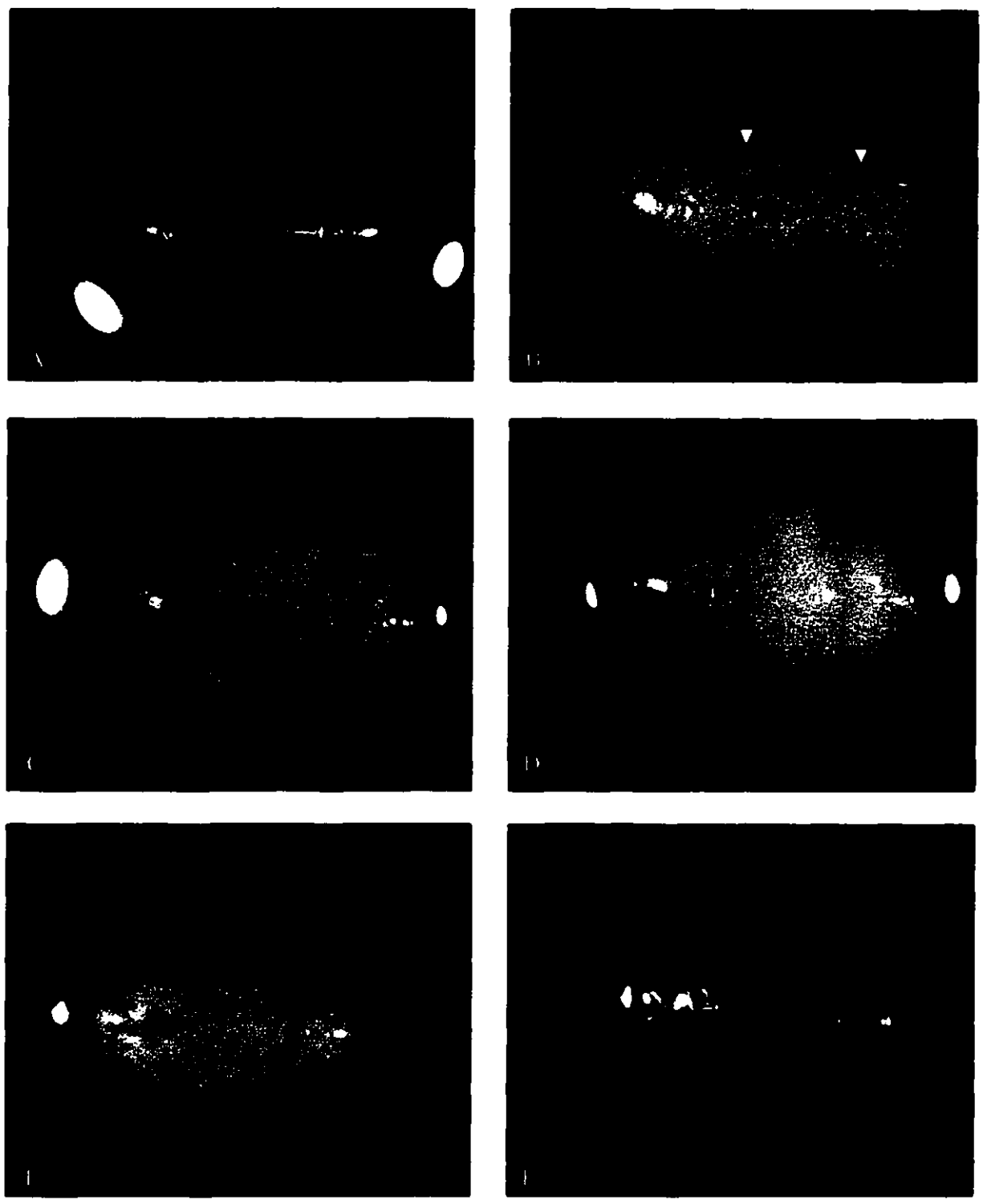

Figure 1. Morphological changes of horn fly pupae during nondiapause development at 25°C. (A) Puparium on the day of puparation (day 0). (B-F) Pupae removed from puparium. (B) 1 day after puparation. Arrows indicate divisions between head, thorax, and abdomen. (C) 2 days after puparation. (D) 3 days after puparation. (E) 4 days after puparation. (F) 5 days after puparation. Eyes (e), wing (w), respiratory horns (rh), antennae (an).

Table 1. Characteristics of horn fly during pupal development.

Stage	Age	Body color	Abdominal Segmentation	Wings	Eye color	Respiratory horns	Antennae
1	24-48 h	yellow		visible	yellow		
2	48-72 h	yellow		visible	yellow	visible	visible
3	72-96 h	yellow	visible	visible	yellow	visible	enlarged
4	96-120 h	yellow	visible	visible	red	visible	enlarged
5	120-144 h	darkened	visible	visible	red		enlarged

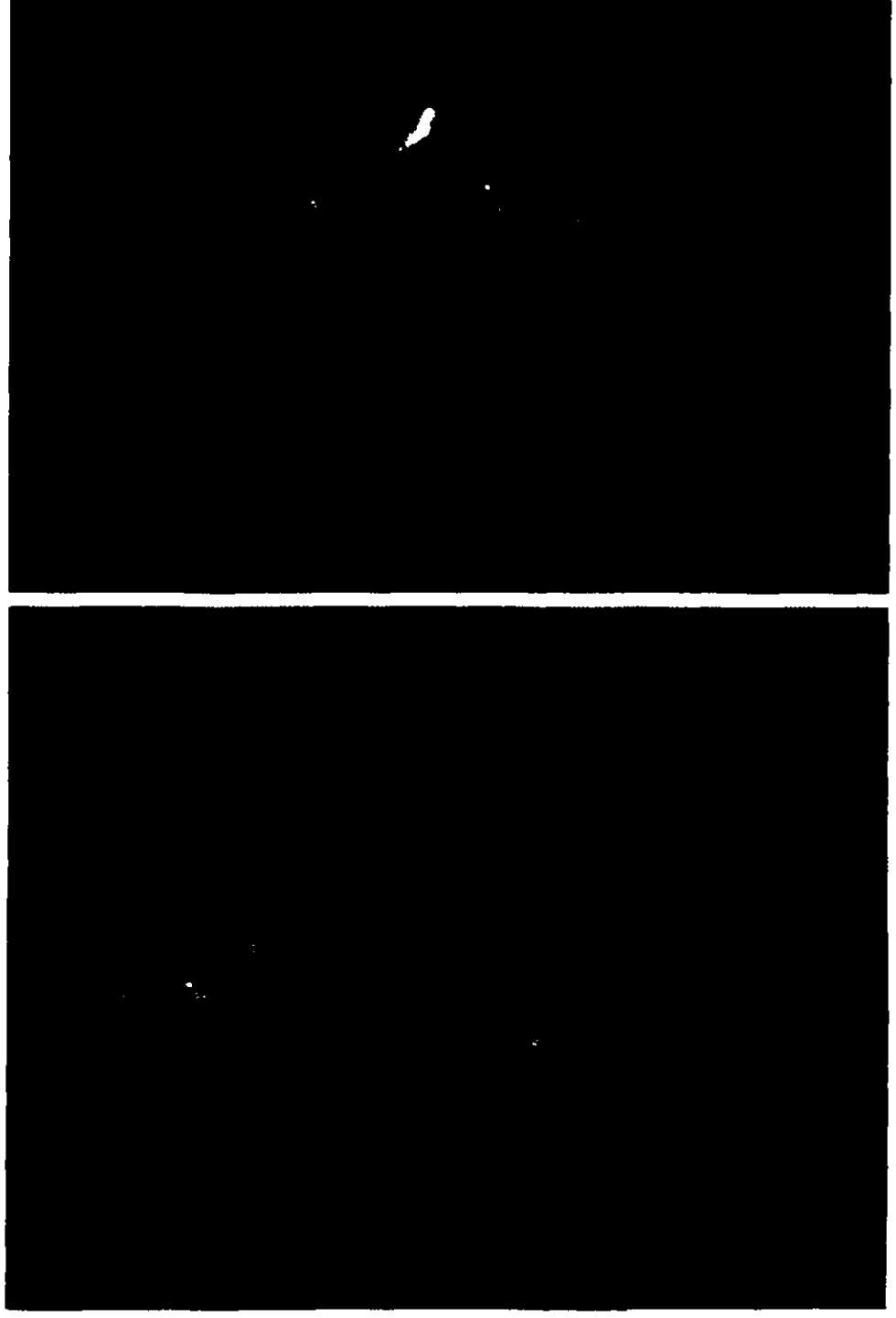

Figure 2. Frontal view of horn fly pupal head showing antennae (an). (A) Antennae are visible in nondiapause pupa 2 days after pupation. (B)Diapause pupa 15 days after pupation showing a lack of visible antennal development.

4.2 Morphological characteristics of horn fly central nervous system during pupal development.

The horn fly CNS was dissected and photographed daily during the pupal-adult metamorphosis (Fig. 3). The stages and characteristics of the horn fly CNS during pupal development are summarized in Table 2. During stage 0, less than 24 hours after pupation, the optic lobes and antennal discs are visible (Fig. 3A). The subesophageal, thoracic, and abdominal ganglia are fused to form a single ventral ganglion. During stage 1, 24-48 hours after pupation, the optic lobes have separated slightly and eye discs are noticeable (Fig. 3B). The subesophageal, thoracic, and abdominal ganglia have differentiated. During stage 2, 48-72 hours after pupation, eye discs are enlarged (Fig. 3C). During stage 3, 72-96 hours after pupation, eye discs have continued to enlarge and ommatidia are visible in the eye disc (Fig. 3D). Cornea lenses and hairs are visible. The subesophageal and thoracic ganglia have separated, and are connected by the cephalothoracic cord (not shown). This separation makes it difficult to keep the thoracic ganglia attached to the brain/optic lobes during dissection, thus the thoracic ganglia is not shown for stages 3 and 4 (Fig. 3D-F). During stage 4, 96-120 hours after pupation, the eye discs have differentiated to form part of the head hypodermis, and are shown attached in Figure 3E and 3F.

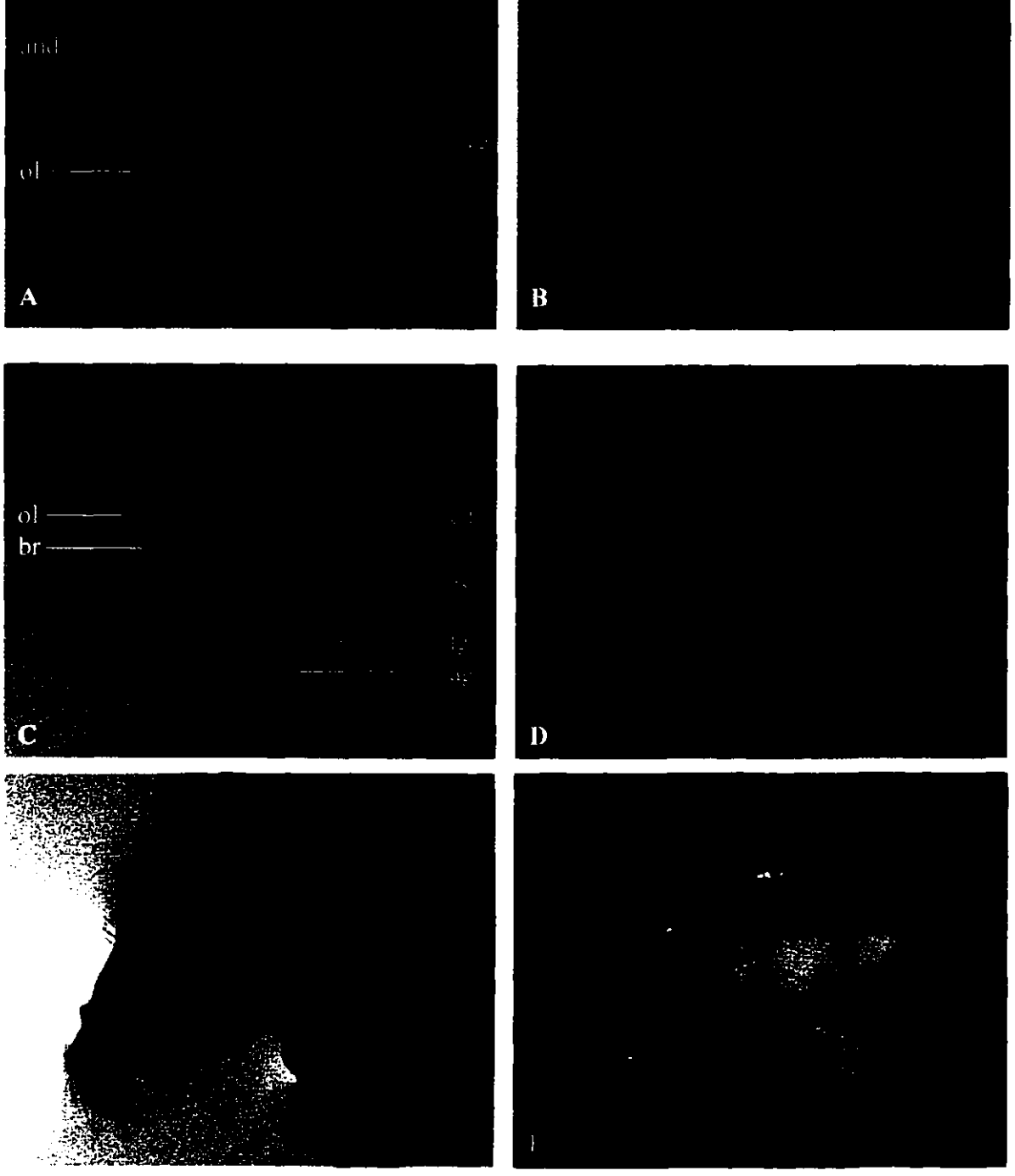

Figure 3. Morphological changes of pupal horn fly central nervous system during nondiapause development at 25°C. (A) Day of pupation (day 0). (B) 1 day after pupation. (C) 2 days after pupation. (D) 3 days after pupation. (E) 4 days after pupation. (F) 4 days after pupation, incident lighting to show red eyes. Antennal disc (and), optic lobe (ol), ventral ganglion (vg), brain (br), eye disc (ed), subesophageal ganglion (seg), thoracic ganglion (tg), abdominal ganglion (ag).

Table 2. Characteristics of horn fly CNS during pupal development.

Stage	Age	Optic lobe	antennal disc	eye disc	corneal lense	eye hairs	ommatidia	ganglia
0	< 24h	visible	visible					fused
1	24-48 h	split		visible				differentiation of segments
2	48-72 h	split		enlarged				differentiation of segments
3	72-96 h	split		enlarged	visible	visible	visible	differentiation from thoracic ganglion
4	96-120 h	split		attached to epidermis				differentiation from thoracic ganglion

4.3 Morphological characteristics of horn fly pupae and central nervous system during diapause.

Diapausing pupae are characteristically yellow in color, and are indistinguishable from day 1 nondiapausing pupae (Fig. 4). Antennae (Fig. 2) and respiratory horns are not visible, indicating that diapause pupae are not as developed as day 2 nondiapause pupae. In the CNS of diapausing pupae (Fig. 4), eye discs are similar in size to those of day 1 nondiapausing pupae and smaller than eye discs of day 2 non-diapausing pupae.

Diapausing pupae remained similar in appearance to day 1 nondiapausing pupae during 16 weeks of storage at 5°C. The body and eyes remained yellow, and antennae were not visible throughout storage. In the CNS, eye discs remained similar in size to eye discs of day 1 nondiapausing pupae throughout storage.

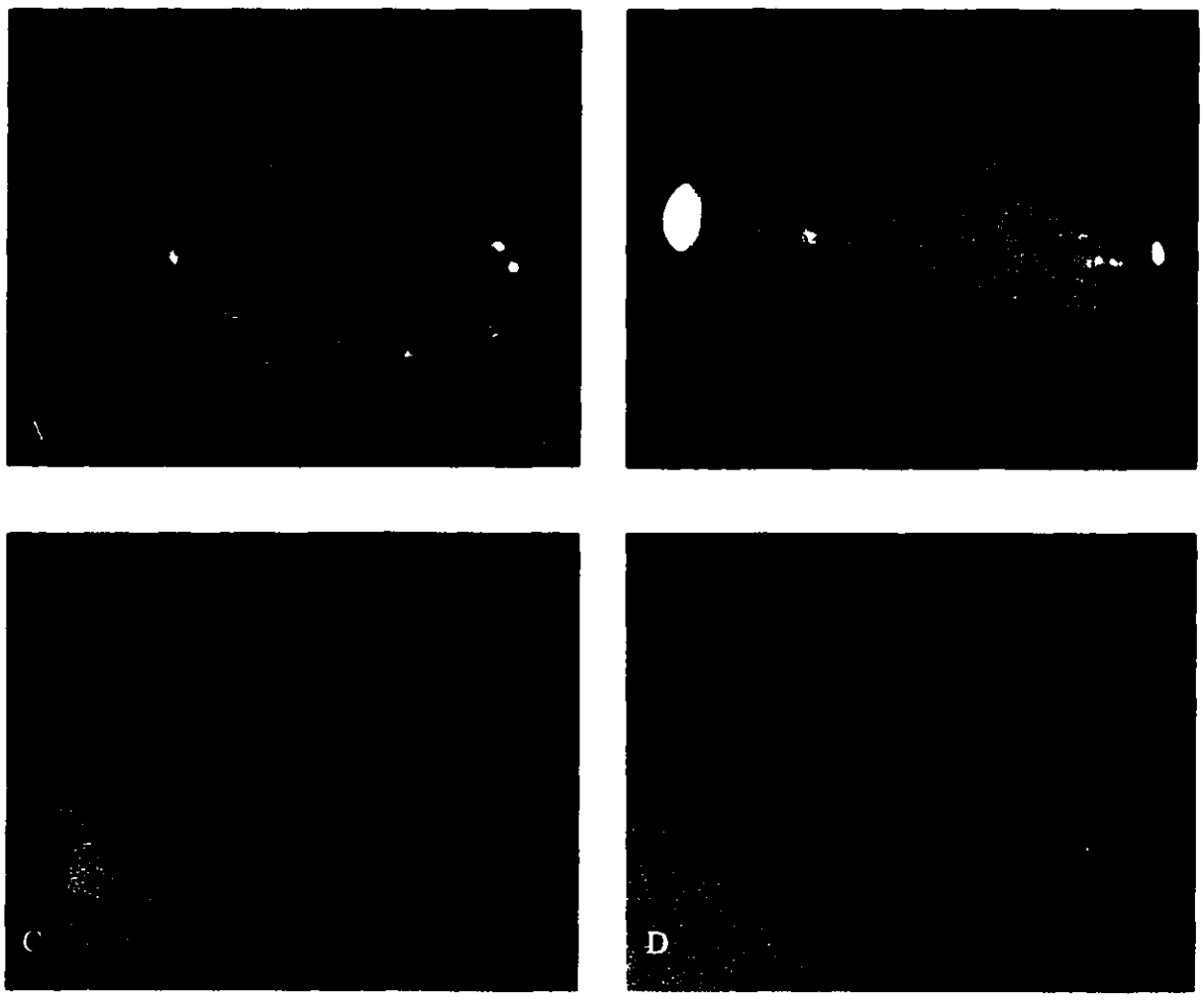

Figure 4. Developmental arrest during pupal diapause of the horn fly. Diapausing pupae were held at 15°C for 16 days prior to dissection. (A) Diapausing pupa removed from puparium. (B) Day 2 nondiapausing pupa removed from puparium. (C) Central nervous system of diapausing pupa. (D) Central nervous system of day 2 nondiapausing pupa.

4.4 Identification and expression of putative diapause up-regulated cDNAs from horn fly pupal cDNA library.

Subtracted diapause up-regulated cDNA fragments were used as probes to screen a diapausing pupal cDNA library. Screening of 15 000 plaques resulted in approximately 1500 plaques (10%) that hybridized with the diapausing pupal cDNA library. Twenty-five of these plaques were picked as putative up-regulated clones. Nine of these plaques (designated as HiD1, 2, 3, 4, 5, 6, 7, 9, 10) were excised into pBluescript, cloned and sequenced. Clones HiD2 and HiD6 had identical sequences, as did clones HiD4 and HiD7, thus clones 4 and 6 were selected for further study. Sequence identities and sizes are summarized in Table 3.

Northern blot analysis was used to determine transcript expression of each clone at pupation (day 0) in whole body total RNA from nondiapause and diapause destined pupae and throughout pupal-adult metamorphosis and early diapause

4.4.1 **HiD1.** A 405 nucleotide fragment was sequenced for HiD1(Fig. 5). The deduced open reading frame represents 135 amino acids. Clone HiD1 does not show significant DNA or protein homology to entries from GenBank, EMBL, or Berkeley *Drosophila* Genome Project databases. The closest amino acid homology is to Sec-independent protein translocase TATA/E-like protein 1 from the thermophilic chemotrophic bacterium *Aquifex aeolicus,* GenBank accession number O66478 (Deckert et al., 1998) (Fig. 6). The sequence homology comparison is considered insignificant as only a small section of HiD1 has homology with *A. aeolicus*. For a stretch of 41 amino acids, 34% (14/41) are identical and 49% (20/41) are either identical and/or functionally similar. The properties of HiD1 deduced amino acid

Table 3. Homology search results for diapause up-regulated cDNAs.

cDNA clone	cDNA Insert size (bp)	Query sequence size (bp)	GenBank entries with highest homology to cDNAsequence	% homology
HiD1	650	405	Sec-independent protein translocase TATA/E-like protein *Aquifex aeloicus* Accession no. O66478	DNA-No significant homology Protein-No significant homology
HiD3	1900	625	transferrin *Sarcophaga peregrina* Accession no. D28940	DNA- 82% (475/577 nucleotides) Protein- 89% (187/208 amino acids)
HiD4	1700	632	cytochrome c oxidase *Chrysomya albiceps* Accession no. AF083657	DNA- 90% (572/629 nucleotides) Protein- 90% (189/210 amino acids)
HiD5	500	425	Kunitz inhibitor-like protein 2 *Drosophila virilis*	DNA-No significant homology Protein- 47% (32/67 amino acids)
HiD6	1750	606	Accession no. AJ249251 tyrosine hydroxylase *Drosophila melanogaster* Accession no. U14395	DNA- 85% (126/148 nucleotides) Protein- 91% (184/201 amino acids)
HiD9	635	635	ALA-E6 repeat region *Drosophila melanogaster* Accession no. X57624 CG11486 gene product *Drosophila melanogaster* Accession no. AE003477	DNA-65% (470 nucleotides) Protein-63% (139/218 amino acids)
HiD10	700	621	carboxylesterase-5A *Drosophila pseudoobscura* Accession no. AF016135	DNA-No significant homology Protein- 42% (87/206 amino acids)

```
                9          18          27          36          45          54
5'GTA ACC ACT GAC CAA GCC ATG ATA AAG AAT GTT GAT GAA GTC GTG ACA ACA GTT
  Val Thr Thr Asp Gln Ala Met Ile Lys Asn Val Asp Glu Val Val Thr Thr Val

               63          72          81          90          99         108
  AAG CCC ATG GCC AAG GAT GTT ACT GAA GTT GTG GTA CCA GTT CAT GAG GTG ACT
  Lys Pro Met Ala Lys Asp Val Thr Glu Val Val Val Pro Val His Glu Val Thr

              117         126         135         144         153         162
  CCT GTG GTT AAG GAT AGT GTT GAA GGT TCT GCA GTT CCC AAT ATG AAA CCC AAG
  Pro Val Val Lys Asp Ser Val Glu Gly Ser Ala Val Pro Asn Met Lys Pro Lys

              171         180         189         198         207         216
GTC AAT GGC GAG ACA ATG ACC AAA GAA GTC CCA GTT CAA GGA ACT GTT GTA GAG
Val Asn Gly Glu Thr Met Thr Lys Glu Val Pro Val Gln Gly Thr Val Val Glu

              226         234         243         252         261         270
TCA CGA AAA CCT CAT AAA GGT ACC GTG ACT GAG AAA ATC TCC AAT GAT GCA TTG
Ser Arg Lys Pro His Lys Gly Thr Val Thr Glu Lys Ile Ser Asn Asp Ala Leu

              279         288         297         306         315         324
CCC CCC ATA CTC CAT CAT CCT GTA ATT AAA GAT GAA GTT CTG GTA CCT CAT GTC
Pro Pro Ile Leu His His Pro Val Ile Lys Asp Glu Val Leu Val Pro His Val

              333         342         351         360         369         378
TCT CTT TCG CCA CCA ATT ACT GAA GAT GTC AAA GAT AAA TCC TCG GAT GAA GAT
Ser Leu Ser Pro Pro Ile Thr Glu Asp Val Lys Asp Lys Ser Ser Asp Glu Asp

              387         396         405
AGC GAT GAG GAA GAA GAG AAG AAA ACC 3'
Ser Asp Glu Glu Glu Glu Lys Lys Thr
```

Figure 5. Partial nucleotide sequence and deduced amino acid sequence data from putative up-regulated cDNA clone HiD1.

```
HiD1: 133 GSAVPNMKPKVNGETMTKEVPVQGTVVESRKPHKGTVTEKI 255
          G  + N +  ++GET  KEV  +    E RK  K    EK+
Sec:   35 GEGIRNFRKALSGETEVKEVKAEDVKTEERKEEKKEEKEKV 75
```

Figure 6. BLAST alignment (Altschul et al., 1990) of amino acid sequences from HiD1 and Sec-independent protein translocase TATA/E-like protein 1 from *Aquifex aeloicus* (GenBank accession number O66478). Functionally similar amino acids are indicated by "+".

sequence were analyzed using PROPSEARCH (Hobohm and Sander, 1995) in an attempt to determine functional or structural homologues. The deduced amino acid sequence of HiD1 shows no significant property homology to the 58 protein families in the database. The closest protein family homologies (all below 19% reliability) include 40S ribosomal protein S20 from *D. melanogaster*, class II heat shock protein from *D. melanogaster*, and cuticle protein 4 from the giant cockroach, *Blaberus cranifer* Burm.

At pupation (day 0), expression of HiD1 was higher in diapause destined pupae compared to nondiapause pupae (Fig. 7). Throughout development (Fig. 8), expression was highest at pupation (day 0) in both nondiapause and diapause destined pupae. Expression was not detectable in diapausing pupae after pupation (days 1-15). Expression was not detectable in nondiapausing pupae on day 1-3 and increased slightly on day 4 and 5.

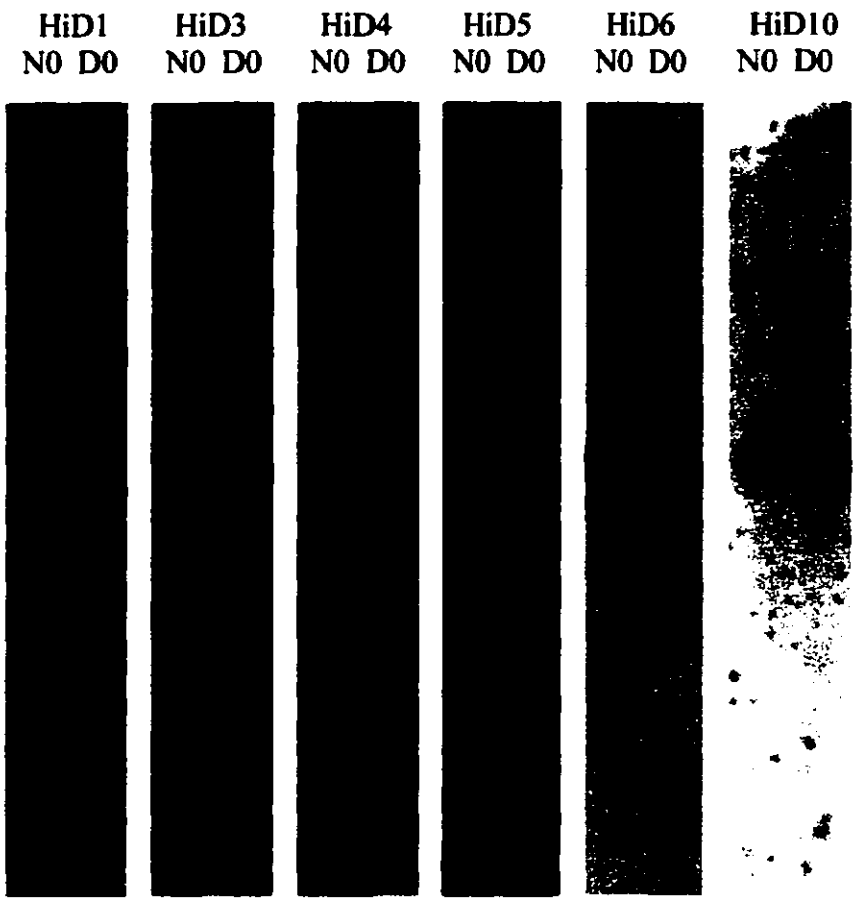

Figure 7. Northern blot analysis of day 0 nondiapause (N0) and diapause destined (D0) pupal RNA probed with HiD1, 3, 4, 5, 6, and 10. Each lane contains 20μg of total RNA isolated from whole body. HiD9 not included due to high background.

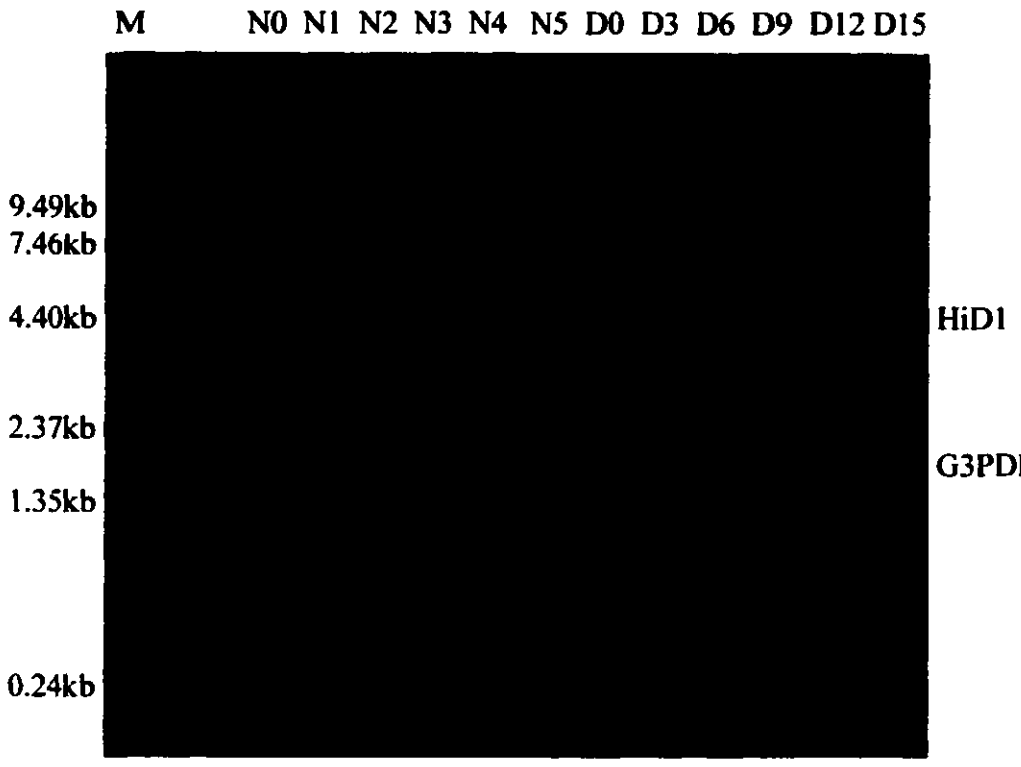

Figure 8. Expression of HiD1 in *H. irritans* pupae reared at nondiapause (N) and diapause (D) inducing temperatures. RNA was extracted from nondiapausing pupae 0, 1, 2, 3, 4 and 5 days after pupation and from diapausing pupae 0, 3, 6, 9, 12 and 15 days after pupation. Each lane contains 20μg of total RNA. M=molecular weight markers.

4.4.2 **HiD3.** A 624 nucleotide fragment was sequenced for HiD3 (Fig.9). There is 82% (475/ 577) nucleotide homology to transferrin from *S. peregrina*, GenBank accession number D28940 (Kurama et al., 1995) (Fig. 10). The deduced open reading frame represents 208 amino acids. For this stretch of sequence, 90% (187/208) of amino acids are identical and 93% (193/208) of the amino acids are identical and/or functionally similar to *S. peregrina* transferrin.

Expression of HiD3 was higher in diapause destined pupae compared to nondiapause pupae at pupation (Fig. 7). Throughout non-diapause development, the highest expression of HiD3 was in nondiapausing pupae on days 3, 4 and 5 (Fig 11). In diapause pupae, expression was reduced in day 0 and 3, and was not detectable after day 3.

4.4.3 **HiD4.** A 632 nucleotide fragment was sequenced for HiD4 (Fig. 12). There is 90% (572/629) nucleotide homology to cytochrome c oxidase from the fly, *Chrysomya albiceps* (Wiedemann), GenBank accession number AF083657 (Wells and Sperling, 1999) (Fig. 13). The deduced open reading frame represents 210 amino acids. For this stretch of sequence, 90% (188/210) of amino acids are identical and 97% (204/210) of amino acids are identical and/or functionally similar to *C. albiceps* cytochrome c oxidase. The next highest homologies are to several dipteran species including *Drosophila*, *Phaenicia*, and *Lucilia*. This sequence is not dipteran specific as significant homologies are also noted for mammalian species including rat, mouse, and bovine.

```
            9          18          27          36          45          54
5'GCC TTC CGT TAT GAG GGC ATT ATT TTG GTT AAG AAA AAC TCC CAT ATT AAA TCC
  Ala Phe Arg Tyr Glu Gly Ile Ile Leu Val Lys Lys Asn Ser His Ile Lys Ser

           63          72          81          90          99         108
  TTA AGA GAT TTA CCT GGA GCC AAA TCT TGT CAT ACA GGA TTC GGT CGT AAT GTT
  Leu Arg Asp Leu Pro Gly Ala Lys Ser Cys His Thr Gly Phe Gly Arg Asn Val

          117         126         135         144         153         162
  GGT TTC AAA ATC CCT GTT ACC AAA TTG AAA AAT CAT CGT ATC TTG AAG GTG TCT
  Gly Phe Lys Ile Pro Val Thr Lys Leu Lys Asn His Arg Ile Leu Lys Val Ser

          171         180         189         198         207         216
  ATG GAT CCT GAA TTA ACA GCC ACT GAA CGT GAA CTT AAG GCC TTA TCG GAA TTC
  Met Asp Pro Glu Leu Thr Ala Thr Glu Arg Glu Leu Lys Ala Leu Ser Glu Phe

          225         234         243         252         261         270
  TTC AGT CAA TCT TGT TTG GTT GGA ACT TAT TCA CCA TAT CCT GAT ACT GAT CGT
  Phe Ser Gln Ser Cys Leu Val Gly Thr Tyr Ser Pro Tyr Pro Asp Thr Asp Arg

          279         288         297         306         315         324
  TTG TTG AAG AAA AAA TAC TCC AAC TTG TGT GCT TTG TGT GAG AAA CCC GAA CAA
  Leu Leu Lys Lys Lys Tyr Ser Asn Leu Cys Ala Leu Cys Glu Lys Pro Glu Gln

          333         342         351         360         369         378
  TGT AAC TAT CCT GAT AAA TAT TCT GGA TAT GAT GGG GCC ATT CGT TGT TTG GAT
  Cys Asn Tyr Pro Asp Lys Tyr Ser Gly Tyr Asp Gly Ala Ile Arg Cys Leu Asp

          387         396         405         414         423         432
  AAG GGT AAG GGT GAA GTT GCC TTC ACC AAG GTT CAA TAC ATC AAG AAA TAC TTT
  Lys Gly Lys Gly Glu Val Ala Phe Thr Lys Val Gln Tyr Ile Lys Lys Tyr Phe

          441         450         459         468         477         486
  GGG TTG GTT CCT GGA ACT ACA GCT GAG GGT GAT CCA TCG AAT TTC GAA TAT TTA
  Gly Leu Val Pro Gly Thr Thr Ala Glu Gly Asp Pro Ser Asn Phe Glu Tyr Leu

          495         504         513         522         531         540
  TGT GAA GAT GGT AGT AGA CGC CCC ATC ACT GGT CCC GCA TGC TCT TGG GCT CAG
  Cys Glu Asp Gly Ser Arg Arg Pro Ile Thr Gly Pro Ala Cys Ser Trp Ala Gln

          549         558         567         576         585         594
  CGT CCA TGG ACT GGT TAT ATT TCC AAT ACT GAT GCT GTT AAT GGT GAC GTA AAA
  Arg Pro Trp Thr Gly Tyr Ile Ser Asn Thr Asp Ala Val Asn Gly Asp Val Lys

          603         612         621
  CTA CAT AAT TTG CAA AAA CGT TTG GAA AAA 3'
  Leu His Asn Leu Gln Lys Arg Leu Glu Lys
```

Figure 9. Partial nucleotide sequence and deduced amino acid sequence data from putative up-regulated cDNA clone HiD3.

Nucleotide sequence

```
HiD3   :4    ttccgttatgagggcattattttggttaagaaaaactcccatattaaatccttaagagat  63
             |||||||||||||| |||||||||| || || |||||  | ||| | ||  |||  ||
TRANSF:382 ttccgttatgagggtattattttggtcaaaaagaactctaacattcactctctaaaggaa 441

HiD3   :64   ttacctggagccaaatcttgtcatacaggattcggtcgtaatgttggtttcaaaatccct 123
             || | ||| |||||||||| || || || |||||||| |||||||| ||||||||||||
TRANSF:442 ttgcgtggtgccaaatcttgccacaccggtttcggtcgcaatgttggcttcaaaatccct 501

HiD3   :124  gttaccaaattgaaaaatcatcgtatcttgaaggtgtctatggatcctgaattaacagcc 183
             || |||||||||| |||   | ||||||||||||||| |||||||||||||| || ||
TRANSF:502 gtcaccaaattgaagaatgcccatatcttgaaggtgtccatggatcctgaattgactgct 561

HiD3   :184  actgaacgtgaacttaaggccttatcggaattcttcagtcaatcttgtttggttggaact 243
             || ||||||||| | || ||||| || |||||||     |||| ||||||||||| |||
TRANSF:562 accgaacgtgaattgaaagccttgtccgaattcttttcagaatcctgtttggttggcact 621

HiD3   :244  tattcaccatatcctgatactgatcgtttgttgaagaaaaaatactccaacttgtgtgct 303
             || || |||||||| || ||||||||||||||||| || ||||| ||||||||||||||
TRANSF:622 tactcgccatatcccgaaactgatcgtttgttgaaaaagaaatatcccaacttgtgtgct 681

HiD3   :304  ttgtgtgagaaacccgaacaatgtaactatcctgataaatattctggatatgatggggcc 363
             |||||||||||||||||||| |||||||||| |||||| ||||| |||||||| ||
TRANSF:682 ttgtgtgagaaacccgaacagtgtaactatcccgataaattctctggctatgatggcgct 741

HiD3   :364  attcgttgtttggataagggtaagggtgaagttgccttcaccaaggttcaatacatcaag 423
             || |||||||||| ||||| || |||||||| ||||| ||||| || |||| |||||||
TRANSF:742 atacgttgtttggacaagggcaaaggtgaagtggcctttaccaaagtacaattcatcaag 801

HiD3   :424  aaatactttgggttggttcctggaactacagctgagggtgatccatcgaatttcgaatat 483
             |||||||||||  |||| || ||   |||||||| |||||||| ||  | ||||||||
TRANSF:802 aaatactttggcatggtacccggtgttacagctgaaggtgatccttctgaattcgaatac 861

HiD3   :484  ttatgtgaagatggtagtagacgccccatcactggtcccgcatgctcttgggctcagcgt 543
             | || |||||||    ||||| ||  | | |||||||| |||||||||| || |||
TRANSF:862 ctttgcgaagatggctccagacgtcctcttaatggtcccgcctgctcttgggcacaacgt 921

HiD3   :544  ccatggactggttatatttccaatactgatgctgtta 580
             || |||||||||| |||||||| ||||||||||
TRANSF:922 ccctggactggttacatttccaatgttgatgctgtta 958
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Amino acid sequence HiD3

```
HiD3   :1    AFRYEGIILVKKNSHIKSLRDLPGAKSCHTGFGRNVGFKIPVTKLKNHRILKVSMDPELT 180
             AFRYEGIILVKKNS+I SL++L GAKSCHTGFGRNVGFKIPVTKLKN  ILKVSMDPELT
TRANSF:108 AFRYEGIILVKKNSNIHSLKELRGAKSCHTGFGRNVGFKIPVTKLKNAHILKVSMDPELT 167

HiD3   :181  ATERELKALSEFFSQSCLVGTYSPYPDTDRLLKKKYSNLCALCEKPEQCNYPDKYSGYDG 360
             ATERELKALSEFFS+SCLVGTYSPYP+TDRLLKKKY NLCALCEKPEQCNYPDK+SGYDG
TRANSF:168 ATERELKALSEFFSESCLVGTYSPYPETDRLLKKKYPNLCALCEKPEQCNYPDKFSGYDG 227

HiD3   :361  AIRCLDKGKGEVAFTKVQYIKKYFGLVPGTTAEGDPSNFEYLCEDGSRRPITGPACSWAQ 540
             AIRCLDKGKGEVAFTKVQ+IKKYFG+VPG TAEGDPS FEYLCEDGSRRP+ GPACSWAQ
TRANSF:228 AIRCLDKGKGEVAFTKVQFIKKYFGMVPGVTAEGDPSEFEYLCEDGSRRPLNGPACSWAQ 287

HiD3   :541  RPWTGYISNTDAVNGDVKLHNLQKRLEK 624
             RPWTGYISN DAV+GD KLHNLQ RLEK
TRANSF:288 RPWTGYISNVDAVSGDEKLHNLQHRLEK 315
```

Figure 10. BLAST alignments (Altschul et al., 1990) of nucleotide and amino acid sequences from HiD3 and flesh fly transferrin (GenBank accession number D28940). Functionally similar amino acids are indicated by "+".

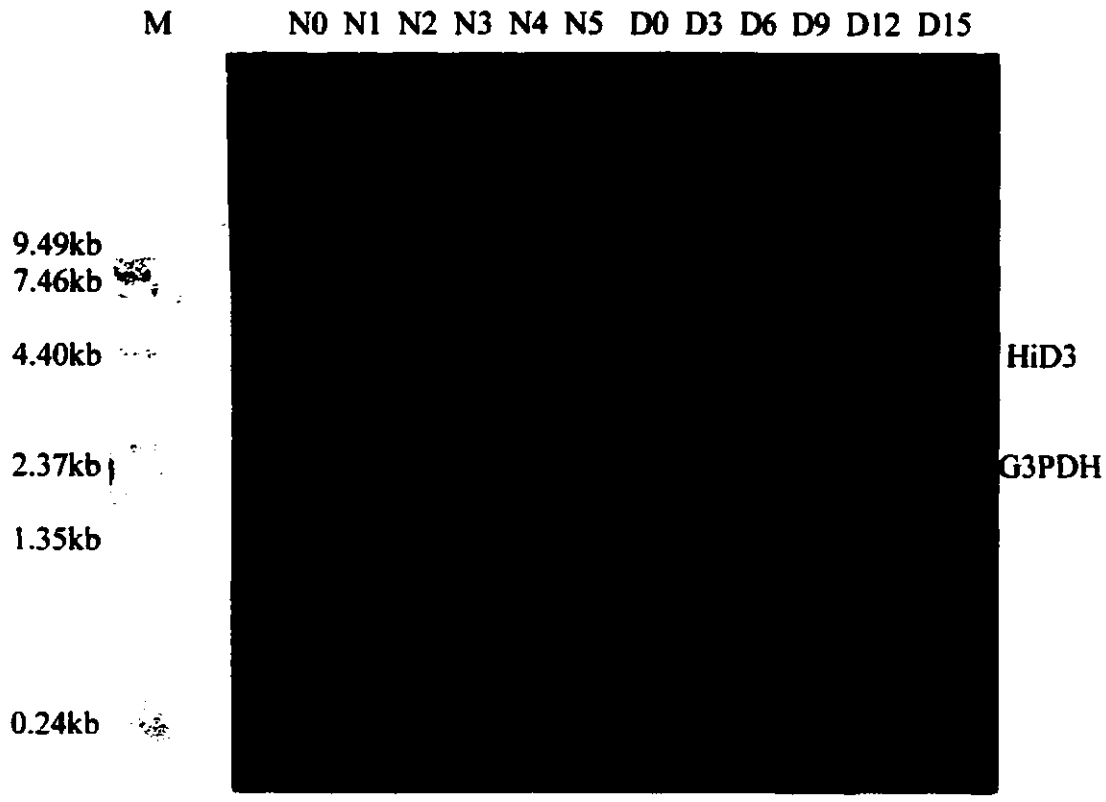

Figure 11. Expression of HiD3 in *H. irritans* pupae reared at nondiapause (N) and diapause (D) inducing temperatures. RNA was extracted from nondiapausing pupae 0, 1, 2, 3, 4 and 5 days after pupation and from diapausing pupae 0, 3, 6, 9, 12 and 15 days after pupation. Each lane contains 20μg of total RNA. M=molecular weight markers.

```
           9           18           27           36           45           54
5'AAG GAT ATT GGT ACT TTA TAT TTT ATT TTT GGA GCT TGA TCT GGA ATA ATT GGA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  Lys Asp Ile Gly Thr Leu Tyr Phe Ile Phe Gly Ala *** Ser Gly Ile Ile Gly

          63           72           81           90           99          108
  ACT TCA TTA AGA ATT TTA ATT CGA GCT GAA TTA GGA CAT CCT GGA GCT TTA ATT
  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  Thr Ser Leu Arg Ile Leu Ile Arg Ala Glu Leu Gly His Pro Gly Ala Leu Ile

         117          126          135          144          153          162
  GGT GAT GAT CAA ATT TAT AAT GTA ATT GTT ACA GCT CAT GCA TTT ATT ATA ATT
  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  Gly Asp Asp Gln Ile Tyr Asn Val Ile Val Thr Ala His Ala Phe Ile Ile Ile

         171          180          189          198          207          216
TTC TTT ATA GTT ATA CCT ATT ATA ATT GGA GGA TTT GGA AAT TGA TTA GTT CCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Phe Phe Ile Val Ile Pro Ile Ile Ile Gly Gly Phe Gly Asn *** Leu Val Pro

         225          234          243          252          261          270
TTA ATA TTA GGA GCT CCT GAT ATA GCA TTC CCT CGA ATA AAT AAT ATA AGT TTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Leu Ile Leu Gly Ala Pro Asp Ile Ala Phe Pro Arg Ile Asn Asn Ile Ser Phe

         279          288          297          306          315          324
TGA TTA CTA CCT CCT GCT TTA ACA TTA TTG TTA GTA AGC AGT ATA GTA GAA AAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
*** Leu Leu Pro Pro Ala Leu Thr Leu Leu Leu Val Ser Ser Ile Val Glu Lys

         333          342          351          360          369          378
GGA GCT GGT ACA GGA TGA ACA GTT TAC CCT CCT TTA TCA TCT AAT ATT GCT CAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Gly Ala Gly Thr Gly *** Thr Val Tyr Pro Pro Leu Ser Ser Asn Ile Ala His

         387          396          405          414          423          432
GGA GGA GCT TCA GTA GAT TTA GCT ATT TTT TCT TTA CAT TTA GCT GGA ATT TCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Gly Gly Ala Ser Val Asp Leu Ala Ile Phe Ser Leu His Leu Ala Gly Ile Ser

         441          450          459          468          477          486
TCT ATT TTA GGA GCT GTA AAT TTT ATT ACA ACT GTA ATT AAT ATA CGA GCT ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ser Ile Leu Gly Ala Val Asn Phe Ile Thr Thr Val Ile Asn Ile Arg Ala Thr

         495          504          513          522          531          540
GGA ATT ACA TTT GAT CGA ATA CCT TTA TTT GTA TGA TCT GTT GTA ATT ACT GCA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Gly Ile Thr Phe Asp Arg Ile Pro Leu Phe Val *** Ser Val Val Ile Thr Ala

         549          558          567          576          585          594
TTA TTA TTA CTT TTA TCT TTA CCA GTA TTA GCT GGA GCT ATT ACT ATA TTA TTA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Leu Leu Leu Leu Leu Ser Leu Pro Val Leu Ala Gly Ala Ile Thr Ile Leu Leu

         603          612          621          630
ACT GAT CGA AAT TTA AAT ACT TCA TTC TTT GAT CCA GC 3'
--- --- --- --- --- --- --- --- --- --- --- --- --
Thr Asp Arg Asn Leu Asn Thr Ser Phe Phe Asp Pro
```

Figure 12. Partial nucleotide sequence and deduced amino acid sequence data from putative up-regulated cDNA clone HiD4. Stop codons are indicated by ***.

Nucleotide sequences

```
HiD4/7:4   gatattggtactttatattttatttttggagcttgatctggaataattggaacttcatta  63
           ||||||||||||||||||| ||||| |||||||||||||||| | ||||||| |||
COI   :40  gatattggtactttatatttcattttcggagcttgatctggaatagtaggaacttcttta  99

HiD4/7:64  agaattttaattcgagctgaattaggacatcctggagctttaattggtgatgatcaaatt 123
           |||||| |||||||||||||||||||||||||||||| |||||| ||||| ||||||
COI   :100 agaattctaattcgagctgaattaggacatcctggagcactaattggagatgaccaaatt 159

HiD4/7:124 tataatgtaattgttacagctcatgcatttattataattttctttatagttatacctatt 183
           |||||||||||||| |||||||||| |||||||||||||||||||| ||||| |||
COI   :160 tataatgtaattgtaacagctcatgcctttattataattttctttatagtaataccaatt 219

HiD4/7:184 ataattggaggatttggaaattgattagttcctttaatattaggagctcctgatatagca 243
           |||||||||||||||||||||| |||||||||||||||||| || ||||||||
COI   :220 ataattggaggatttggaaattgactagttcctttaatattaggagccccagatatagct 279

HiD4/7:244 ttcccctcgaataaataatataagtttttgattactacctcctgctttaacattattgtta 303
           ||||| |||||||||||||||||| ||| | |||||||||| ||||| ||| | |||
COI   :280 ttcccacgaataaataatataagtttctgacttttacctcctgcattaactttactatta 339

HiD4/7:304 gtaagcagtatagtagaaaagggagctggtacaggatgaacagtttaccctcctttatca 363
           ||||| |||||||||||||| |||||||| |||||||||| ||||| || ||||||||||
COI   :340 gtaagtagtatagtagaaaatggagctggaacaggatgaactgtttatccacctttatca 399

HiD4/7:364 tctaatattgctcatggaggagcttcagtagatttagctatttttttctttacatttagct 423
           |||||||||||||||| ||||| ||||| |||||||||||||||||||| ||||||
COI   :400 tctaatattgctcatggtggagcatcagttgatttagctatttttttctttacacttagct 459

HiD4/7:424 ggaatttcttctattttaggagctgtaaattttattacaactgtaattaatatacgagct 483
           |||||||| || |||||||||||||||||||||||||||||||| ||||||||||| ||
COI   :460 ggaatttcatcaattttaggagctgtaaattttattacaactgttattaatatacgatct 519

HiD4/7:484 actggaattacatttgatcgaatacctttatttgtatgatctgttgtaattactgcatta 543
           || ||||| |||||||||||||||||||||||| |||||||||| || |||||| |
COI   :520 acaggaatcacatttgatcgaatacctttattcgtatgatctgtagttattactgctctt 579

HiD4/7:544 ttattacttttatctttaccagtattagctggagctattactatattattaactgatcga 603
           | ||| | ||||| |||||||||||||| || || ||||||||||||||||||||
COI   :580 cttttattattatcattaccagtattagccggtgcaattactatattattaactgatcga 639

HiD4/7:604 aatttaaatacttcattctttgatccagc 632
           ||||||||||||||||||||||||||||
COI   :640 aatttaaatacttcattctttgatccagc 668
```

Amino acid sequences

```
HiD4/7:1   KDIGTLYFIFGA*SGIIGTSLRILIRAELGHPGALIGDDQIYNVIVTAHAFIIIFFIVIP 180
           KDIGTLYFIFGA SG++GTSL ILIRAELGHPGALIGDDQIYNVIVTAHAFI+IFF+V+P
COI   :11  KDIGTLYFIFGAWSGMVGTSLSILIRAELGHPGALIGDDQIYNVIVTAHAFIMIFFMVMP  70

HiD4/7:181 IIIGGFGN*LVPLILGAPDIAFPRINNISF*LLPPALTLLLVSSIVEKGAGTG*TVYPPL 360
           I+IGGFGN LVPL+LGAPD+AFPR+NN+SF LLPPALTLLLVSS+VE GAGTG TVYPPL
COI   :71  IMIGGFGNWLVPLMLGAPDMAFPRMNNMSFWLLPPALTLLLVSSMVENGAGTGWTVYPPL 130

HiD4/7:361 SSNIAHGGASVDLAIFSLHLAGISSILGAVNFITTVINIRATGITFDRIPLFV*SVVITA 540
           SSNIAHGGASVDLAIFSLHLAGISSILGAVNFITTVIN+R+TGITFDR+PLFV SVVITA
COI   :131 SSNIAHGGASVDLAIFSLHLAGISSILGAVNFITTVINMRSTGITFDRMPLFVWSVVITA 190

HiD4/7:541 LLLLLSLPVLAGAITILLTDRNLNTSFFDP 630
           LLLLLSLPVLAGAIT+LLTDRNLNTSFFDP
COI   :191 LLLLLSLPVLAGAITMLLTDRNLNTSFFDP 220
```

Figure 13. BLAST alignment (Altschul et al., 1990) of nucleotide and amino acid sequences from HiD4 and *Chrysomya albiceps* cytochrome oxidase subunit 1 gene (GenBank accession number AF083657). Functionally similar amino acids are indicated by "+".

Expression of HiD4 was similar on day 0 for nondiapause and diapause destined pupae (Fig. 7). Throughout development of nondiapausing pupae, expression of HiD4 was similar from day 0 to 3, and was up-regulated on days 4 and 5 (Fig. 14). In diapausing pupae, HiD4 was expressed at similar levels on day 0 and 3, and was slightly down-regulated from day 6 to 15.

4.4.4 **HiD5.** A 425 nucleotide fragment was sequenced for clone HiD5 (Fig. 15). The deduced open reading frame represents 120 amino acids, and there are two stop codons at the end of the open reading frame. There was no significant homology of the nucleotide sequence to entries in GenBank. The highest amino acid homology is to a Kunitz inhibitor like protein 2 from *Drosophila virilis* Sturtevant, GenBank accession number AJ249251 (unpublished) (Fig. 16). For this stretch of sequence 47% (32/67) of amino acids are identical and 55% (38/67) of amino acids are identical and/or functionally similar to *D. virilis* Kunitz inhibitor like protein 2. Clone HiD5 contains the consensus pattern observed for the bovine pancreatic trypsin inhibitor (BPTI) Kunitz family of serine protease inhibitors (PROSITE: Hofmann et al., 1999) and this is indicated by asterisks in Figure 16. The consensus pattern for this protein family is F-x(3)-GC-x(6)-[FY]-x(5)-C, where x is any amino acid, (#) is the number of amino acids, and [FY] is either of the amino acids F or Y. Homology of HiD5 is also noted for other proteins containing the consensus pattern including collagen chain precursor from human (GenBank accession number X52022), thrombospondin from *Haemonchus contortus* (GenBank accession number AF043121), and amyloid protein A4 from human (GenBank accession number X06989). A multiple sequence alignment program, CLUSTAL W (Thompson et al.,

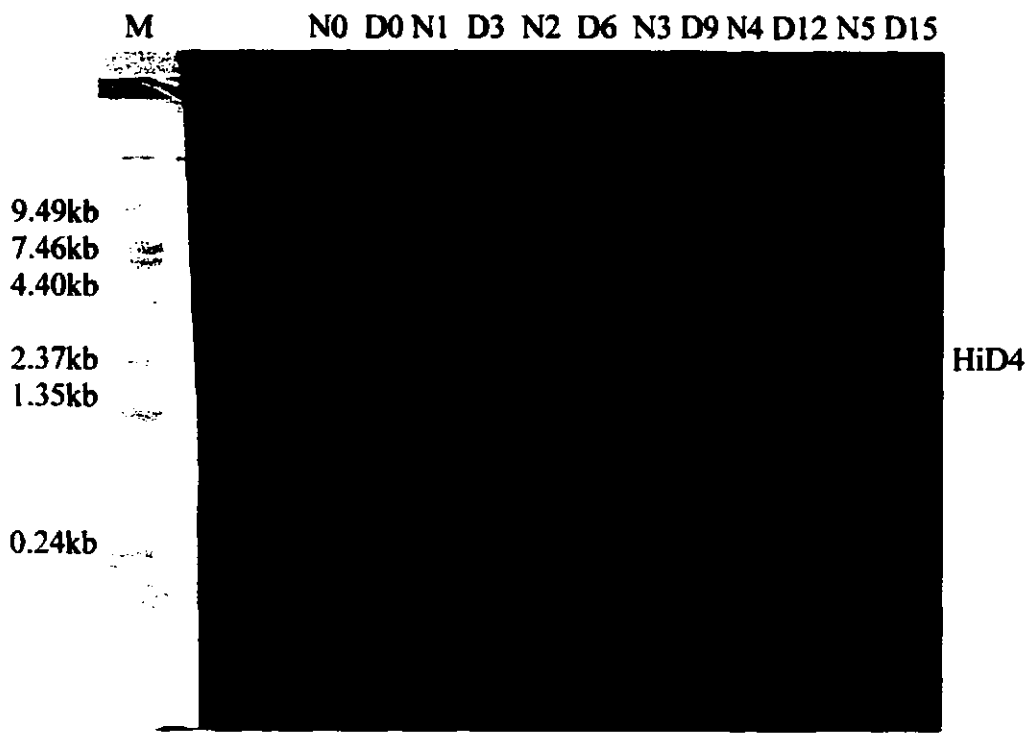

Figure 14. Expression of HiD4 in *H. irritans* pupae reared at nondiapause (N) and diapause (D) inducing temperatures. RNA was extracted from nondiapausing pupae 0, 1, 2, 3, 4 and 5 days after pupation and from diapausing pupae 0, 3, 6, 9, 12 and 15 days after pupation. Each lane contains 20μg of total RNA. M=molecular weight markers.

```
           9          18          27          36          45          54
5'AAA ATT ACT CAA ATA TAC ATC ATG AAA ATC TTT ATT GGA ATT GTA ATT TTC GCA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Lys Ile Thr Gln Ile Tyr Ile Met Lys Ile Phe Ile Gly Ile Val Ile Phe Ala

          63          72          81          90          99         108
 ACA TTC CTT TTA GCA GTC CAC AGC CAG TGT CGC AAT GCT CCA GCT CAA CCC AGA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Thr Phe Leu Leu Ala Val His Ser Gln Cys Arg Asn Ala Pro Ala Gln Pro Arg

         117         126         135         144         153         162
 TGC AGA GGT CCA CGT AAT TTA GGA CAA GCC AGA CGT GGT TGT CGT ATG GCG ACC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Cys Arg Gly Pro Arg Asn Leu Gly Gln Ala Arg Arg Gly Cys Arg Met Ala Thr

         171         180         189         198         207         216
 AGA TGG TGG TAT GAC ACC TCA TCG GGA TCT TGT AAA TCC TTT AAA TAT CGT GGA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Arg Trp Trp Tyr Asp Thr Ser Ser Gly Ser Cys Lys Ser Phe Lys Tyr Arg Gly

         225         234         243         252         261         270
 TGT GGA GGT AAT GCA AAT CGT TTT TGC ACA AAA GAG GCA TGC GAG GAA CGC TGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Cys Gly Gly Asn Ala Asn Arg Phe Cys Thr Lys Glu Ala Cys Glu Glu Arg Cys

         279         288         297         306         315         324
 GAA CGT CAC TGG CGC CAT TAA CTT AGT TAC ATA TTC GAA ATG ATT GTG TTC AGA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Glu Arg His Trp Arg His *** Leu Ser Tyr Ile Phe Glu Met Ile Val Phe Arg

         333         342         351         360         369         378
 TCG TTT AGT TGT ATT TTA AGG TGT AGA AAA TAT TTC TAA TAA ATT TTG GAG ATT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Ser Phe Ser Cys Ile Leu Arg Cys Arg Lys Tyr Phe *** *** Ile Leu Glu Ile

         387         396         405         414         423
 TCA AAA TAG CTT AGT AAA AAA AAA AAA AAA AAA CTC GAG GGG GGG GC 3'
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --  --
 Ser Lys *** Leu Ser Lys Lys Lys Lys Lys Lys Leu Glu Gly Gly
```

Figure 15. Partial nucleotide sequence and deduced amino acid sequence data from putative up-regulated cDNA clone HiD5. Stop codons are indicated by ***.

A. BLAST alignment (Altschul et al., 1990) of amino acid sequences from HiD5 and Kunitz inhibitor like protein 2 from *Drosophila virilis* (GenBank accession number AJ249251).

```
                                                    *   **      *     *
HiD5:  2  APAQPRCRGPRNLGQARR----GCRMATRWWYDTSSGSCKSFKYRGCGGNANRFCTKEAC 57
          A +QP C G ++ G A      G      WWYDT S SCK   Y GCGGN NRFCTK  C
Kunitz:29 ADSQPVCIGGKSEGHANETVCYGNANIYMWWYDTRSRSCKRLSYNGCGGNKNRFCTKSLC 88

HiD5:   58 EERCERH 64
           + +C R+
Kunitz: 89 KSKCRRN 95
```

B. CLUSTAL W (Thompson et al., 1994) multiple sequence alignment of HiD5, Kunitz inhibitor like protein 2 from *Drosophila virilis* (GenBank accession no. AJ249251), collagen chain precursor from human (GenBank accession no. X52022), thrombospondin from *Haemonchus contortus* (GenBank accession no. AF043121), and amyloid protein A4 from human (GenBank accession no. X06989).

```
                                                    *   **      *     *
HiD5        NAPAQPRCRGPRNLGQARRGCRMATR----WWYDTSSGSCKSFKYRGCGGNANRFCTKEACEERCERH
Kunitz      -ADSQPVCIGGKSEGHANETVCYGNANIYMWWYDTRSRSCKRLSYNGCGGNKNRFCTKSLC
collagen    -------CKLPKDEGTCRDFIL-------KWYYDPNTKSCARFWYGGCGGNENKFGSQKECEKVC
thrombo     SAKSRQSCHLPLDVGKCQGSFD-------SWYYEMATGSCVEFKYSGCSGNANRFASREECENTCRRN
amyloid     ----------QAETGPCRAMIS-------RWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVC
```

Figure 16. A. BLAST alignment (Altschul et al., 1990) and B. CLUSTAL W multiple sequence alignment (Thompson et al., 1994) of HiD5 and amino acid sequences with highest degree of homology. Functionally similar amino acids are indicated by "+".
* denotes the PROSITE (Hofmann et al., 1999) consensus pattern of the Kunitz family of serine protease inhibitors

1994) was used to compare homology of HiD5 to these proteins (Fig. 16).

At pupation (day 0), expression of HiD5 was higher in diapause-destined pupae compared to nondiapause pupae (Fig. 7). Throughout development, expression was highest at pupation (day 0) in both nondiapause and diapause destined pupae (Fig. 17). Expression was not detectable after pupation in either diapausing or nondiapausing pupae.

4.4.5 **HiD6.** A 605 nucleotide fragment was sequenced for clone HiD6 (Fig. 18). Two sections of this sequence have high homology with *D. melanogaster* tyrosine hydroxylase (TH), GenBank accession number U14395 (Birman et al., 1994) (Fig. 19). A 266 nucleotide section (from nucleotide 10 to 275) and 148 nucleotide section (from nucleotide 335 to 482) have 80% (213/266) and 85% (126/148) homology to *D. melanogaster* TH. The deduced open reading frame represents 201 amino acids. In this stretch of sequence, 91% (184/201) of amino acids are identical and 97% (197/201) of amino acids are identical and/or functionally similar to *D. melanogaster* TH (Fig. 19).

In *D. melanogaster*, two isoforms of tyrosine hydroxylase are produced through alternate splicing of a single copy gene (Birman et al., 1994). One isoform is associated with the nervous system and the other with non-nervous tissue such as the epidermis, which secretes the cuticle. The isoform corresponding to clone HiD6 cannot be determined from the partial sequence. As both isoforms are highly homologous, suppression subtractive hybridization is unlikely to distinguish between them, and will enrich for increased expression of total TH. Relative expression of TH

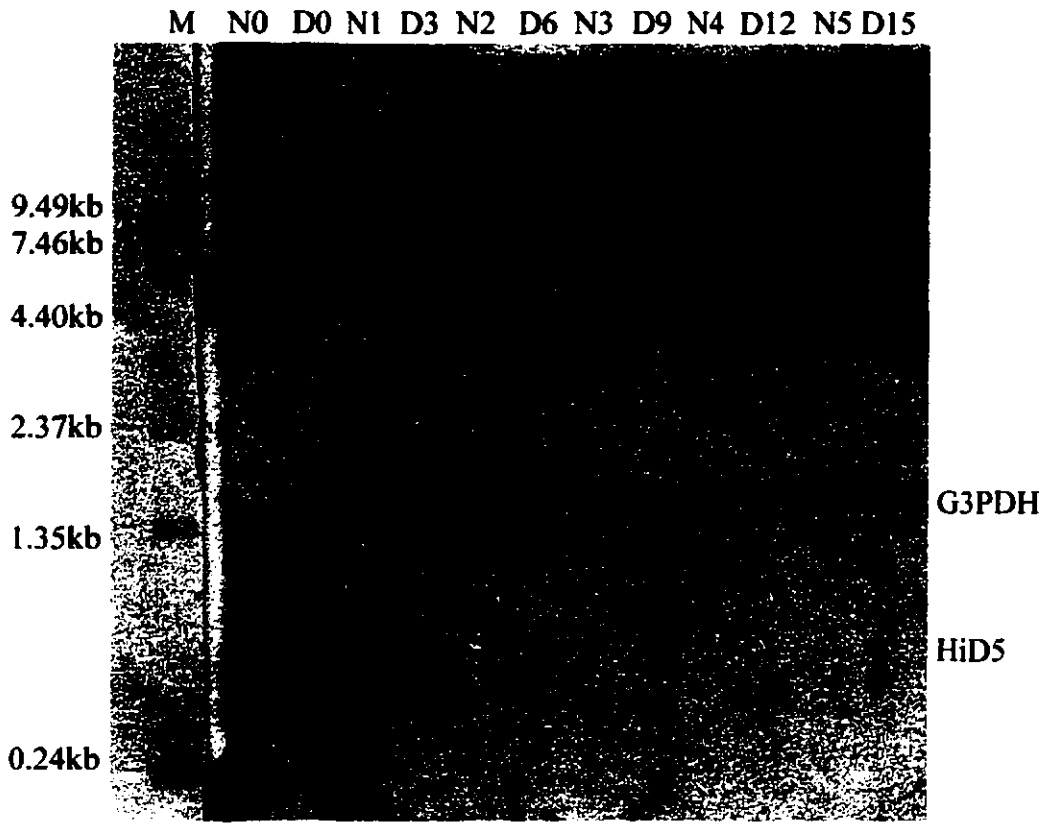

Figure 17. Expression of HiD5 in *H. irritans* pupae reared at nondiapause (N) and diapause (D) inducing temperatures. RNA was extracted from nondiapausing pupae 0, 1, 2, 3, 4 and 5 days after pupation and from diapausing pupae 0, 3, 6, 9, 12 and 15 days after pupation. Each lane contains 20μg of total RNA. M=molecular weight markers.

```
                9          18          27          36          45          54
5'TTG GCT TCG TTG GCT TTC CGT ATC TTC CAG AGC ACC CAA TAT GTG CGT CAT GTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Leu Ala Ser Leu Ala Phe Arg Ile Phe Gln Ser Thr Gln Tyr Val Arg His Val

           63          72          81          90          99         108
 AAC TCA CCC TTC CAC ACT CCA GAA CCT GAT TGC ATT CAT GAA TTG TTG GGT CAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Asn Ser Pro Phe His Thr Pro Glu Pro Asp Cys Ile His Glu Leu Leu Gly His

         117         126         135         144         153         162
 ATG CCT TTG TTG TCT GAT CCC AGC TTT GCT CAA TTC TCT CAG GAA ATT GGT TTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Met Pro Leu Leu Ser Asp Pro Ser Phe Ala Gln Phe Ser Gln Glu Ile Gly Leu

         171         180         189         198         207         216
 GCC TCA TTG GGT GCT TCT GAT GAT GAA ATT GAA AAA TTA TCT ACC GTC TAC TGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Ala Ser Leu Gly Ala Ser Asp Asp Glu Ile Glu Lys Leu Ser Thr Val Tyr Trp

         225         234         243         252         261         270
 TTC ACC GTT GAA TTC GGT CTC TGC AAA GAA CAT GGC GAT GTC AAG GCC TAT GGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Phe Thr Val Glu Phe Gly Leu Cys Lys Glu His Gly Asp Val Lys Ala Tyr Gly

         279         288         297         306         315         324
 GCT GGT CTT CTA TCG GCT TAT GGT GAA CTC TTG CAT GCC ATT TCC GAT AAA TGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Ala Gly Leu Leu Ser Ala Tyr Gly Glu Leu Leu His Ala Ile Ser Asp Lys Cys

         333         342         351         360         369         378
 GAA CAT CGT CCC TTC GAA CCC GCC TCC ACC GCT GTT CAA CCC TAT CAA GAT CAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Glu His Arg Pro Phe Glu Pro Ala Ser Thr Ala Val Gln Pro Tyr Gln Asp Gln

         387         396         405         414         423         432
 GAA TAC CAA CCC ATC TAT TAT GTG GCC GAA AGT TTC GAT GAT GCC AAG GAC AAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Glu Tyr Gln Pro Ile Tyr Tyr Val Ala Glu Ser Phe Asp Asp Ala Lys Asp Lys

         441         450         459         468         477         486
 TTC CGT CGT TGG GTA TCC ACC ATG TCC CGT CCC TTC GAA GTG CGT TTC AAT CCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Phe Arg Arg Trp Val Ser Thr Met Ser Arg Pro Phe Glu Val Arg Phe Asn Pro

         495         504         513         522         531         540
 CAT ACT GAG CGT GTA NAA ATT CTG GAC ACA GTT GAA AAA TTG GAT ACC CTT TTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 His Thr Glu Arg Val Xxx Ile Leu Asp Thr Val Glu Lys Leu Asp Thr Leu Leu

         549         558         567         576         585         594
 CAT CAA ATG AAC ACA GAA ATC CTA CAT CTG ACC AAT GCC ATT AAT AAA ATG CGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 His Gln Met Asn Thr Glu Ile Leu His Leu Thr Asn Ala Ile Asn Lys Met Arg

         603
 CGT CCT TTC TA 3'
--- --- --- --
 Arg Pro Phe
```

Figure 18. Partial nucleotide sequence and deduced amino acid sequence data from putative up-regulated cDNA clone HiD6.

Nucleotide sequence : Identical = 213/266 (80%)

```
HiD6:   10 ttggctttccgtatcttccagagcacccaatatgtgcgtcatgtgaactcacccttccac 69
           ||||| ||||| ||||||||||||||||| |||||||| || || |||||||| | ||||
DTH: 1021 ttggccttccgcatcttccagagcacccagtatgtgcgccacgttaactcaccataccac 1080

HiD6:   70 actccagaacctgattgcattcatgaattgttgggtcatatgcctttgttgtctgatccc 129
           || || || || || | |||||| ||  || ||||||| ||||| || || | ||||||
DTH: 1081 acccccgagcccgactccattcacgagctgctgggtcacatgcccctgctggccgatccc 1140

HiD6: 130 agctttgctcaattctctcaggaaattggtttggcctcattgggtgcttctgatgatgaa 189
           ||||| || || ||||| ||||| ||||| |||||||  ||||||| || || || |||
DTH: 1141 agcttcgcccagttctcgcaggagattggactggcctcgctgggtgcctccgacgaagaa 1200

HiD6: 190 attgaaaaattatctaccgtctactggttcaccgttgaattcggtctctgcaaagaacat 249
           || || ||  | || || || |||||||||||| ||||| |||||||||||||| ||||||
DTH: 1201 atcgagaagctgtccacggtatactggttcactgttgagttcggtctctgcaaggaacat 1260

HiD6: 250 ggcgatgtcaaggcctatggtgctgg 275
           ||  |  |||||||||| ||||||||
DTH: 1261 ggtcagatcaaggcctacggtgctgg 1286
```

Identical= 126/148 (85%)

```
HiD6: 335 ccttcgaacccgcctccaccgctgttcaaccctatcaagatcaagaataccaacccatct 394
           ||||||| ||||| |||||||| || || ||||| || ||||| || ||||| |||||||
DTH: 1346 ccttcgagcccgcatccaccgccgtgcagccctaccaggatcaggagtaccagcccatct 1405

HiD6: 395 attatgtggccgaaagtttcgatgatgccaaggacaaattccgtcgttgggtatccacca 454
           | |||||||||| || ||||| |||||||||||||| |||||||| |||||    |||||
DTH: 1406 actatgtggccgagagcttcgaggatgccaaggacaagttccgtcgctgggtgagcacca 1465

HiD6: 455 tgtcccgtcccttcgaagtgcgtttcaa 482
           |||| ||||| ||||| ||||||||||
DHT: 1466 tgtcgcgtccattcgaggtgcgtttcaa 1493
```

Amino acid sequence

```
HiD6 : 2   LASLAFRIFQSTQYVRHVNSPFHTPEPDCIHELLGHMPLLSDPSFAQFSQEIGLASLGAS 181
           LASLAFRIFQSTQYVRHVNSP+HTPEPD IHELLGHMPLL+DPSFAQFSQEIGLASLGAS
DTH  : 379 LASLAFRIFQSTQYVRHVNSPYHTPEPDSIHELLGHMPLLADPSFAQFSQEIGLASLGAS 438

HiD6 : 182 DDEIEKLSTVYWFTVEFGLCKEHGDVKAYGAGLLSAYGELLHAISDKCEHRPFEPASTAV 361
           D+EIEKLSTVYWFTVEFGLCKEHG +KAYGAGLLS+YGELLHAISDKCEHR FEPASTAV
DTH  : 439 DEEIEKLSTVYWFTVEFGLCKEHGQIKAYGAGLLSSYGELLHAISDKCEHRAFEPASTAV 498

HiD6 : 362 QPYQDQEYQPIYYVAESFDDAKDKFRRWVSTMSRPFEVRFNPHTERVXILDTVEKLDTLL 541
           QPYQDQEYQPIYYVAESF+DAKDKFRRWVSTMSRPFEVRFNPHTERV +LD+V+KL+TL+
DTH  : 499 QPYQDQEYQPIYYVAESFEDAKDKFRRWVSTMSRPFEVRFNPHTERVEVLDSVDKLETLV 558

HiD6 : 542 HQMNTEILHLTNAINKMRRPF 604
           HQMNTEILHLTNAI+K+RRPF
DTH  : 559 HQMNTEILHLTNAISKLRRPF 579
```

Figure 19. BLAST alignments (Altschul et al., 1990) of sequences from HiD6 and *Drosophila melanogaster* tyrosine hydroxylase (GenBank accession number U14395). Functionally similar amino acids are indicated by "+".

in the whole insect body and CNS was assessed by Northern blotting analysis. Expression of HiD6 in whole body and CNS are presented separately.

Expression of HiD6 in whole body. At pupation (day 0), expression of HiD6 was higher in diapause destined pupae compared to nondiapause pupae (Fig. 7). Throughout development, expression was highest at pupation (day 0) in both non-diapause and diapause destined pupae (Fig. 20). Expression was not detectable after pupation in either diapausing or nondiapausing pupae.

Expression of HiD6 in CNS. Expression of HiD6 was similar in the CNS of diapausing and nondiapausing insects on day 0 and appears to decrease slightly in day 15 diapause CNS compared to day 2 nondiapause CNS (Fig. 21).

4.4.6 **HiD9.** A 634 nucleotide fragment was sequenced for clone HiD9 and the deduced open reading frame represents 210 amino acids (Fig. 22). Two sections of this sequence have significant nucleotide homology with *D. melanogaster* ALA-E6 repeat region, GenBank accession number X57624 (Magoulas and Hickey, 1992) (Fig. 23). A 224 nucleotide section (from nucleotide 25 to 233) and a 470 nucleotide section (from nucleotide 173 to 634) have 66% (148/224) and 65% (307/470) homology to *D. melanogaster* ALA-E6 repeat region. The deduced reading frame is 63% (139/218) identical and 78% (171/218) identical and/or functionally similar to *D. melanogaster* CG11486 gene product [alt 1], GenBank accession number AE003477 (Adams et al., 2000) (Fig. 24).

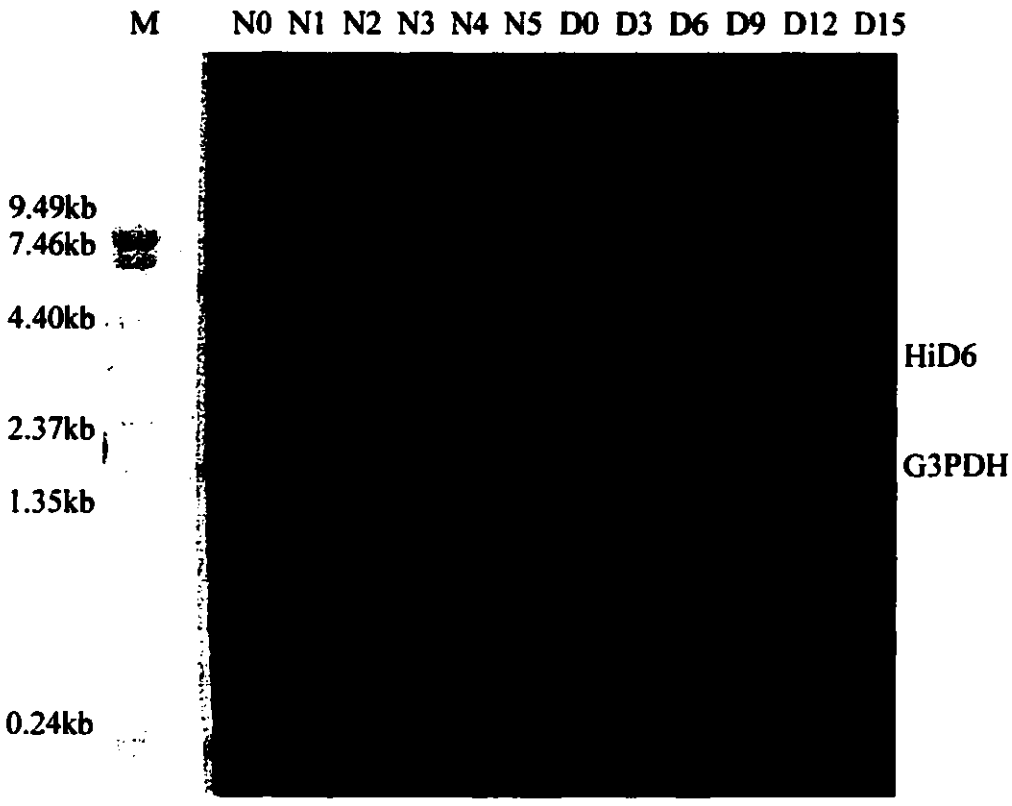

Figure 20. Expression of HiD6 in *H. irritans* pupae reared at nondiapause (N) and diapause (D) inducing temperatures. RNA was extracted from nondiapausing pupae 0, 1, 2, 3, 4 and 5 days after pupation and from diapausing pupae 0, 3, 6, 9, 12 and 15 days after pupation. Each lane contains 20μg of total RNA. M=molecular weight markers.

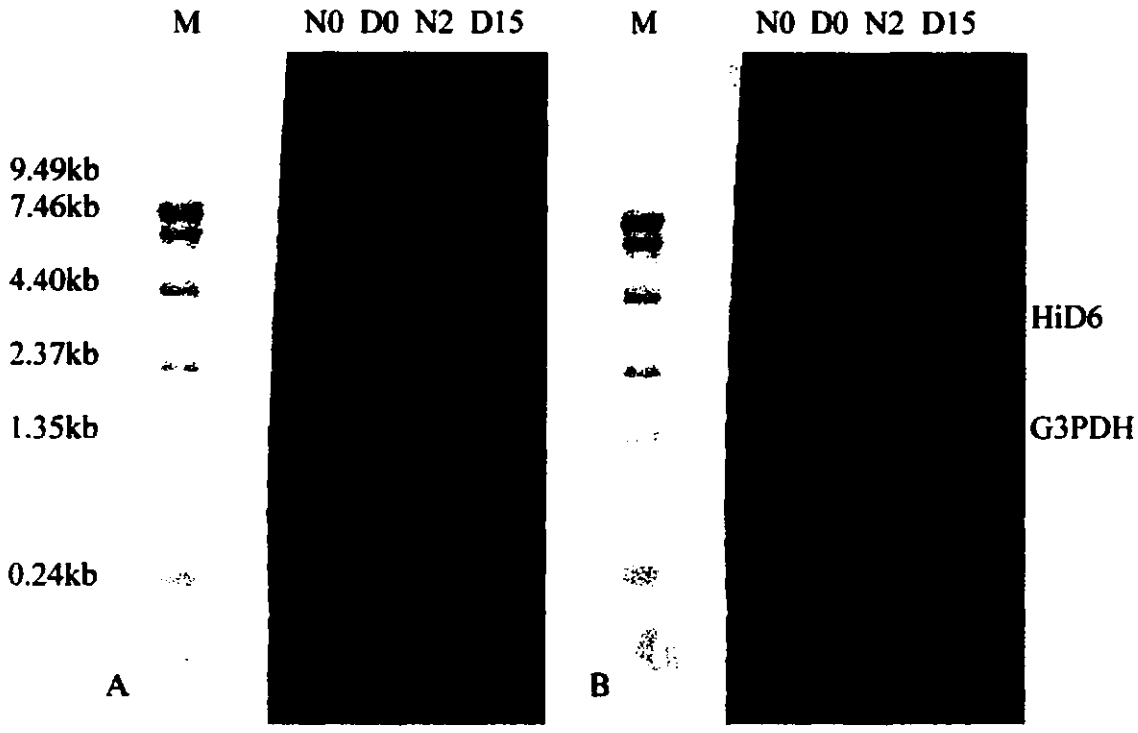

Figure 21. Northern blot analysis of nondiapause (N) and diapausing (D) pupal RNA probed with HiD6. Total RNA extracted from approximately 3-5 pupae whole pupae (A) and CNS of 30 pupae (B) on day 0 and 2 for nondiapausing pupae and day 0 and 15 for diapausing pupae. M=molecular weight markers.

```
            9          18          27          36          45          54
5'CTT AAA GGT GGA CGC AGT AGT TTG CGG CGC AAT GAG TCT CCC TCA AGT GTA GGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Leu Lys Gly Gly Arg Ser Ser Leu Arg Arg Asn Glu Ser Pro Ser Ser Val Gly

           63          72          81          90          99         108
 GAG AAA TCT CCG CCA CGC ATA ACA CCG CAT GCA TCG CCC ATA CCC ACC ACA TTG
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Glu Lys Ser Pro Pro Arg Ile Thr Pro His Ala Ser Pro Ile Pro Thr Thr Leu

          117         126         135         144         153         162
 CCC GGT AGT GTA CAT CAA GAA AAT GTT GGT GGC ACT GTC TAT TTT TAT CCA ACT
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Pro Gly Ser Val His Gln Glu Asn Val Gly Gly Thr Val Tyr Phe Tyr Pro Thr

          171         180         189         198         207         216
 GCC AAT CAC ACA ACC GCC CAG AAT TCC AAT GCC ATT GTT GGC ATT GGT GCC AGC
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Ala Asn His Thr Thr Ala Gln Asn Ser Asn Ala Ile Val Gly Ile Gly Ala Ser

          225         234         243         252         261         270
 GGT GTA GTC GAT GCG GTC TCA TCA CAT CAC TCT GGC CAA ACG GCT GGT GCA TCT
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Gly Val Val Asp Ala Val Ser Ser His His Ser Gly Gln Thr Ala Gly Ala Ser

          279         288         297         306         315         324
 TTG ACG CCC GTC CAA CCT TCA CTT TTG TAT ACG GGT CAT GTG TAT CCG GGT CCT
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Leu Thr Pro Val Gln Pro Ser Leu Leu Tyr Thr Gly His Val Tyr Pro Gly Pro

          333         342         351         360         369         378
 GCG TCA AAT GTA ATA ACA ATG CAA CCG AAA ACA CAA CTG GAA TCG GCA TTC TTT
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Ala Ser Asn Val Ile Thr Met Gln Pro Lys Thr Gln Leu Glu Ser Ala Phe Phe

          387         396         405         414         423         432
 GTG CCG GAT GAA ATG CGT AGC GAA CTT TTG TCG CGC AAT ATG ATC TCG AAT TTA
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Val Pro Asp Glu Met Arg Ser Glu Leu Leu Ser Arg Asn Met Ile Ser Asn Leu

          441         450         459         468         477         486
 TTA ATG GAT GCC AGC GAA GCA GCA CAA CAT GCC CTG CCA ATG GAA ATT GAT AAT
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Leu Met Asp Ala Ser Glu Ala Ala Gln His Ala Leu Pro Met Glu Ile Asp Asn

          495         504         513         522         531         540
 TAT CAT TCA TTG TAT CCA TTG GAA GCG TTA CCC GTA CAA CCA TTA CAT GCC AAG
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Tyr His Ser Leu Tyr Pro Leu Glu Ala Leu Pro Val Gln Pro Leu His Ala Lys

          549         558         567         576         585         594
 TTA ACT CTG CCC TCG TCC ACC TAT AAG GCA ACG AAT AGT ACA ACC GGT ATC AAA
 --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 Leu Thr Leu Pro Ser Ser Thr Tyr Lys Ala Thr Asn Ser Thr Thr Gly Ile Lys

          603         612         621         630
 TAC TGT TTG AGG AGA TTA CAT GGG TTC CGA TTA CAA TCA A 3'
 --- --- --- --- --- --- --- --- --- --- --- --- --- -
 Tyr Cys Leu Arg Arg Leu His Gly Phe Arg Leu Gln Ser
```

Figure 22. Partial nucleotide sequence and deduced amino acid sequence data from putative up-regulated cDNA clone HiD9.

Identical = 148/224 (66%)

```
HiD9:  25 CGGC-GCAATGAGT-CTCCCTCAAGTGTAGGTGAGAAATCTCCGCC--ACGC-ATAACAC 79
          |||| || |||| | |   | || |   |||||||||| || || ||  |||  || || |
ALAE6:430 CGGCAGCCATGATTTCGGGCGCAGGGCCAGGTGAGAAGTCGCCACCGCACGGGATGACGC 489

HiD9:  80 CGCATG---CATCGCCCATACCCACCA-CATTGCCCGGTAGTGTACATCAAGAAAATGTT 135
          |||| |   |||||||||||||| | || |  |||||   || || || || || || ||
ALAE6:490 CGCACGGAGCATCGCCCATACC-ATCAGCGCTGCCCACCAGCGTGCACCAGGAGAACGTA 548

HiD9: 136 GGTGGCACTGTCTATTTTTATCCAACTGCCAAT-CACACAACCGCCCAGAAT-TC--CAA 191
          || |||||  | || || ||||| |||||||| |||| ||| ||| ||    ||  |||
ALAE6:549 GGCGGCACCATATACTTCTATCCGACTGCCAACGCACAGAACAGCC-AGCCCGTCGTCAA 607

HiD9: 192 TGCCATTGTTG-GCATTGGTGC-CAGCGGTGTAGTCGATGCGGT 233
          | |||| || | | || ||  | || | |  |  || |||  ||
ALAE6:608 TTCCATGGTGGTGGAT-GGAACACATCCGGCTC-TCCATGGCGT 649
```

Identical= 307/470 (65%)

```
HiD9: 173 CAACCGCCCAGAATTCCAATGCCATTGTTGGCATTGGTGCCAGCGGTGTAGTCGATGCGG 232
          |||| || |||||   | | |||   || | || |  | |||  ||||  || |||| |
ALAE6:578 CAAC-GCACAGAACAGCCA-GCCC--GTCGTCAAT--T-CCAT-GGTG--GTGGATG-GA 626

HiD9: 233 TCTCATCACATCACTC--TGGCCAAACGGCTGGTGCATCTTTGAC-GCC--CGT-CCAAC 286
          | |||| |  | |||  ||||   |  |||| ||  |  ||| ||   ||| || |
ALAE6:627 ACACATC-CGGCTCTCCATGGCGTGAGTGCTGTGGCGCCGATGAGTGCGGGCGTTCCGGC 685

HiD9: 287 CTTCACTTTTGTATACGGGTCATGTGTATCCGGGTCCTGCGTCAAATGTAATAACAATGC 346
            || |  |||| ||||| |||||||||||||| ||  | || || ||  | || ||||
ALAE6:686 GGCCA-TGATGTACACGGGGCATGTGTATCCGGGACCGTCCTCCAACGTGGTCACCATGC 744

HiD9: 347 AACCGAAAACACAACTGGAATCGGCATTCTTTGTGCCGGATGAAATGCGTAGCGAACTTT 406
          | || || || |  ||||| ||||| || ||| |||| || || |||||   |||| |
ALAE6:745 AGCCAAAGACGCTGCTGGAGTCGGCCTTTTTTATGCCCGACGAGATGCGCGCCGAAGTCC 804

HiD9: 407 TGTCGCGCAATATGATCTCGAATTTATTAATGGATGCCAGCGAAG-CAGCACAACATGCC 465
          |  | |||||   |||||| ||  |  | ||||| || |||| || | || || || ||
ALAE6:805 TCGCCCGCAACGAGATCTCTAACCTGATCATGGACGC-AGCGGAGGCTGCGCAGCACGCT 863

HiD9: 466 CTGCCAATGGAAATTGATAATTATCATTCATTGTATCCATTGGAAGCGTTACCCGTACAA 525
          || ||  |||| | || || || ||| |  |||||||| ||||| ||   || |  ||
ALAE6:864 CTACCGCTGGAGGTGGAGAACTACCATGCCCTGTATCCACTGGAACCG---CCGGCGCAG 920

HiD9: 526 CCATTACATGCCAAGTTAACTCTGCCCTCGTCCACCTATAAGGCAACGAATAGTACAACC 585
          || || || |||||| | ||  | ||| |  |||| |||| ||| ||| | |  || ||
ALAE6:921 CCGTTGCACGCCAAGCTCACGTTCCCCGCCACCACTTATAGGGCCACGCACAACACGACG 980

HiD9: 586 GG-TATCAAATACTGTTTGAGGAGATTACATGGGTTCCGATTACAATCAA 634
          || || ||| |||||  || | ||| |||||||| |||||  | || || |
ALAE6:981 GGCTA-CAAGTACTGCCTGCGAAGAATACATGGTTTCCGCCTGCAGTCGA 1029
```

Figure 23. BLAST alignment (Altschul et al., 1990) of nucleotide sequence from HiD9 and *Drosophila melanogaster* ALA-E6 repeat region (GenBank accession number X57624)

```
HiD9:    10 GRSSLRRNESPSSV--------GEKSPPR-ITPH-ASPIPTTLPGSVHQENVGGTVYFYPT 162
            GR ++ R ESP++         GEKSPP  +TPH ASPIP+ LP SVHQENVGGT+YFYPT
CG11486:174GRGAMMRQESPTAAMISGAGPGEKSPPHGMTPHGASPIPSALPTSVHQENVGGTIYFYPT 233

HiD9:   163ANHTTAQNSNAIVGIGASGVVDAVSSHHSGQTAGASLTPVQPS-LLYTGHVYPGPASNVI 339
            AN    AQNS  +V     S VVD        G +A A ++    P+ ++YTGHVYPGP+SNV+
CG11486:234AN---AQNSQPVVN---SMVVDGTHPALHGVSAVAPMSAGVPAAMMYTGHVYPGPSSNVV 287

HiD9:   340TMQPKTQLESAFFVPDEMRSELLSRNMISNLLMDASEAAQHALPMEIDNYHSLYPLEALP 519
            TMQPKT LESAFF+PDEMR+E+L+RN  SNL+MDA+EAAQHALP+E++NYH+LYPLE  P
CG11486:288TMQPKTLLESAFFMPDEMRAEVLARNEISNLIMDAAEAAQHALPLEVENYHALYPLEP-P 346

HiD9:    520VQPLHAKLTLPSSTYKATNSTTGIKYCLRRLHGFRLQS 633
             QPLHAKLT P++TY+AT++TTG KYCLRR+HGFRLQS
CG11486: 347AQPLHAKLTFPATTYRATHNTTGYKYCLRRIHGFRLQS 384
```

Figure 24. BLAST alignment (Altschul et al., 1990) of HiD9 and *Drosophila melanogaster* CG11486 gene product [alt 1] (GenBank accession number AE003477). Functionally similar amino acids are indicated by "+".

The functions of ALA-E6 repeat region and CG11486 gene product [alt 1] are unknown. The top 20 BLAST results for CG11486 gene product [alt 1] are shown in Table 4. The closest sequence homology (highest score) is to *D. melanogaster* CG11486 gene product [alt 4] (GenBank accession number AAF47713). This protein is almost identical to CG11486 gene product [alt 1] and its function is also unknown. Most of the matches sharing sequence homology with HiD9 appear to be hypothetical proteins from a number of organisms. Properties of HiD9 were analyzed using PROPSEARCH (Hobohm and Sander, 1995) in an attempt to determine functional or structural homologues. The top 20 results for the PROPSEARCH analysis are shown in Table 4. Entries with a distance from 12.5 to 13.7 have greater than a 53% chance of belonging to the same protein family. Entries with a distance from 13.7 to 14.9 have greater than a 41% chance of belonging to the same protein family. The highest match is to DREG-5 from *D. melanogaster* (SwissProt ID reg5_drome). There is greater than a 53% chance that DREG-5 and HiD9 belong to the same protein family. Almost all of the other matches are transcription factors, including paired-box proteins and homeobox proteins.

Temporal expression patterns of clone HiD9 are hard to interpret due to extremely high background levels on Northern blots (Fig. 25).

4.4.7 **HiD10.** A 621 nucleotide fragment was sequenced for clone HiD10 (Fig. 26). Clone HiD10 does not have significant nucleotide homology to entries in GenBank, EMBL or Berkeley *Drosophila* genome database. The deduced open reading frame represents 206 amino acids. The highest amino acid homology is to carboxylesterase-5A from *D. pseudoobscura* Frolova, GenBank accession number

Table 4. Top 20 BLASTP results (Altschul et al., 1990) for *Drosophila melanogaster* CG11486 gene product [alt 1] (GenBank accession number AE003477) and Top 20 PROPSEARCH results (Hobohm and Sander, 1995) for HiD9.

Top 20 BLASTP results for *Drosophila melanogaster* CG11486 gene product [alt 1]

	SCORE	ACCESSION	PROTEIN DESCRIPTION
1.	3798	AAF47713	CG11486 gene product [alt 4] [*Drosophila melanogaster*]
2.	872	P34653	HYPOTHETICAL 76.2 KDA PROTEIN ZK632.7 IN CHROMOSOME III
3.	756	BAB28221	putative [*Mus musculus*]
4.	496	CAA81860	ORF YKL025c [*Saccharomyces cerevisiae*]
5.	369	CAB58166	hypothetical protein [*Schizosaccharomyces pombe*]
6.	358	CAB11083	hypothetical protein [*Schizosaccharomyces pombe*]
7.	144	AAB30051	Hkr1p [*Saccharomyces cerevisiae*, YNN295, Peptide, 1802 aa]
8.	142	CAB88653	hypothetical protein [*Neurospora crassa*]
9.	137	AAB64857	Hkr1p; YDR420W; CAI: 0.10 [*Saccharomyces cerevisiae*]
10.	134	CAB46680	proteophosphoglycan [*Leishmania major*]
11.	128	CAA98194	hypothetical protein Rv2082 [*Mycobacterium tuberculosis*]
12.	126	BAA34474	KIAA0754 protein [*Homo sapiens*]
13.	123	AAG21430	L8453.1 [*Leishmania major*]
14.	120	VGBEX1	glycoprotein X precursor - equine herpesvirus 1 (strain Ab4p)
15.	116	AAF48196	Smr gene product [*Drosophila melanogaster*]
16.	116	CAA63845	SOX3 protein [*Mus musculus*]
17.	115	AAC48525	gastric mucin [*Sus scrofa*]
18.	114	AAD39760	transcriptional activator SRCAP [*Homo sapiens*]
19.	114	AAB03322	transcription factor HOXA13 [*Mus musculus*]
20.	114	CAA86176	mal5, sta1, len: 1367, [*Saccharomyces cerevisiae*]

Top 20 PROPSEARCH results for HiD9.

	DIST.	SWISSPROT ID	PROTEIN DESCRIPTION
1	13.19	reg5_drome	RHYTHMICALLY EXPRESSED GENE 5 PROTEIN (DREG-5).
2	13.63	vp40_mabvm	MATRIX PROTEIN VP40.
3	13.93	pax9_chick	PAIRED BOX PROTEIN PAX-9 (FRAGMENT).
4	13.94	vp40_mabvp	MATRIX PROTEIN VP40 (VP3).
5	13.97	px8a_human	PAIRED BOX PROTEIN PAX-8, ISOFORMS 8A/8B.
6	14.05	pax8_rat	PAIRED BOX PROTEIN PAX-8.
7	14.06	dlx1_brare	HOMEOBOX PROTEIN DLX-1.
8	14.18	pax8_mouse	PAIRED BOX PROTEIN PAX-8.
9	14.30	vhel_shvx	PROBABLE HELICASE (ORF 2).
10	14.42	dlx1_mouse	HOMEOBOX PROTEIN DLX-1.
11	14.45	px8a_canfa	PAIRED BOX PROTEIN PAX-8, ISOFORM 8A.
12	14.46	dsg1_mouse	DESMOGLEIN 1 (DG1) (FRAGMENT).
13	14.49	ydfc_schpo	HYPOTHETICAL 34.0 KD PROTEIN C17C9.12 IN CHROMOSOME I.
14	14.53	vhel_p1amv	PUTATIVE HELICASE (ORF 2).
15	14.58	pax2_brare	PAIRED BOX PROTEIN PAX[ZF-B] (PAX-2).
16	14.62	sox2_chick	TRANSCRIPTION FACTOR SOX-2.
17	14.65	sox3_xenla	TRANSCRIPTION FACTOR SOX-3 (SOX-11).
18	14.71	otx3_brare	HOMEOBOX PROTEIN OTX3 (ZOTX3).
19	14.82	movp_cmvq	CELL-TO-CELL MOVEMENT PROTEIN (MP) (3A PROTEIN).
20	14.87	ga1b_xenla	GATA BINDING FACTOR-1B (TRANSCRIPTION FACTOR).

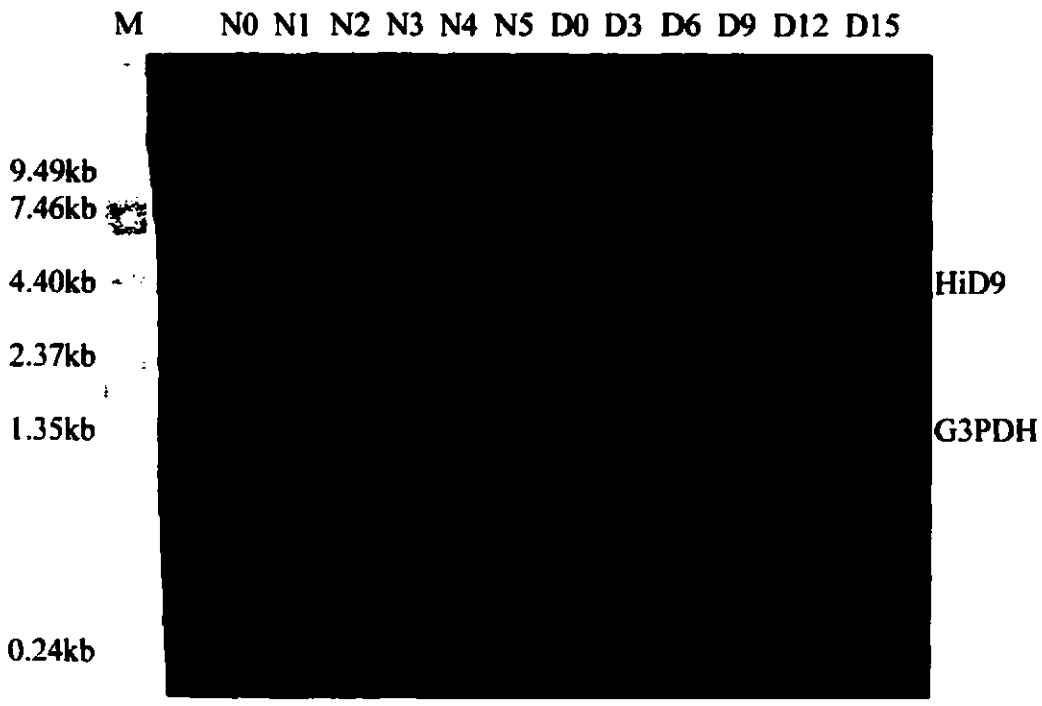

Figure 25. Expression of HiD9 in *H. irritans* pupae reared at nondiapause (N) and diapause (D) inducing temperatures. RNA was extracted from nondiapausing pupae 0, 1, 2, 3, 4 and 5 days after pupation and from diapausing pupae 0, 3, 6, 9, 12 and 15 days after pupation. Each lane contains 20μg of total RNA. M= molecular weight markers.

```
            9          18          27          36          45          54
5'TTA AGA CCC TCC TTG GCC AGT ATT GCT AAG GCT GTA GTG TCA ATG AGT GGA GTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  Leu Arg Pro Ser Leu Ala Ser Ile Ala Lys Ala Val Val Ser Met Ser Gly Val

           63          72          81          90          99         108
  GCT CTA AAT CCT TGG GCC ATA ACC ACA AAT GCC CCA GAT AAT GCT AAA CGT TTG
  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  Ala Leu Asn Pro Trp Ala Ile Thr Thr Asn Ala Pro Asp Asn Ala Lys Arg Leu

          117         126         135         144         153         162
  GCG GCT GCT GTT AAT TGC ACC AAC ACC AAT ACC AGT CAG GAT ATA AAA GCC TGT
  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  Ala Ala Ala Val Asn Cys Thr Asn Thr Asn Thr Ser Gln Asp Ile Lys Ala Cys

          171         180         189         198         207         216
TTA AAG GCC GCC AAA CCC GAA GAT GTT GTG GGG GCT GTC CAA TCT TTG CTA AAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Leu Lys Ala Ala Lys Pro Glu Asp Val Val Gly Ala Val Gln Ser Leu Leu Asn

          225         234         243         252         261         270
TGG GGT TAT AAT CCC TTT ACC ACA TTT GGG CCA GTA ATA GAA TCG CCC AAT ACG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Trp Gly Tyr Asn Pro Phe Thr Thr Phe Gly Pro Val Ile Glu Ser Pro Asn Thr

          279         288         297         306         315         324
CCT AAT GCC TTT CTA ACC GAG CAA CCG GAA GCT ATC ATA CGA TCC GGT GCT TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Pro Asn Ala Phe Leu Thr Glu Gln Pro Glu Ala Ile Ile Arg Ser Gly Ala Phe

          333         342         351         360         369         378
AGT CAT ATT CCT TGG CTG GCC TCA TAC ACC ACC AAT GAT GGA GCT TTC AAT GCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ser His Ile Pro Trp Leu Ala Ser Tyr Thr Thr Asn Asp Gly Ala Phe Asn Ala

          387         396         405         414         423         432
GCC GAA CTA TTG CGT ATA AAC TCT CGC ACT GGT ACC GAA TTC CTT AAT GAA ATG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ala Glu Leu Leu Arg Ile Asn Ser Arg Thr Gly Thr Glu Phe Leu Asn Glu Met

          441         450         459         468         477         486
AAT GAC AAT TGG CTG GAC ATG GCT CCA GAA AAT CTA TTC CTT AAA AAT CTC CAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Asn Asp Asn Trp Leu Asp Met Ala Pro Glu Asn Leu Phe Leu Lys Asn Leu His

          495         504         513         522         531         540
GAC AAC CCC CGG GAC TAT GCC CAA AGT CTA AAG GAT ATC TAT ATA GGC AAA GAA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Asp Asn Pro Arg Asp Tyr Ala Gln Ser Leu Lys Asp Ile Tyr Ile Gly Lys Glu

          549         558         567         576         585         594
AAT TTC ACT GTG GAA AAT TAT CTG GAA ATT CAA ACG ATG TAC ACT GAT GTC CTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Asn Phe Thr Val Glu Asn Tyr Leu Glu Ile Gln Thr Met Tyr Thr Asp Val Leu

          603         612
ATG CGA TTG GGT GTC CTA AAG GCT AT 3'
--- --- --- --- --- --- --- --- --
Met Arg Leu Gly Val Leu Lys Ala
```

Figure 26. Partial nucleotide sequence and deduced amino acid sequence data from putative up-regulated cDNA clone HiD10.

AF016135 (unpublished) (Fig. 27). In this stretch of sequence, 42% (87/206) of amino acids are identical and 59% (123/206) of amino acids are identical and/or functionally similar *to D. pseudoobscura* carboxylesterase-5A.

Homology of HiD10 and juvenile hormone esterase precursor from *Heliothis virescens* (F.) (GenBank accession number J04955) is considered insignificant as they share homology for only a very short segment of the sequences (Fig. 27). For a 44 amino acid stretch of sequence, 31% (14/44) of amino acids are identical and 56% (25/44) of amino acids are identical and/or functionally similar.

At pupation (day 0), expression of HiD10 was higher in diapause destined pupae compared to nondiapause pupae (Fig. 7). Expression increased throughout development in nondiapausing pupae, and remained at a constant low level in diapausing pupae (Fig 28).

HiD10 and *D. pseudoobscura* carboxylesterase-5A.

```
HiD10: 2   LRPSLASIAKAVVSMSGVALNPWAITTNAPDNAKRLAAAVNCTNTNTSQDIKACLKAAKP 181
           LR    + +AKA +S SG AL+PW I        A  L   V C    N S  +K CLK+
Est5A: 221 LREDFSKLAKAAISFSGNALDPWVIQQGLRGRAFELGRIVGCGQANDSVTLKKCLKSKPA 280

HiD10: 182 EDVVGAVQSLLNWGYNPFTTFGPVIESPNTPNAFLTEQPEAIIRSGAFSHIPWLASYTTN 361
            ++V AV+S L + Y PFT FGP IESP+ P AF+T+ P  II+SG FS +PW  +YTT
Est5A: 281 SEIVSAVRSFLVFSYVPFTPFGPAIESPDAPEAFITQHPIDIIKSGKFSQVPWAVTYTTE 340

HiD10: 362 DGAFNAAELLRINSRTGTEFLNEMNDNWLDMAPENLFLKNLHDNPR---DYAQSLKDIYI 532
           DG +NAA LL   + +G E + ++ND W D AP  LF ++     +   DY++ L+  Y+
Est5A: 341 DGGYNAALLLEKQASSGRELIVDLNDRWFDWAPYLLFYRDSMTTIKDMDDYSRKLRQEYL 400

HiD10: 533 GKENFTVENYLEIQTMYTDVLMRLGV 610
           G   F+VE+Y ++Q M+TD+L +  V
Est5A: 401 GDRRFSVESYWDVQRMFTDLLFKNSV 426
```

HiD10 and *H. virescens* juvenile hormone esterase precursor.

```
HiD10:  77 TTFGPVIESPNTP-NAFLTEQPEAIIRSGAFSHIPWLASYTTND 119
           TTF P++ESP       + + PE +I  G   ++P L  +T+++
JH Est:308 TTFLPIVESPLPGVTTIIDDDPEILIAEGRGKNVPLLIGFTSSE 351
```

Figure 27. BLAST alignments (Altschul et al., 1990) of amino acid sequences from HiD10 and *Drosophila pseudoobscura* carboxylesterase-5A (GenBank accession number AF016135) and HiD10 and *Heliothis virescens* juvenile hormone esterase precursor (GenBank accession number J04955).

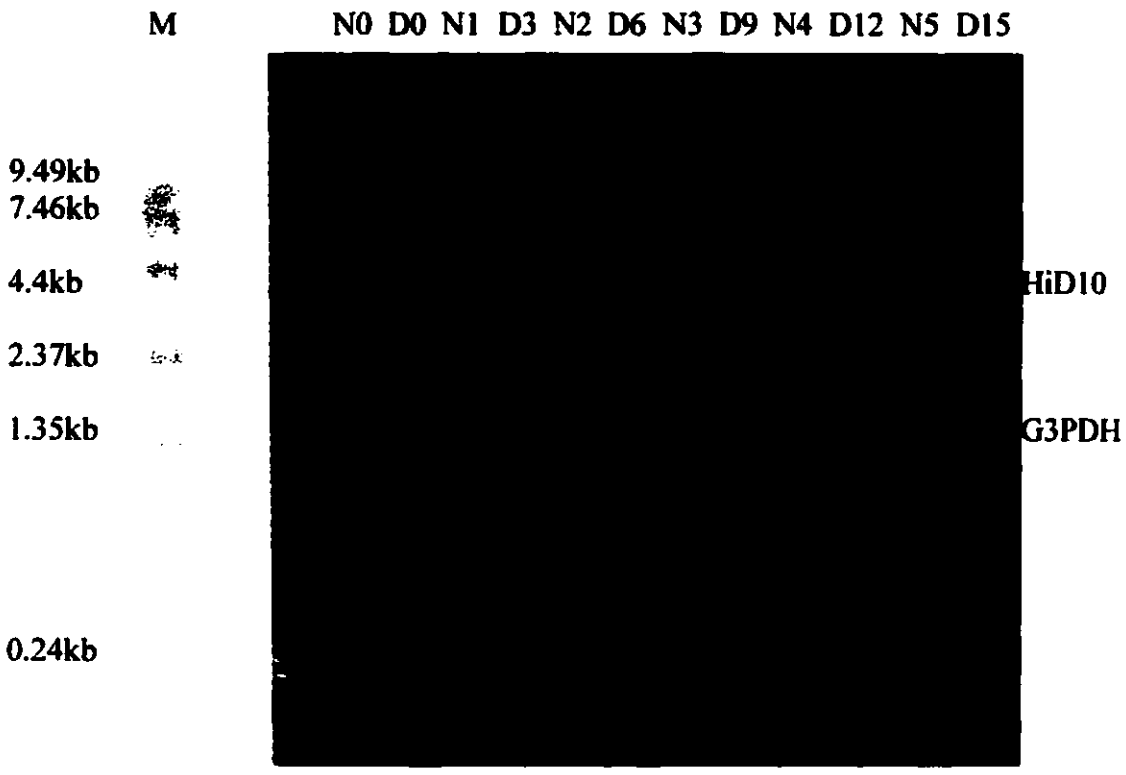

Figure 28. Expression of HiD10 in *H. irritans* pupae reared at nondiapause (N) and diapause (D) inducing temperatures. RNA was extracted from nondiapausing pupae 0, 1, 2, 3, 4 and 5 days after pupation and from diapausing pupae 0, 3, 6, 9, 12 and 15 days after pupation. Each lane contains 20μg of total RNA. M=molecular weight markers.

4.4.8 **Summary of temporal expression patterns.** A summary of temporal expression pattern of the putative diapause up-regulated genes is shown in Table 5. Expression of the putative diapause up-regulated genes tended to fall into two patterns. HiD4 showed a unique pattern of expression. This gene was expressed equally at pupation (day 0) in total RNA extracted from nondiapausing and diapause destined pupae. Expression increased before moulting (day 4 and 5) in nondiapausing pupae, and decreased throughout diapause.

The rest of the genes had slightly varied patterns of expression, but showed some basic trends. Highest expression occurred at pupation in diapause destined pupae. For HiD3, 5 and 6, that is transferrin, serine protease inhibitor and tyrosine hydroxylase, the difference in expression between nondiapause and diapause was very pronounced, whereas the difference is less evident for HiD1 and 10. Expression often increased in the last days of pupal development (closer to moult) in nondiapausing pupae. This was the case for HiD1, 3, 6 and 10.

Table 5. Summary of temporal expression of putative diapause up-regulated genes.

Clone	Identity	Expression during Nondiapause (day)						Expression during Diapause (day)					
		0	1	2	3	4	5	0	3	6	9	12	15
HiD1	none	+				+	+	+					
HiD3	transferrin	+			+	+	+	++					
HiD4	cytochrome c oxidase	++	++	++	++	+++	+++	++	++	+	+	-	+
HiD5	serine protease inhibitor	+						++					
HiD6	tyrosine hydroxylase	+						++					
HiD10	carboxylesteras	+	+	+	+	+	+	+	+	-+	++	--	+-+

CHAPTER 5

DISCUSSION

5.1 Developmental arrest during diapause.

The stage of pupation in which diapause occurs in horn flies appears similar to that of two other flies, *Sarcophaga argyrostoma* (Robineau-Desvoidy) and *S. bullata* (Parker). Developmental arrest of *S. argyrostoma* and *S. bullata* occurs soon after head eversion, before the pupal-adult moult (apolysis), and in most instances, before any visible signs of antennal development (Fraenkel and Hsiao, 1968). During metamorphosis at 23°C, horn flies undergo head eversion (larval-pupal ecdysis) approximately 24 hr after pupation, and pupal-adult apolysis occurs during the period of 40 hr to 65 hr after pupation (Thomas, 1985). Morphology of diapausing horn flies indicates that development arrests between 24 hr to less than 48 hr after pupation. Thus like *S. argyrostoma* and *S. bullata,* horn flies diapause in the interval between head eversion and pupal-adult apolysis.

Put into perspective of Thomas's (1985) schematic representation of the stages of metamorphosis of horn fly pupae, horn flies diapause during the phanerocephalic pupal stage. This is consistent with the claim that diapausing horn fly pupae have yellow eyes (Depner, cited in Kunz and Miller, 1985) but not with the claim made by Thomas that horn flies diapause as red-eyed pharate adults (Thomas, 1985).

There are two peaks in ecdysone levels during pupal-adult metamorphosis of the Australian sheep blowfly, *Lucilia cuprina* (Weidemann) (Barritt and Birt, 1971), fruit fly, *D. melanogaster* (Handler, 1984) and stable fly, *Stomoxys calcitrans* (L.)

(O'Neill et al., 1977). The first peak occurs during the larval-pupal apolysis and is associated with pupation. The second peak occurs during the pupal-adult apolysis, and is associated with histogenesis of imaginal tissues during metamorphosis. Considering the possibility of similar patterns of ecdysone production in a broad range of flies, it may be reasonable to assume that there are two peaks of ecdysone in the horn fly, and the second peak occurs during pupal-adult apolysis. Significantly, developmental arrest in the horn fly may be speculated to occur before the second peak.

Both body and CNS appear to arrest at similar developmental stages, that is, prior to pupal-adult apolysis. It is unknown whether all tissues arrest simultaneously in the horn fly. During diapause induction of *Chymomyza costata* larvae, imaginal discs and CNS immediately slow development whereas body development slows after 3 days (Kostal et al., 2000).

The CNS of diapausing horn fly pupae remain developmentally arrested throughout prolonged storage at 5°C (Table 1). Similarly, brain cells of *S. crassipalpis* remain arrested at the G0/G1 phase of the cell cycle during diapause (Tammariello and Denlinger, 1998a). Developmental and cell cycle arrest of the CNS may be a good indicator of diapause status.

5.2 Isolation and identification of putative diapause up-regulated cDNAs from horn fly pupal cDNA library.

A subtracted library containing putative hornfly diapause up-regulated cDNAs hybridized with approximately 10% of diapausing pupal cDNAs. Although this number is likely high due to false positives, the percentage of diapause up-

regulated clones is similar to other percentages reported in the literature. In the brain of *S. crassipalpis*, four percent of expressed genes (Flannagan et al., 1998) and nine percent of expressed proteins (Joplin et al., 1990) are up-regulated during diapause. The percentage of false positives may vary from 5% to 95% for suppression subtractive hybridization (Diatchenko et al., 1996; Gurskaya et al., 1996; Clonetech PCR-Select cDNA subtraction kit user manual).

5.2.1 HiD 6: Tyrosine hydroxylase.

Expression of TH in the whole body. Tyrosine hydroxylase (TH) catalyzes the first and rate-limiting step in dopamine (DA) synthesis. This enzyme catalyzes the hydroxylation of tyrosine to L-dopa (L-3, 4-dihydroxyphenylalanine), which is then converted to dopamine.

TH gene expression in whole bodies of horn fly pupae appears similar to the expression of dopa decarboxylase (DDC) in whole bodies the cabbage armyworm, *Mamestra brassicae* (L.). DDC is an enzyme in the dopamine biosynthetic pathway that catalyzes the transformation of L-dopa to dopamine. Diapause destined *M. brassicae* have significantly higher levels of dopamine compared to nondiapausing pupae (Noguchi and Hayakawa, 1997). This increase in dopamine is attributed to the expression of DDC. The mechanism by which haemolymph dopamine inhibits development of *M. brassicae* remains unknown (Noguchi and Hayakawa, 1997). TH was also measured in *M. brassicae*, and in contrast to the horn fly, TH expression may be similar between nondiapause and diapause destined *M. brassicae* pupae (Noguchi and Hayakawa, 1997).

Increased dopamine levels have also been noted in other developmentally arrested insects. Dopamine levels are higher in the whole body of diapause destined 2nd and 3rd instar larvae of the fly, *C. costata*, compared to continuously developing larvae (Kostal et al., 1998). Developmentally delayed, parasitized armyworm larvae, *Pseudaletia separata* Walker, have increased levels of dopamine in the integument, haemolymph and CNS (Noguchi and Hayakawa, 1997).

TH expression in the whole body is primarily associated with pupation (day 0) in both diapause and non-diapause destined horn fly pupae. The pattern of TH expression in horn flies is similar to the levels of dopamine in *M. brassicae*. The highest levels of dopamine occur during pupation, in both nondiapausing and diapause destined *M. brassicae*, but are significantly higher in diapause destined pupae (Noguchi and Hayakawa, 1997).

The cuticular pool contributes the majority of dopamine in whole insects (Kostal et al., 1998), and is not involved in diapause in the fly, *C. costata* (Kostal et al., 1999). Northern blots also indicate that whole body RNA contributes the majority of TH in the horn fly (Figure 21). Cuticular dopamine is a precursor for sclerotization and melanization (reviewed by Wright, 1987). Products derived from dopamine are incorporated into the cuticle where they cross-link proteins and chitin, resulting in a hardened and strengthened cuticle. Increased levels of TH expression during pupation of diapause-destined horn flies may ultimately result in increased sclerotization and melanization of the puparium.

Increased melanization of the cuticle of diapausing adult insects has been well documented for a number of species (reviewed by Tauber et al., 1986).

Typically, overwintering coloration is an adaptation to camouflage these insects from predators throughout diapause. Increased melanization of the horn fly puparium, which is already highly melanized, is not likely to provide increased protection from predators. During sclerotization, dopamine is incorporated into the cuticle, oxidized into quinone, and then cross-linked to protein and/or chitin (reviewed by Wright, 1987). Sclerotization results in strengthening and hardening of the cuticle. During diapause, increased dopamine could result in increased sclerotization and may ultimately result in increased strength that could provide extra protection from either parasitism or the environment. Parasitism of pupae by *Spalangia* wasps before the onset of cold weather is a significant mortality factor of diapausing horn fly pupae (Thomas and Kunz, 1986). To the best of my knowledge, there are no references of increased sclerotization of the cuticle or puparium during diapause. However, there are other changes associated with the puparium of diapausing *S. crassipalpis*. The puparium of diapausing *S. crassipalpis* has increased hydrocarbon (Yoder et al., 1992, 1995). The puparium of diapause and nondiapause horn flies should be compared for possible differences in structure and properties.

Expression of TH in the CNS. Dopamine is a candidate for the regulation of diapause induction by acting as a neurohormone in the insect nervous system (Houk and Beck, 1977). Dopamine modulates expression of diapause hormone in *B. mori* (Noguchi and Hayakawa, 2001). As well, dopamine levels in diapause-destined *M. brassicae* pupae may inhibit brain neurosecretion of PTTH, leading to diapause induction (Noguchi and Hayakawa, 1997).

In CNS tissues of nondiapause and diapause destined horn fly pupae (day 0), levels of TH expression are comparable. Nondiapause and diapause destined, *C. costata* larvae also produce similar levels of dopamine in the CNS during the sensitive period for induction (Kostal et al., 1999).

Comparable levels of TH expression in the CNS of nondiapause and diapause destined horn flies suggest that increased levels of dopamine are not involved in diapause induction. However, it must be noted that Northern blotting compares the level of TH transcripts and is therefore not necessarily indicative of dopamine content. Direct measurement of dopamine in the CNS (i.e. by HPLC) is necessary to determine if increased levels of dopamine are involved in diapause induction in the horn fly. It is also important to note that it may not be the total dopamine content of the CNS, but the dopamine activity at specific sites in the CNS that is important for diapause induction Kostal et al. (1999). Changes in dopamine at these sites may occur without requiring an increase in the overall content. These changes could be determined by *in-situ* hybridization studies.

During early diapause (day 15) there is a slight decrease in TH expression in the CNS of the horn fly (Fig. 20). Similarly, in the CNS of *P. brassicae*, dopamine levels are lower during the first days of diapause (Puiroux et al., 1990). Lower dopamine level may modulate neurosecretory cell activity by the reduction of stimulating activity (Puiroux et al., 1990). Decreased expression in diapausing horn fly pupae appears consistent with an overall decrease in brain activity during diapause.

In addition to its roles in melanization and sclerotization of the puparium and as a neurotransmitter, neuromodulator and neurohormone in the insect nervous system, dopamine participates in a number of physiological processes. Of particular importance to diapause may be the involvement of dopamine in defense and immune response. In *D. melanogaster*, dopamine functions in the defense response of melanotic and sclerotic encapsulation of parasites (Nappi, 1991). As a component of the insect's defensive mechanism, HiD6 may in part provide protection against invading foreign organisms, including bacteria and parasitoids. This may be especially important during early fall when warm temperatures may allow the proliferation of bacterial and fungal pathogens, and movement of parasitic wasps.

5.2.2 **HiD3: Transferrin.** The expression of HiD3 is associated with pupation in both non-diapause and diapause destined pupae, but is significantly higher in diapause-destined pupae, suggesting that HiD3 may be involved in either pupation or diapause induction. Expression of a juvenile hormone-repressible transferrin-like protein has also been documented in fat body, haemolymph, and ovary of diapausing *Riptortus clavatus* (Hirai et al., 1998, 2000). The function of transferrin during diapause in *R. clavatus* remains unknown.

Transferrin is up-regulated during immune response and cellular defense. In *D. melanogaster*, transferrin is up-regulated during bacterial infection (Yoshiga et al., 1999) and in the mosquito *Aedes aegypti* (L.), transferrin is up-regulated, upon infection with bacteria (Yoshiga et al., 1997) and during encapsulation of filarial worms (Beerntsen et al., 1994). In the mosquito, transferrin may inhibit growth of invading organisms by sequestering iron (Yoshiga et al., 1997). HiD3 may be up-

regulated during diapause to provide protection against invading foreign organisms by sequestering iron from these organisms.

Transferrin may also be involved in melanization. Tyrosine hydroxylase (TH) is an iron dependent metalloenzyme (Hoeldtke and Kaufman, 1977; Haavik et al., 1991). Perhaps transferrin provides the iron required for TH. Transferrin and tyrosine hydroxylase may be part of the same pathway for melanization during pupation, immune response and cellular defense. The involvement of transferrin in melaniziation remains to be proved. However, if this in fact does happen, HiD3 may be up-regulated during diapause to provide increased ability to melanize invading organisms.

5.2.3 **HiD5: Serine protease inhibitor.** HiD5 does not have a high degree of protein homology with entries in GenBank, however, it contains a signature pattern specific for the Kunitz family of serine protease inhibitors. Serine protease inhibitors are grouped into families based on structural characteristics. The Kunitz family consists of low molecular weight serine protease inhibitors (PROSITE: Hofmann et al., 1999). The small size of HiD5 as indicated by Northern blot analysis is consistent with this transcript belonging to this family of proteins.

The expression of HiD5 is associated with pupation in both non-diapause and diapause destined pupae, but is significantly higher in diapause destined pupae. This suggests that HiD5 may be involved in either pupation or diapause induction. Several serine protease inhibitors were isolated from the desert locust *Schistocerca gregaria* (Forskal) (Vanden Broeck et al., 1998). These inhibitors are expressed in different

tissues and expression is dependent on developmental stage, implying multiple functions.

During cuticle sclerotiztion and melanization, serine proteases are involved in the proteolytic cleavage of prophenol oxidases (reviewed by Wright, 1987). A serine protease inhibitor found in haemolymph of *Sarcophaga* (Sugumaran et al., 1985) prevents melanization (in the presence of prophenol oxidase, serine protease, and catecholamine precursors). As overall gene expression is decreasing during diapause, the components of the cellular defense system (i.e. those involved in melanization) may already be present at induction, ready to launch an immune response. During diapause, HiD5 may function to keep the reaction in check, that is, to inhibit undesirable melanization in the presence of increased TH (increased dopamine).

Serine protease inhibitors are also involved in immune response and cellular defense. Several serine protease inhibitors were isolated from haemolymph of the greater wax moth, *Galleria mellonella* (L.), after inoculation with a fungal cell wall preparation (Frobius et al., 2000). One of these inhibitors exhibits homology to the Kunitz family. Protease inhibitors may be used to inhibit cell destruction or bacterial proteolytic enzymes. The serine protease inhibitor-like protein, HiD5, may be part of the insect's defense mechanism, and may be up-regulated during diapause to provide protection against invading foreign organisms in early fall, before the onset of cold temperatures.

HiD5 could also function to inhibit the differentiation and destruction of imaginal tissues during diapause. During metamorphosis, serine proteases are involved in the differentiation of imaginal disc (Ohtsuki et al., 1994) and tissue

restructuring (larval gut disintegration) in *S. peregrina* (Nakajima et al., 1997). A serine protease inhibitor prevents the differentiation of imaginal disc during metamorphosis of *S. peregrina* (Ohtsuki et al., 1994). During diapause, HiD5 may prevent differentiation and destruction of imaginal tissues by inhibiting serine proteases.

Another possibility is that HiD5 is a structural protein of the puparium that serves to protect against microbial invasion and dehydration. Amyloid fibrils play a protective role in the chorion of *B. mori* (Iconomidou et al., 2000) and the egg envelope of diapausing fish embryo, ***Austrofundulus limnaeus*** (Podrabsky et al., 2001). The egg envelope of A. *limnaeus* is composed of amyloid fibrils similar to those associated with human disease such as Alzheimer's disease. Amyloid fibrils serve as a barrier against microbes and low molecular weight solutes, and are in part responsible for resistance to desiccation in fish. Amyloid proteins like those involved in Alzheimer's disease contain a consensus sequence for serine protease inhibitor. HiD5 also shares protein homology with amyloid proteins.

5.2.4 **HiD10: Carboxylesterase.** The expression of HiD10 is associated with pupation in both nondiapause and diapause destined pupae, but is slightly higher in diapause destined pupae. Similarly, juvenile hormone (JH)-esterase and general carboxylesterase activities in the haemolymph of Colorado potato beetle, *Leptinotarsa decemlineata* Say, are highest in 4th instar larvae and during diapause induction (Kramer and DeKort, 1975).

HiD10 may be involved in either metamorphosis and/or diapause. One possible function of HiD10 may be the metabolism of juvenile hormone (JH) by

ester hydrolysis during metamorphosis or diapause. Low JH titers during pupal metamorphosis regulate developmental programming to the adult. JH esterases increase during metamorphosis, presumably to metabolize JH, ensuring proper adult development. JH may also be involved in oxygen consumption during diapause. Juvenile hormone activity cycles during pupal diapause of *S. crassipalpis*, and is thought to drive cyclical oxygen consumption (Denlinger and Tanaka, 1989). JH-esterase activity also cycles, and may function to partially regulate cyclical JH levels. Note however, homology between JH esterase from *H. virescens* and HiD10 is insignificant suggesting functions other than metabolism of JH.

A number of esterases have been identified in insects. HiD10 has the closest homology to Est-5A. Besides the role in JH ester hydrolysis, the functions of these esterases in development are largely unknown. Expression of specific esterases varies depending on tissue and life stage. In *Drosophila mojavensis* Patterson and Crow and *D. buzzattii* Patterson and Crow, expression of Est-4 limited to cuticle of late larvae, and probably functions during pupation, whereas Est-5 is expressed in haemolymph and fat body throughout all life stages (Zouros et al., 1982). Est-P (similar in structure to Est-6) is expressed during late larval stage (Collet et al, 1990).

5.2.5 **HiD9.** HiD9 has significant sequence homology to a *D. melanogaster* cDNA with unknown function. Instead of relying on sequence homology, PROPSEARCH (Hobohm and Sander, 1995) was used to generate a list of proteins with similar properties to the protein coded by HiD9 (Table 4). These proteins likely have common domains or motifs and may be in the same family as HiD9, implying a similar function. The highest scoring protein is *D. melanogaster* Dreg-5 (Van Gelder

and Krasnow, 1996). This protein is notable because it is expressed in a circadian rhythm and is dependent on the *period* gene (see Chapter 2) for its rhythmic expression. It is thought that diapause is ultimately controlled by the circadian system. Note however, that HiD9 does not have significant sequence homology to Dreg-5 but has a greater than 53% chance of belonging to the same protein family. Property comparisons with other known protein families suggest HiD9 may also code for a transcription factor. As a potential transcription factor, HiD9 may have a regulatory function and therefore warrants further investigation.

5.2.6 **HiD1.** HiD1 appears to code for a novel protein as it has no significant sequence homology to known sequences (DNA or protein) or property homology to 58 protein families. This cDNA displays similar expression pattern to other HiD isolates, suggesting that it may be involved in multiple processes including diapause and pupation.

5.2.7 **HiD4: Cytochrome oxidase.** HiD4 is not a diapause up-regulated cDNA as expression on day 0 is similar for both nondiapausing and diapause destined pupae. Expression patterns suggest that the mitochondrial gene, cytochrome oxidase, may be an indicator of metabolic activity. Decreasing expression of HiD4 as diapause progressed may reflect the arrest of cellular function. Increased expression during days 4 and 5 in nondiapausing pupae may reflect increasing cellular function coinciding with moulting.

5.2.8 **G3PDH and the use of an internal standard for Northern blotting.** Northern blot analysis is used for measuring and comparing the expression of specific mRNAs between tissues. There is a decrease in overall mRNA expression

throughout the diapause developmental process (Wigglesworth, 1972). Therefore accurate comparison of non-diapausing and diapausing mRNA requires the use of an internal standard. The internal standard is an mRNA expressed at equal levels in all samples and is used as a reference against which the mRNA of interest can be normalized. The use of an internal standard minimizes error due to loading or sample variation. For this study, the ideal internal standard is an mRNA whose expression is constant between non-diapause and diapause pupae, and is expressed equally throughout all stages of diapause development.

A commonly used internal standard is actin. During diapause, the expression of actin is down-regulated in the CNS of *L. dispar* (Lee et al., 1998). As well, *D. melanogaster* has several actin genes that are differentially expressed in different tissues, and throughout development (Fyrberg et al., 1983). Therefore actin may not be an appropriate internal standard for diapausing insects.

This study utilized glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) as the internal standard. G3PDH is a key enzyme in glycolysis and is constitutively expressed in many tissues. Expression in *D. melanogaster* has been shown to vary in some tissues and developmental stages (Sun et al., 1988). Expression of G3PDH appears to decrease during diapause and is likely a reflection of decreased metabolism. G3PDH also increases during nondiapause development and is likely a reflection of increased energy requirements during moulting. As a consideration for future research, this study recommends the use of a marker not associated with metabolism.

CHAPTER 6

CONCLUSIONS

Suppression subtractive hybridization (Diatchenko et al., 1996; Gurskaya et al., 1996) is a useful technique for identifying differentially expressed cDNAs in diapause destined horn fly pupae. Success of this technique depends on having non-diapause and diapause tissues of comparable developmental stages. There are conflicts in the literature regarding the stage of developmental arrest of diapausing horn fly pupae, and the stage of developmental arrest is not immediately apparent. It therefore becomes necessary to define morphologically comparable stages of development.

Morphology of pupae and CNS indicate that developmental arrest of the horn fly occurs early in metamorphosis, in the interval between head eversion and pupal-adult apolysis. This study defines the morphology of diapausing horn fly pupae, which will allow for more accurate and meaningful comparisons of gene expression in future research.

The exact functions of proteins encoded by the diapause up-regulated genes remain undefined. Comparisons of homology suggest they may play a role in a number of processes including melanization and sclerotization of the puparium, metamorphosis, cellular defense and immune response. The insects collected for this study were simultaneously undergoing pupation, metamorphosis, and diapause induction. These up-regulated genes may be involved in either or all of these processes. Particularly intriguing is the potential involvement of tyrosine

hydroxylase, transferrin and serine-protease inhibitor in immune and cellular defense during diapause. These proteins may provide protection against invading foreign organisms during early diapause when warm fall temperatures are permissive for the growth of bacterial and fungal pathogens or parasitic insects. Synthesis of these proteins at diapause induction would also allow for the establishment of a defense mechanism prior to the arrest of gene expression. Further study is necessary to establish the role of these genes and their products during diapause.

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