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ABSTRACT

The tobacco hornworm, Manduca sexta, was reared under laboratory conditions on an artificial wheat-germ diet under diapause-averting and diapause-inducing photoperiods. Two types of fluorescent light sources (Indorsun and Blacklight blue) were used for each of the photoperiods. Observations were made on larval growth, pupal weight and selected biomolecules. Proteins, free amino acids and sugars were characterized in the 5th larval and pupal instars. Osmolality of the hemolymph was also monitored as a function of the experimental regimen used in this study.

The results demonstrate that diapause-inducing photoperiod combined with Blacklight blue promote maximum larval growth as compared to other rearing conditions. Pupal weight is significantly influenced by photoperiod. Pupae in diapause (short-day photoperiod) are heavier than their non-diapause (long-day photoperiod) counterparts.

The age of instar is the determining factor in influencing the protein concentration of the hemolymph of M. sexta. Protein concentration of the hemolymph generally rises throughout the progression of the 5th larval instar, falls at molting and early pupal life, followed by a rise under non-diapause conditions and a decline under diapause condi-

tions. The qualitative distribution of hemolymph proteins in M. sexta is variously influenced by photoperiod and light quality as evidenced in terms of the concentration of bands detected in the hemolymph.

The light quality regimen was slightly modified by using filters and free amino acids were then determined from the hemolymph. Serine, glycine, glutamine, alanine, proline, ornithine and citrulline show changes in response to photoperiodic diapause induction in M. sexta. Particularly, levels of serine were higher in diapause-programmed insects. Arginine and lysine were influenced by light quality. Levels of arginine and lysine were higher in the early pupae reared under LD and SD IND-UV conditions.

Levels of trehalose and glucose in the hemolymph of M. sexta were monitored using HPLC. The results show that, trehalose levels in the hemolymph were consistently higher in diapause-programmed tobacco hornworms, but also show variations with respect to light quality. Glucose levels in the hemolymph change with respect to the age of instar. Levels of glucose fall by the end of the 5th instar, rise in the early pupae and again decline in the late pupae.

Changes in the osmolality values of the hemolymph of M. sexta do not reflect the changes in the biomolecules characterized in this study in response to various rearing conditions since factors such as metabolites and other components of the hemolymph were not characterized.

This study has demonstrated that light quality is an important dimension of insect photoperiodism and diapause physiology of insects. This work adds a new dimension to our understanding of the light requirements of insects.

RESUME

Le sphynx du tabac, Manduca sexta, a été élevé en laboratoire sur un régime artificiel à base de germe de blé. On a utilisé deux photopériodes, une prévenant la diapause et l'autre la favorisant; et deux types de lumière fluorescente (Indorsun et Blacklight blue). On a étudié le développement des larves, mesuré le poids des pupes et observé certaines biomolécules. Les protéines, les acides aminés libres et les sucres ont été caractérisés dans l'hémolymphe des larves de 5ème stade et des pupes. L'osmolalité de l'hémolymphe a été aussi caractérisée en fonction des conditions expérimentales.

Les résultats démontrent que la photopériode favorisant la diapause combinée avec la lumière Blacklight blue a favorisé le développement larvaire de M. sexta au maximum. Le poids des pupes a été influencé significativement par la photopériode. Le poids des pupes en diapause était plus élevé que celles qui ne l'était pas.

L'âge du stade a été le facteur déterminant envers la composition des protéines de l'hémolymphe de M. sexta. Les protéines de l'hémolymphe ont été généralement abondantes durant le 5ème stade larvaire, elles ont diminuées à la mue larve-pupe pour ensuite ~~augmenter~~ sous des conditions d'ab-

sence de diapause alors qu'on observait une réduction sous les conditions de diapause. Le distribution qualitative des protéines de l'hémolymphe de M. sexta a été influencée par la photopériode et la qualité de la lumière comme l'indique la concentration de bandes protéiques détectées dans l'hémolymphe.

On a modifié la qualité de la lumière à l'aide de filtres après quoi on a déterminé le profil des acides aminés libres de l'hémolymphe de M. sexta. La sérine, la glycine, la glutamine, l'alanine, la proline, l'ornithine et la citrulline ont réagi à l'induction de la diapause photopériodique chez M. sexta. Les niveaux de sérine ont été particulièrement élevés sous des conditions de diapause. L'arginine et la lysine ont été plus élevés dans les jeunes pupes élevés sous des conditions LD et IND-UV.

Le composition du tréhalose et du glucose dans l'hémolymphe de M. sexta a été déterminée par l'HPLC. Les résultats indiquent que la concentration du tréhalose dans l'hémolymphe a été élevée sous des conditions de diapause provoquée; cependant les niveaux de tréhalose ont également changé en fonction de la qualité de la lumière. La concentration du glucose dans l'hémolymphe a varié en fonction de l'âge du stade. La concentration du glucose diminué à la fin du 5ème stade, augmente au début du stade pupes et diminué à nouveau vers la fin de ce stade.

Les variations dans l'osmolalité de l'hémolymphe de M. sexta ne reflètent pas les changements de biomolécules caractérisées dans cette étude en fonction des conditions d'élevage puisque les différents métabolites et autres composants de l'hémolymphe n'ont pas été étudiés.

Cette étude a démontré que la qualité de la lumière est une importante dimension du photopériodisme chez les insectes et pour la physiologie de la diapause. Ce travail ajoute une nouvelle dimension à notre compréhension des besoins en lumière des insectes.

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Chapter I
INTRODUCTION

The effects of light and photoperiod on insect development have been extensively investigated, yet not much is known about the fundamental changes that occur at the hemolymph level under various light regimes. The predictive rather than indicative nature of the action of photoperiod on the developmental pattern of insects is of particular significance with respect to forecasting upcoming unfavorable environmental conditions. This makes it necessary for the animal to adjust its behaviour and physiology to an optimal state which will help survival as well as ensure successful adult emergence and hence reproductive success. This can be achieved by behavioural adaptations such as burrowing in the soil and by metabolic adaptations.

The present study was undertaken to elucidate the effects of diapause-inducing and development-promoting photoperiods on some vital biomolecules such as proteins, free amino acids and sugars in the tobacco hornworm, Manduca sexta (Lepidoptera, Sphingidae). It was expected that photoperiod, by virtue of its predictive clue for anticipatory regulation in M. sexta, would bring about physiological changes in these biomolecules and thus additional informa-

tion would be generated on the physiological aspects of diapause in this species. Although a lot of information is available on the physiology, biochemistry and endocrinology of non-diapause tobacco hornworms and on the endocrinology of diapause in M. sexta, there have been surprisingly no reports on other aspects of diapause physiology of this insect species.

Another dimension of the photobiology of this insect considered in this study was light quality. Most photoperiod controlled experiments done in the laboratory use fluorescent lighting as the sole source of illumination, a source which varies widely, with respect to spectral quality and intensity from solar light. Hence, a solar simulating light was compared with an ultraviolet emitting light source in experiments to study the influence of light quality on some physiological aspects of M. sexta.

The information generated by this thesis should give some idea of the physiological effects of photoperiod and light quality and help elucidate the effects of light quality in this process.

The objectives of this study were, therefore, to determine the effects of (a) non-diapause and diapause-inducing photoperiods and (b) light quality on the growth of M. sexta and more specifically on variations in protein, free amino acid and sugar levels.

Chapter II LITERATURE REVIEW

Light is the most important environmental factor, after food, in controlling bodily functions of many organisms. Some animals have an absolute requirement for light and its various facets like intensity, periodism and quality in order to sustain development and reproduction. This is particularly true for insects (Beck, 1980) and birds (Menaker, 1972). On the other hand, animals that live in complete darkness are not equipped to cope with direct solar radiation.

Insects vary in their photoresponses because of their ecological diversity (Philogène and McNeil, 1984). Depending on their respective niches, even insects from closely related taxa may have very different light requirements.

2.1 Artificial Light

Although the sun is a good bright source of visible radiation, for practical reasons, artificial light sources such as incandescent, fluorescent, and arc lamps are best suited for experimental work. The information available today in insects' reaction to light is largely based on experiments using artificial sources of light, particularly

in photoperiod controlled experiments (Philogène and McNeil, 1984). Fluorescent lamps are widely used for illumination, which, with respect to spectral quality and intensity differ widely from solar light. However, the dissimilarity between normal and artificial light has far-reaching consequences for the interpretation and extrapolation of experiments carried out with incandescent and fluorescent light sources (Shields, 1980). Yet experimenters generally fail to characterize the spectrum of their light source or the energy flux (Philogène, 1982). Thus, it is necessary that light quality and intensity of the artificial light source should not be ignored as the latter's effects on photoresponses at the behavioral, physiological and biochemical levels are largely unknown. Even within the types of fluorescent lamps available to the experimenter, the spectral output of different fluorescent lamps is quite different. The spectral biases of some fluorescent lamps available in the market are shown in Figure 1. Recently some manufacturers have developed fluorescent lamps that can simulate, to some extent, natural outdoor light. For example, Vita-Lite (Duro-Test Corp., North Bergen, N.J.) and Indorsun F20T12/1DS (Verd-A-Ray Corp., Hightstown, N.J.).

Figure 1: The spectral distribution of some fluorescent light sources.

(From Thoms and Philogène, 1979).

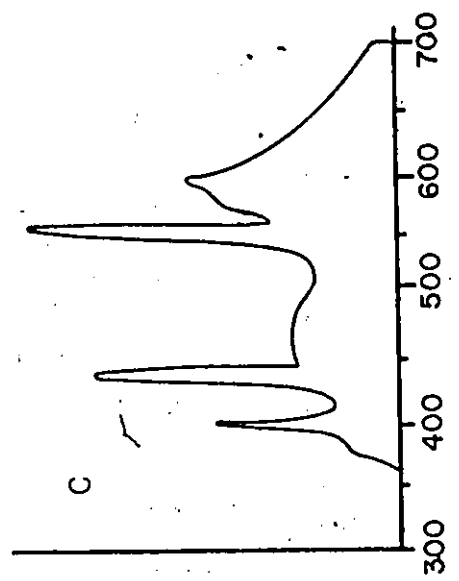
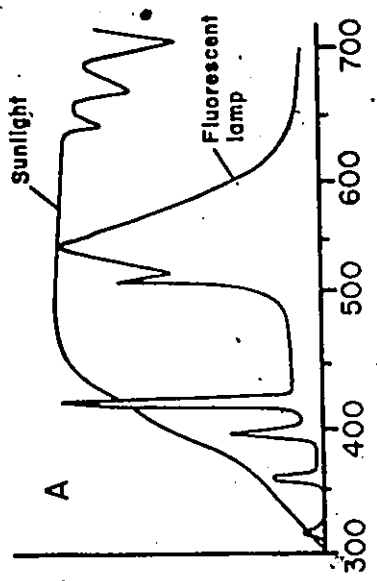
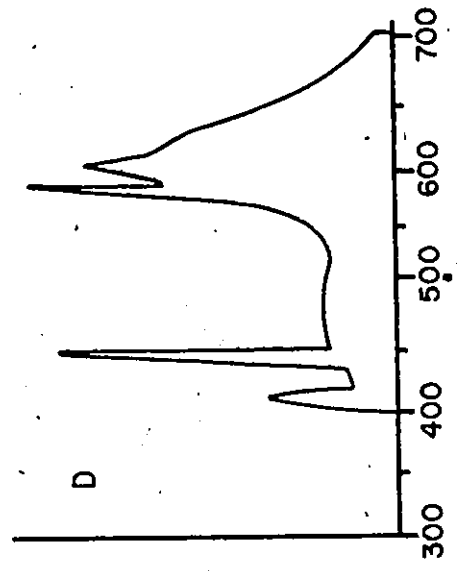
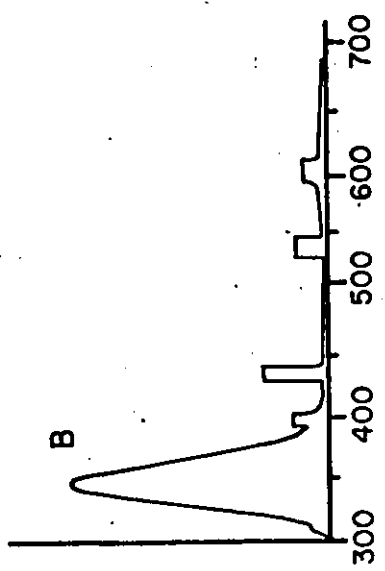
A: Natural light, compared with fluorescent lamp.

B: Blacklight blue fluorescent.

C: Daylight fluorescent.

D: Cool White fluorescent.

The spectral distribution of Indorsun fluorescent is not available.



Relative energy

Wavelength in nanometers

2.2 Influence of Light on Insect Development

Insects possess photoreceptors such as the unicorneal simple eyes (ocelli and stemmata) and the compound eyes. But extraretinal photoreception is also present such as the neurosecretory cells of various parts of the brain, the suboesophageal ganglion and the abdominal ganglia (Saunders, 1982). Pigments such as carotenoids, carotenoproteins, flavins and polycyclic quinones have been implicated in extraocular photoreception (Philogène and McNeil, 1984). Action spectra studies have shown that these compounds are generally most sensitive to the blue portion of the light spectrum (Truman, 1976; Lees, 1981). Spectral sensitivity for the photoperiodic response has been reported in many insect species (Saunders, 1982). Most terrestrial insects are subjected to the presence of light throughout their life cycle. Light periodicity (photoperiod), quality and intensity have been shown to affect insect development in various ways, although very little work has been done on this aspect of insect physiology. Most evidence on the effects of light on insects available to date concerns insect photoperiodism and details pertaining to the circadian rhythms and biological clocks (Beck, 1980; Saunders, 1982).

Photoperiod comprises a period of illumination, the photophase, followed by a period of relative darkness, the scotophase, measured as the number of hours and minutes elapsing between sunrise and sunset (Beck, 1980).

2.3 Diapause

Diapause may be defined as a state of arrested development usually at species specific stages in the insect's life cycle, the onset of which is brought about by the environmental factors which, although signalling the approach of unfavorable conditions, are not, in themselves adverse (Saunders, 1982). It is thus a case of anticipatory regulation (Philogène, 1983).

Although many definitions of diapause have been coined (Wheeler, 1893; Henneguy, 1904; Shelford, 1929; Way, 1962; Muller, 1965, 1970; Mansingh, 1971) there has been much confusion about defining the phenomenon (Philogène, 1983). Shelford's (1929) definition of diapause as the spontaneous arrest in development which does not respond immediately to a change in the environment and the term 'quiescence' for cases in which development is temporarily inhibited due to immediate unfavorable environment has found some acceptance and use (Andrewartha, 1952; Danilevskii et al., 1970 Harvey, 1962; Lees, 1956; Tauber and Tauber, 1973, 1976; deWilde, 1962). However, Shelford's definition is actually inadequate (Philogène, 1983).

On the other hand, quiescence is the state of dormancy directly imposed by the adverse conditions, and recovery occurs soon after these restrictions are removed (Saunders, 1982).

2.3.1 Factors Inducing Diapause

2.3.1.1 Photoperiod

The major factor inducing diapause is photoperiod. Beck (1980) has classified insect diapause into four broad categories depending on the type of diapause induction curve they exhibit : Type I or long-day response; Type II or the short-day response; Type III or the short-day-long-day response and Type IV or the absence of diapause over a very restricted range of long photophases.

Another factor which seems to be neglected in most studies on diapause is the spectral quality of light. Most photoperiodic experiments conducted in the laboratory use artificial lighting which, as described earlier, with respect to spectral quality and intensity differ widely from solar light. Light quality has been shown to have an influence on insect diapause. Kogure, as early as 1933, reported that 98% of adult silkworms, raised under the influence of blue-violet radiation (350-410 nm) laid diapause eggs. Recently, Thoms and Philogène (1979) found that in the cabbage white butterfly Pieris rapae, long photoperiod diapause (15L:9D) can be completely averted under high ultraviolet light conditions.

2.3.1.2 Temperature

The other factor influencing diapause induction in insects is temperature (Beck, 1980). Temperature exerts a two-fold effect on diapause induction, depending on whether it is constant or fluctuating through a daily cycle. At constant environmental temperature, the critical daylength displayed by populations of experimental insects is changed depending on the temperature employed. The exact effects of different constant temperatures on critical daylengths differ widely among species. The diapause response may be eliminated at high temperatures while lower temperatures exert an opposite effect (Saunders, 1980). On the other hand, very few insects experience constant temperature conditions within their natural environment. The environmental temperatures tend to fluctuate through a daily cycle and the substitution of thermoperiodic signals for photoperiodic signals or the interaction between the two has been shown to influence diapause induction in some insects (Beck, 1980).

2.4 Physiology of Diapause

2.4.1 Insect Growth and Development in Relation to Diapause

The effects of diapause-inducing photoperiod on insect growth and development has been studied by many workers (reviews by Beck, 1980; Saunders, 1982). Relatively slow growth is frequently associated with diapause programming

photoperiods, but as Beck (1980) has pointed out, there are many exceptions to warrant any generalization. Thus, under diapause-inducing photoperiod, that is, short days, larval development of the viceroy butterfly, Limenitis archippus is much slower as compared to their non-diapause programmed counterparts (Clark and Platt, 1969). Similar results have been obtained for the flesh flies, Sarcophaga spp. (Denlinger, 1972; Saunders, 1972), the rice stem borer, Chilo suppressalis (Fukaya and Mitsuhashi, 1961), the redbanded leafroller, Argyrotaenia velutinana (Glass, 1963), the cabbage butterfly, Pieris brassicae (Claret, 1968) and the sunflower moth, Homoeosoma electellum (Chippendale and Kikukawa, 1983). On the other hand, diapause-inducing short-days have been shown to hasten development in insects such as the southwestern corn borer, Diatraea grandiosella (Kikukawa and Chippendale, 1983), the tussock moth, Dasychira pudibunda (Geyspitz, 1953), the webworm, Crambus tutillii (Kamm, 1972) and the odonatan, Tetragoneuria cynosura (Lutz, 1974). Moreover, insects whose life cycle include a facultative, photoperiodically induced diapause display unaffected growth irrespective of photoperiod (Beck, 1980). This has been reported in insects such as the tobacco hornworm, Manduca sexta (Bell et al., 1975). Photoperiod (short-days) may accelerate larval growth in species in which diapause is obligatory, that is, not induced by photoperiod (Beck, 1980). The relationship between diapause-inducing

photoperiod and growth is far from clear. That photoperiod can act on developmental rate, independently from diapause induction has been shown in the flesh flies, Sarcophaga argyrostoma (Saunders, 1976). The specific physiological mechanism(s) by which diapause-inducing or diapause-averting photoperiods can influence developmental rate remains to be researched.

Although, studies have been done on the effects of diapause-inducing photoperiod on the developmental rate of insects, very few studies have concerned themselves with growth and development measured, in terms of weight gain. This is important because rate of weight gain is a good indicator of growth (measured as live weight). Parameters such as the number of days required to attain a specific growth stage and the number of instars occurring in a particular stage may not be adequate to represent growth.

Thus, the full grown pre-diapause larvae of the sunflower moth, Homoeosoma electellum are reported to be significantly heavier than the equivalent non-diapause larvae (Chippendale and Kikukawa, 1983), but this effect in the former is achieved by a decrease in the growth rate. Diapause-programmed larvae of the cabbage butterfly, Pieris brassicae are heavier than their non-diapause programmed counterparts (Claret, 1968), and this is achieved by lengthening of the 3rd and 4th larval instars. Obviously, this aspect of insect development needs careful examination.

2.4.2 Proteins in Insects

Proteins are the most abundant organic molecules in cells, constituting 50 per cent or more of their dry weight. Insects contain a variety of proteins in their hemolymph and tissues which have been studied extensively in attempts to relate their presence to particular events in the insect's life cycle. The literature on insect hemolymph has been very well reviewed by Wyatt (1961, 1980), Gilbert and Schneiderman (1961), Florin and Jeuniaux (1974), Agosin (1978), Wyatt and Pan (1978), and Hagedorn and Kunkel (1979). The physicochemical properties of various insect proteins have been described and, with the advent of sensitive and efficient analytical techniques, much progress is being made in characterizing the proteins found in insect hemolymph and tissues. Some of the well characterized insect proteins include various contractile proteins, fibroins, integumentary proteins, collagen, chromosomal proteins, interstitial proteins, carrier proteins, storage proteins, vitellogenins, enzymes and some peptide hormones (Agosin, 1978).

The protein content of the hemolymph, both quantitative-ly and qualitatively, has been a subject of many extensive investigations (Wyatt and Pan, 1978). The number of different protein fractions is highly variable, according to the species, caste (Lue and Dixon, 1965), sex (Stephen and Steinhauer, 1957; Kulkarni and Mehrotra, 1970), diet (Bodnaryk

and Morrison, 1966; Dahlman, 1969); starvation (Feir and Krzywda, 1969), and ontogenetic stage (Florkin and Jeuniaux, 1974). Much of the work done in Lepidoptera is on Saturniidae and the results indicate that the total protein content of the hemolymph increases during the successive larval stages, but is decreased in the adults. These changes have been observed in electropherograms as an increase in the number and concentration of discrete bands which reach a maximum in the pupae. Some modification due to the larval molt cycle have also been reported by Laufer (1960).

Chippendale (1970) reported that the protein concentration of the hemolymph of the southwestern corn borer, Diatraea grandiosella increased markedly during the last two larval stages before falling sharply during the pharate pupal stage before declining at the onset of metamorphosis. The fat body and midgut are major larval tissues that are capable of high rates of protein synthesis and both have large surface areas exposed to hemolymph for chemical exchanges (Chippendale and Kilby, 1970). Similar results have been reported for the fifth instar Bombyx mori and Manduca sexta larvae (Wyatt et al., 1956 Dahlman, 1969).

The existence of cyclic changes in hemolymph proteins due to molting is a characteristic feature of insects. A regular increase in the number of protein fractions in the pharate stage and a reduction after molting have been observed in the tomato hornworm, Protoparce quinquemaculata

in the 3rd, 4th and 5th larval instars (Hudson, 1966). Specific instar proteins have been reported in a number of insects (Loughton and West, 1965). Also, specific larval stage proteins are known to occur; for example, calliphorin in the blowfly, Calliphora erythrocephala (Greville et al., 1967; Munn and Greville, 1969), manducin in the tobacco hornworm, Manduca sexta (Kramer et al., 1980) and arylphorin in the giant silkmoth, Hyalophora cecropia and the silkmoth, Bombyx mori (Tojo et al., 1978, 1980; Telfer et al., 1983). The synthesis and uptake of storage proteins have been shown to be under hormonal control (juvenile hormone and ecdysone) in some insects (Butterworth et al., 1979; Sass and Kovacs, 1980).

2.4.2.1 Proteins in Relation to Diapause

Appreciable stability in the protein composition of the hemolymph is typical of insect diapause. Telfer and Williams (1953, 1960) found that there was very little change in the protein composition of the hemolymph of Hyalophora cecropia pupae in diapause. They observed a lower incorporation of labelled glycine into the hemolymph proteins of diapause pupae than into that of developing adults. Their results suggested that amino acid incorporation of the pupae in diapause was either in equilibrium with protein degradation, or that it resulted from energy-requiring exchange reactions (Chippendale and Beck, 1966). Consistency in the protein composition of the hemolymph was also reported in

the pupae of Pieris brassicae (Chippendale and Kilby, 1969), and prepupal Ostrinia nubilalis in diapause (Chippendale and Beck, 1966).

It is interesting to note that RNA and protein synthesis declined during the first two weeks of diapause in Heliothis zea pupae, but only RNA synthesis increased during sustained diapause (Keeley, 1983). In contrast, protein synthesis was low throughout diapause and increased in correspondence with diapause termination. Keeley (1983) suggested that biosynthetic processes were suspended at the translational level during diapause in H. zea, whereas the capacity for transcriptional activities were only briefly interrupted shortly after pupation, then continued at an only slightly reduced level during the remainder of diapause.

In the diapause-programmed third-instar larvae of the flesh fly, Sarcophaga crassipalpis, hemolymph protein concentration was nearly twice as high than in non-diapause-programmed larvae of the same age (Adedokun and Denlinger, 1985). They suggested that these differences in protein synthesis or storage were linked to the developmental fate of the larvae. Luzev and Belozarov (1978) reported that there was an increase in the concentration of hemolymph proteins as well as an increase in the density of many protein fractions in the pine moth, Dendrolimus pini larvae toward the end of pre-diapause development. With the onset of dia-

pause, there was a stability of the protein profile, which remained constant throughout diapause. Moreover, these authors reported that the concentration of proteins in the hemolymph increased appreciably during diapause. Luzev and Belozarov (1978) attributed this to cessation of larval growth during diapause and the associated inhibition of protein utilization in the hemolymph of D. pini larvae.

Specific diapause associated proteins have been reported in some insects in diapause. Three diapause-associated proteins have been found in the females of the Colorado potato beetle, Leptinotarsa decemlineata, an insect with adult diapause (deLoof and deWilde, 1970). Recently, a dominant fat body protein has been associated with larval diapause in the southwestern corn borer, Diatraea grandiosella (Brown and Chippendale, 1978). This low molecular weight diapause-associated protein was found to be a single polypeptide chain with compact globular structure (Dillwith and Chippendale, 1984). A dominant soluble protein in the hemolymph of diapause larvae of the codling moth, Cydia pomonella has been reported by Brown (1980).

In the codling moth and the Colorado potato beetle, the diapause-associated proteins were shown to be controlled by photoperiod (Brown, 1980; Dortland, 1978). Furthermore, data suggests that in the Colorado potato beetle, the southwestern corn borer and the codling moth, regulation of diapause-associated proteins is mediated by juvenile hormone (deLoof, 1972; Brown, 1980; Yin and Chippendale, 1976).

The primary functions of diapause-associated proteins are storage reserves (Brown, 1980; Dillwith and Chippendale, 1984; Turunen and Chippendale, 1979), maintenance and regulation of diapause (as a juvenile hormone binding protein) (Brown and Chippendale, 1978; Turunen and Chippendale, 1980), and as antifreeze and nucleators (Horwath and Duman, 1983; Duman and Horwath, 1983). Photoperiodic and thermal regulation of the latter have been shown in Meracantha contracta (Duman, 1977), Tenebrio molitor (Patterson and Duman, 1978) and Dendroides canadensis (Duman, 1980; Horwath and Duman, 1983).

2.4.3 Amino Acids in Insects

One of the characteristics of insect hemolymph is its high concentration of free amino acids. In fact, insect hemolymph contains more amino acids than in any other animal studied so far (Mansingh and Baqaya, 1971). The term "aminoacidemia" has been used in the literature to describe this characteristic of insect hemolymph (Duchateau and Florkin, 1958). This aminoacidemia is especially pronounced in the order Lepidoptera (Florkin and Jeuniaux, 1974). Proline, glutamine and one of the positively charged amino acids (arginine, histidine and lysine) are usually present in high titres in the hemolymph of holometabolous insects (Florkin and Jeuniaux, 1974; Jungreis, 1980). Although the major function of free amino acids is in protein synthesis, it is evident that they may have other important roles to

play because, for the most part, the supply of free amino acids is in large excess of the demand for them (Florkin and Jeuniaux, 1974). Amino acids can be important in various aspects of intermediary metabolism in insects.

Free amino acids such as arginine may function to store energy in the form of the phosphagen, arginine phosphate in some insects when it accepts a phosphate group from ATP (Corrigan, 1970). Tyrosine serves as a precursor for compounds which are involved in sclerotization and melanization of the cuticle (Richards, 1978). Glutamate and γ -amino butyric acid (GABA) are putative transmitters at the insect neuromuscular junction (Pichon, 1974). Glycine and some other amino acids have been implicated in detoxification of xenobiotic acids by conjugation (Dauterman and Hodgson, 1978). Proline has been implicated in energy production (Bursell, 1978; Sacktor, 1976).

Buck (1953) has suggested that amino acids might make a small contribution to the buffering capacity of the insect hemolymph, but histidine is the only amino acid which may be a significant buffer in the physiological pH range (pH 6-8) (Lehninger, 1975). Amino acids may also be involved in osmoregulation in some aquatic insects (Buck, 1953; Moens, 1975). An inverse relationship of fluctuations in the hemolymph concentration of free histidine, and the sericigenous amino acids such as glycine, asparagine, glutamine, serine, threonine and proline during silk spinning in the

silkworm, Bombyx mori, has been suggested to maintain the osmotic pressure of the hemolymph at a fairly constant level (Jeuniaux, 1971).

The free amino acids of insect hemolymph are in a dynamic state influenced by demand and supply. Jungreis (1980) has stated that amino acids in the hemolymph are regulated and that although some amino acids such as histidine appear to be "stored" in the hemolymph, most are dynamically maintained. Rapid exchange of amino acids must occur between those present in the hemolymph and those localized intracellularly (Jungreis, 1980). In general, arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are essential and must be present in the diet of insects (Corrigan, 1970). The non-essential amino acids can be synthesized by the insect in sufficient quantities for normal growth and development.

Attempts have been made to relate free amino acid titres in the hemolymph or the body of insects to factors such as developmental changes (Uhler et al., 1971), the formulation of chemical based tissue culture media for insects (Stevens, 1961), the determination of an insect's physiological condition (Pickett and Friend, 1966), the role of amino acids in the blood osmotic pressure regulation (Leader and Bedford, 1972) and to insect taxonomy and phylogeny (Sutcliffe, 1963).

However, the significance of high concentration of free amino acids in insect hemolymph is still not fully understood. The titres of free amino acids in the insect hemolymph are maintained in a dynamic state not only by supply and demand but also by interconversion between various amino acids in the hemolymph. A homeostatic mechanism for the maintenance of the hemolymph free amino acids has been suggested by some authors. Recent evidence includes that of Bosquet (1977) on the saturniid, Philosamia cynthia walkeri and of Collett (1976) on the blowfly, Calliphora erythrocephala. Hemocytes have also been implicated in the homeostasis in regards to plasma glutamate and aspartate in the blowfly, C. vicina (Evans and Crossley, 1974). In some insects, the total concentration of free amino acids declines during larval development while in others it is maintained at a relatively constant level (Chen, 1971). In insects, the excess nitrogen must be excreted or stored in a non-toxic form, and uric acid is the major end product of nitrogen metabolism. Ammonia, urea and amino acids may also be excreted by some species (Bursell, 1967; Cochran, 1975).

2.4.3.1 Amino Acids in Relation to Diapause

The levels of free amino acids as a causal factor in the initiation or termination of diapause have been taken into consideration in at least two studies. Wiygul et al. (1974) stated that adult boll weevils, Anthonomus grandis grandis fed on cotton bolls had higher levels of certain amino acids

than had those that had fed on cotton squares. Because boll-feeding contributes to diapause initiation (dietary effect)(Lloyd et al., 1967), high levels of these amino acids were implicated in diapause initiation. In another report, MacFarlane and Hogan (1966) stated that the levels of glutamine increases in the diapause eggs of the cricket, Teleogryllus commodus following diapause development- accelerating treatments of ammonia or exposure to 12 °C. This led them to suggest that glutamine levels were critical for diapause termination.

Amino acids might play some special role in the survival of insects in diapause. LaFage et al.(1974) reported that the proportions of many amino acids in whole body hydrolyzates of diapause and non-diapause larvae of the pink bollworm, Pectinophora gossypiella, were significantly different. These authors suggested that amino acids are involved in some aspect of diapause physiology, perhaps in energy production. Duchateau and Florkin (1958) conducted microbiological amino acid determinations on the hydrolyzed dialysate of the hemolymph of many insects and concluded that there was less variability in the levels of amino acids in the hemolymph of diapause pupae of different species than there was in that of non-diapause (growing) larvae.

The titres of alanine in particular were quite variable among pupae in diapause. Somme (1966,1967) reported that alanine accumulates during low temperature storage or during

anoxia in several insects including diapause larvae of a grain moth, Nemapogon personellus and diapause pupae of the cabbage butterfly, Pieris brassicae. Mansingh (1967) determined the concentrations of free amino acids in the various developmental stages of both non-diapause and diapause programmed Antheraea pernyi and reported that only proline and histidine differed in their pattern of quantitative changes during the metamorphosis of non-diapause and diapause generations. Although histidine was at its lowest level in non-diapause pupae and proline was lower in the non-diapause pupae than in the pharate adults, both of these amino acids increased during diapause. Levels of alanine, arginine, glutamine, lysine and threonine also increased during diapause. The increase in alanine and proline accounted for the major portion of the increase in the total free amino acid concentration. Mansingh (1967) suggested that the increase in alanine was a result of anaerobic metabolism.

Accumulation of alanine in cold exposed insects in diapause has also been reported (Morgan and Chippendale, 1983; Hansen and Viik; 1975), and it has been suggested that at low temperature, alanine may accumulate due to a stress-related increase in anaerobic energy metabolism (Meyer, 1974; Mansingh, 1967). Suzuki et al. (1984) reported a large increase in alanine content at the initiation of embryonic diapause in the eggs of Bombyx mori. The levels of alanine decreased at the termination of diapause. Alanine and glutamine show

a significant change in diapause eggs of the cricket, Teleogryllus commodus (MacFarlane and Hogan, 1966).

High titres of some amino acids have been reported in overwintering insects in diapause. Particularly, serine has been reported to accumulate at moderate temperatures in the European corn borer, Ostrinia nubilalis and the southwestern corn borer, Diatraea grandiosella larvae in diapause (Morgan and Chippendale, 1983). In many of the early studies of amino acids in the hemolymph of insects in diapause, the titre of serine was however not reported. Nevertheless, higher titres of serine in the hemolymph of diapause larvae of the pink bollworm, P. gossypiella and diapause pupae of Antheraea pernyi and Heliothis armigera have been recorded (Rostom et al. 1972; Mansingh, 1967; Boctor, 1981). Notable observations were those of Mitsuhashi (1978), who reported a very high titre of serine (65 mM) in the hemolymph of diapause larvae of the rice stem borer, Chilo suppressalis and a high titre of serine (30 mM) in the hemolymph of post-diapause pupae of Mamestra brassicae. Morgan and Chippendale (1983) have suggested that large amounts of serine in overwintering diapause larvae or pupae of D. grandiosella, O. nubilalis, C. suppressalis and M. brassicae may be used during post-diapause development for the synthesis of uric acid and other purines. Their suggestion followed from the observations of Tojo and Hirano (1966, 1968) who reported that uric acid is synthesized at a high rate during post-

diapause development of C. suppressalis and M. brassicae. It has also been reported that cleavage of β -carbon from serine yields glycine and a one carbon fragment which is the source of formic acid (Cochran, 1975; Corrigan, 1970). De novo synthesis of 1 mol of uric acid requires 2 mol of formic acid and 1 mol of glycine. Additionally required nitrogen may be derived from glutamine and aspartic acid (Cochran, 1975; Corrigan, 1970).

Accumulation of proline has been observed in the hemolymph of overwintering Ostrinia nubilalis larvae in diapause (Morgan and Chippendale, 1983). Cold-hardened O. nubilalis can survive freezing (Henec and Beck, 1960). Storey et al. (1981) stated that in the freeze-tolerant Eurosta solidaginis proline accumulated at -5°C to 57 mmol/kg body weight, whereas alanine accumulated to only 3 mmol/kg. Proline and alanine also accumulated in the hemolymph of A. pernyi, and the wax moth, Galleria mellonella during exposure to low temperature (Marek, 1979; Mansingh, 1967). However, according to Suzuki et al. (1984), proline content was low during the initiation and maintenance of embryonic diapause of B. mori, but increased during termination. Proline accumulated during the period of diapause termination could be oxidized throughout embryogenesis (Suzuki et al., 1984). The latter also suggested that the accumulation of alanine or proline may also lower the supercooling point of B. mori eggs, but in non-diapause eggs, concentra-

tions increased during early embryogenesis and they approached the same level as in non-diapause eggs at the end of diapause (Suzuki et al., 1984). However, these authors could not offer any conclusion about the chemophysiological role of this amino acid in the egg diapause of B. mori. It is possible that high concentrations of some amino acids in the hemolymph of insects in diapause are of adaptive advantage for survival during adverse environmental conditions which normally prevail during diapause.

2.4.4 Carbohydrates in Insects

The carbohydrates, or saccharides, are most simply defined as polyhydroxy aldehydes or ketones and their derivatives. Insects, in addition to "pure" polyhydroxy aldehydes and ketones present as monomers (monosaccharides) or polymers (di-, oligo-, and polysaccharides), contain derived carbohydrates, in which the monomer has been modified chemically to various degrees; and conjugated carbohydrates, in which pure and derived carbohydrates are combined with other non-carbohydrate molecules. Carbohydrates form one of the principal classes of organic compounds that are found in insects and contribute to the structure and function of their tissues, and can be found in the nuclei, cytoplasm, and membranes of cells, as well as in the extracellular hemolymph and supporting tissues (Chippendale, 1978).

The most abundant monosaccharide in insects is D-glucose while the most abundant disaccharide is the nonreducing tre-

halose (α -D-glucopyranosyl- α -D-glucopyranoside) which contains two D-glucose residues. It is non-reducing, because the anomeric carbon atoms of both glucose moieties are bound in the glycosidic linkage. The existence of trehalose in insect hemolymph was first reported by Wyatt and Kalf (1956, 1957), Howden and Kilby (1956) and Evans and Dethier (1957). According to Wyatt (1961), trehalose has been identified in every insect species that has been appropriately examined, although it could not be detected in some developmental stages of some species.

Generally, levels of trehalose are higher than the levels of glucose in the insect hemolymph, but there are exceptions (Wyatt, 1961). The absence of trehalose from the hemolymph of larval Phormia regina (Evans and Dethier, 1957; Wimer, 1969), trace amounts in Agria affinis (Barlow and House, 1960), and its probable absence in the earwig, Anisoblabis littorea (Leader and Bedford, 1972) suggest that trehalose may not be an all inclusive characteristic of insects as suggested by Florin and Jeuniaux (1974) while its probable occurrence in the scorpion, Androctonus australis (Bricteux-Gregoire et al., 1963) and the centipede, Cormocephalus rubriceps (Bedford and Leader, 1975) suggest that it may not be exclusive. Other trehalose containing organisms besides insects are bacteria, algae, yeasts, fungi, nematodes, annelids, and crustaceans. A few ferns and seed plants have also been shown to contain trehalose (Elbein, 1974).

There have been many studies on the biosynthesis of trehalose in insects (Treherne, 1958; Friedman, 1967; Jungreis and Wyatt, 1972; Wyatt, 1961). Fat body glycogen and dietary glucose are its two important substrates. Treherne (1958) demonstrated that when D-glucose was injected into adult Schistocerca spp., over 90% of the radioactivity recovered from the hemolymph 15 min later are found in trehalose and this indicates a very efficient conversion mechanism. Candy and Kilby (1961) further showed that glucose is readily converted into trehalose by the fat body homogenates of Schistocerca and described the enzymatic steps involved in the synthesis of trehalose. It has been shown that the highest rate of trehalose synthesis occurs in the fat body, particularly in the cytosol of fat body cells (Bailey, 1975).

There are various mechanisms operating in insects to regulate biosynthesis of trehalose. Trehalose 6-phosphate synthetase is controlled by various factors such as the concentration of glucose 6-phosphate, intracellular Mg^{2+} , and trehalose (Steele, 1981). Glucose derived from the diet is converted either into glycogen or trehalose; when the trehalose 6-phosphate synthetase reaction is not subject to feedback control by trehalose, glucose 6-phosphate is preferentially converted into trehalose. With increasing synthesis of trehalose, the trehalose 6-phosphate synthetase reaction is gradually shut-off because of feedback inhibition

(Steele, 1981; Friedman, 1967, 1978). This causes the concentration of both glucose 6-phosphate and UDP to rise and this activates glycogen synthesis. Trehalose levels in the hemolymph are also regulated homeostatically by metabolic and hormonal controls operating on its synthesis and hydrolysis (Chippendale, 1978). Trehalose is an important reserve disaccharide because it is readily hydrolyzed to glucose which is in turn oxidized to provide energy for the various energy requiring processes in insects.

2.4.4.1 Trehalose in Relation to Diapause

It is generally accepted that, in many insects in diapause, glycogen is converted into sugar alcohols such as glycerol and/or sorbitol (Asahina, 1969); however, this may not be a general adaptive mechanism in insects overwintering in diapause. In some insects, sugar alcohols do not accumulate appreciably during diapause although hemolymph trehalose concentration is considerably elevated concomitant with a decrease in fat body glycogen (Asahina and Tanno, 1964; Hayakawa and Chino, 1981). The examples of insects overwintering in diapause which accumulate or have higher levels of trehalose include: the poplar saw fly, Trichiocampus populi (Asahina and Tanno, 1964), the beetle, Popilius disjunctus (Rains and Dimock, 1978), the Antarctic midge, Belgica antarctica (Baust and Edwards, 1979), the cabbage butterfly, Pieris brassicae (Moreau et al., 1981), the goldenrod gall fly, Eurosta solidaginis (Storey et al., 1981), the

silkmoth, Philosamia cynthia (Hayakawa and Chino, 1982) and the soybean pod borer, Leguminivora glycinivorella (Shimada et al., 1984). However, accumulation of trehalose may not correlate well with diapause in some insects (Wyatt, 1961). Examples are diapause eggs of the silkmoth, Bombyx mori (Yamashita, 1965) and diapause pupae of the giant silkmoth, Hyalophora cecropia (Wyatt, 1961).

The primary role of trehalose in overwintering insects is cryoprotection, as has been demonstrated in the overwintering prepupae of T. populi (Asahina and Tanno, 1964) and other insect species studied so far. In some insects, trehalose is not the sole cryoprotectant, but is present in association with cryoprotective molecules such as glycerol and sorbitol. High levels of trehalose in insects in diapause can also function as a reserve disaccharide (energy reserve) to meet the limited energy demands of diapause maintenance and also for post-diapause development (Chippendale, 1978). Trehalose can be readily broken down by the enzyme trehalase into glucose which can be utilized as a substrate for polyol synthesis.

Hayakawa and Chino (1981) proposed two categories for insects in diapause in terms of carbohydrate metabolism: the sugar alcohol accumulating type and the trehalose accumulating type. There are also insects which accumulate neither polyols nor sugars (Baust and Morrissey, 1975).

Numerous papers have appeared on carbohydrate metabolism and regulation in insects (Steelé, 1983). However, there has been no attempt to correlate the changes in the levels of trehalose to diapause-inducing environmental triggers such as short-day photoperiods. This is important due to the fact that photoperiodic cues are predictive in nature while temperature cues are indicative in nature (Baust, 1982).

2.5 MANDUCA SEXTA

The tobacco hornworm, Manduca sexta (Lepidoptera : Sphingidae) is a multivoltine leaf feeder that grows rapidly up to 10 g in the larval stage and is considered to be a serious pest of commercial tobacco plants in the United States, southern Ontario and Quebec (Stewart, 1981). There are one to four generations per year, depending on the latitude (Davidson and Lyon, 1979). M. sexta enters diapause as a pupa in the soil and adults emerge in the spring, mate and oviposit (Davidson and Lyon, 1979). The life cycle lasts 60 to 70 days (Bell and Joachim, 1976). The larvae of M. sexta undergo five instars (four moults) during their development. Larval development requires about 30 days, a period during which larvae will consume about 35 g artificial diet (Bell and Joachim, 1976). The bulk of this consumption occurs during the fourth and fifth instars. Fifth instar larvae can weigh between 5 and 10 g. Prepupae construct a pupation

chamber and pupate within 12 to 24 hours (Nijhout and Williams, 1974). In non-diapause insects, the life cycle require about 60 days, whereas insects in diapause require about 130 days to complete development (Bell et al., 1975).

The tobacco hornworm has become a choice experimental insect because of its large size and rapid rate of development (Bell and Joachim, 1976). Many physiological studies have been done on the tobacco hornworm because of its ease of rearing on synthetic diet. Manduca sexta is particularly suited for studies on the physiology of diapause because it enters diapause facultatively in response to short day photoperiods (Bell et al., 1975). The large size of the larvae during the final instar facilitates surgical manipulations of the nervous and endocrine system and provides large quantities of experimental material such as the hemolymph and body tissues which are usually required in adequate quantities for biochemical analyses. M. sexta has also been used for extensive endocrinological and ultrastructural studies.

2.5.1 Diapause in Manduca sexta

Photoperiod is the primary factor inducing diapause in M. sexta. Rabb (1966) studied the photoperiodic response in laboratory populations reared on tobacco and found that diapause could be induced by exposing only the last two larval instars to short days (13L : 11D), whereas long day (15L : 9D) treatment during the last instar only was sufficient to prevent diapause. Thus, determination of diapause

in M. sexta appeared also to be dependent on photoperiod encountered during the last one or two larval instars, indicating extraordinary photoperiodic responsiveness (Bell et al., 1975). The intensity or duration of photoperiodically induced diapause in M. sexta varied with the inductive photoperiod in which the hornworms were reared during the sensitive period (Bell et al., 1975; Denlinger and Bradfield, 1981). Insects reared in longer diapause-inducing photoperiods within a range of 12 to 13.5 hours remained in diapause longer than those reared in shorter photoperiods (Bell et al., 1975). It was reported that the shortest photoperiod used (12L : 12D) induced diapause with a mean duration of 105 days as compared to 140 days in the group reared under 13.25L : 10.75D. On the basis of reciprocal transfers between short- and long-day photoperiods, Denlinger and Bradfield (1981) reported that short daylength throughout embryonic and larval development results in a high incidence of diapause of short duration. Hornworms transferred from long- to short-day conditions at later stages of larval development enter diapause at a lower rate, but the resulting diapause is of longer duration (Denlinger and Bradfield, 1981).

Manipulations such as partial starvation and low rearing temperature (20 °C) to increase developmental time (and thus increase period of exposure to light regime) consistently supported the model that diapause duration is inversely

related to the number of short-day cycles received by the hornworms (Denlinger and Bradfield, 1981). However, Bell et al. (1975) reported that temperatures of 26 to 30 °C were most favorable for the photoperiodic induction of diapause. At a lower temperature (21 °C), the critical photoperiod and diapause incidence were decreased. There was no difference in the rate of larval development of hornworms reared in diapause-inducing and diapause-preventing photoperiods (presumably between 26 and 30 °C). Photoperiodic sensitivity for diapause was reported to extend from the late embryo to the end of larval feeding but showed considerable fluctuation during development with maximum sensitivity occurring just before egg hatch and during larval growth (Bell et al., 1975). Thus, the tobacco hornworm displays the type-I or long-day response to photoperiod (Beck, 1980).

The endocrinology of diapause in M. sexta has been thoroughly investigated (Bowen et al., 1984, 1985 Bradfield and Denlinger, 1980). However, there have been no studies done on the physiology of diapause in M. sexta. To date there is no information available about the biochemical changes or metabolic adjustments associated with the state of diapause in M. sexta and thus the current study is of primary significance.

Chapter III

MATERIALS AND METHODS

3.1 Experimental Conditions and Insect Rearing

The tobacco hornworms, M. sexta used for this study were from our laboratory culture originally obtained from Carolina Biological Supply Co., N. Carolina. Eggs were collected from the undersurface of the leaves of potted tobacco plants, Nicotiana tabacum or potted tomato plants, Lycopersicon esculantum. The eggs were placed in petri dishes (100mm dia. X 15mm ht.) lined with moistened filter paper which is required to prevent dessication and thus facilitate hatching. Insects were reared according to methods described by Bell and Joachim (1976) and Stewart (1981) with modifications in the lighting schedule as shown in Table 1. Under diapause-inducing conditions, all hornworms entered diapause. The spectral distribution of Indorsun light is akin to sunlight while the Blacklight-blue has a high portion of long wave ultraviolet (>350 nm). There was some spectral overlap between these two light sources. This experimental protocol was thus slightly modified for insects used for hemolymph free amino acid analysis.

In order to determine the effects of ultraviolet light on the hemolymph free amino acids of M. sexta, the Indorsun

Table 1: The experimental conditions used for rearing the tobacco
hornworm, Manduca sexta.

Rearing conditions used for the tobacco hornworm, Manduca sexta.

Photoperiod (L/D, hr.)	Light (Fluorescent)	Intensity (W/m ²)	Temperature (°C)
18/6 (Diapause-averting)	Indorsun [®]	2.5	26 ± 1
6/18 (Diapause-inducing)	Indorsun [®]	2.5	26 ± 1
18/6 (Diapause-averting)	Blacklight blue [®]	2.23	26 ± 1
6/18 (Diapause-inducing)	Blacklight blue [®]	2.23	26 ± 1

Long day: 18/6

Short day: 6/18

fluorescent was supplemented with a Blacklight blue fluorescent, thus eliminating the spectral overlap in the near-UV region of the spectrum. This lighting situation is referred to as Indorsun+UV. Ultraviolet was excluded from the Indorsun+UV regimen by placing Kodak Wratten 2B filters (400 nm UV cutoff) (Eastman Kodak Co., Rochester, N.Y.). This is referred to as Indorsun-UV.

Newly emerged larvae were individually transferred to diet cubes placed in clear, polystyrene Drosophila culture vials (10 cm ht. X 3.3 cm dia.) with foam plugs to facilitate ventilation and prevent condensation of moisture. When laid on their sides, these vials were large enough to accommodate all instars of the tobacco hornworm. Larvae were transferred to the vials using fine camel-hair brush or broad pliable forceps to minimize damage to the early instar insects. The vials were then placed in enamel trays (60 cm X 30 cm). The diet used was a wheat germ based meridic diet as described by Stewart (1981), but without dried tobacco leaves (Appendix A).

Fifty insects were reared under each of the appropriate experimental conditions in Environator incubators. Neonates were weighed daily from day of hatching until they were late prepupae (about 15 days) on a Mettler balance. Periodically, the incubators, working area and the balance were disinfected with Hinks-Byers solution (Hinks and Byers, 1976) to reduce the incidence of microbial contamina-

tion. Initially, every two days, unused diet and frass were removed from the vials and the larvae were given a fresh cube of diet. Later on, for the 5th instar larvae, diet was given and frass removed everyday. If any fungal or microbial contamination was observed on the unused diet, frass or larva, the insect was transferred to a new container.

Prepupal hornworms were provided with vermiculite for pupation. The vials were wrapped in thick paper since hornworm prepupae require darkness to complete the pupation process (Bell and Joachim, 1976). The vials were checked at two day intervals for completely tanned and sclerotized pupae. Fully formed pupae were transferred to their original experimental condition or 60 X 60 X 48 cm screened cages for adult emergence. A tobacco or tomato plant was provided as an oviposition site. The adults were fed with 20% sucrose solution. At two day intervals, eggs were collected, the sucrose solution replaced, and the pupal cases and dead adults were removed from the cages.

3.2 Insect Sampling and Hemolymph Collection

Larvae were sampled randomly from a synchronous population of insects of the same instar and the same "day of instar" as defined by Nijhout and Williams (1974) and Nijhout (1981). The "day of instar" will from now on be referred to as the "age of instar". Selected larvae were usually uniform in their size. Larvae were first wiped clean with Kleenex tissues.

Pupae were also randomly sampled from a synchronously pupated population of M. sexta. The age of instar was counted from the day of pupal moult. Pupae were usually uniform in size and free from any deformities.

Hemolymph was collected in 15 ml glass centrifuge tubes precooled in an ice bath. Larval hemolymph was collected by clipping off the tip of the horn with a pair of fine surgical scissors. Hemolymph was very gently expressed in the centrifuge tubes on ice. Only clear hemolymph was collected and care was taken to avoid any contamination from the gut contents of the larvae. Pupal hemolymph was collected by clipping off the tongue case (proboscis) and the hemolymph was expressed in centrifuge tubes on ice. Again, care was taken to prevent contamination from the gut contents and the meconium which consists of metabolic/nitrogenous wastes.

About 1.0 to 2.5 ml of hemolymph was collected from the insects depending on the instar, the age of instar and the number of insects sampled. A few crystals of phenylthiourea (0.5-1.0 μg) were added to inhibit tyrosinases which bring about melanization of the hemolymph thus altering its physiological status and rendering it unfit for physiological work. Hemolymph was centrifuged at 800 Xg for 15 min at room temperature to remove any hemocytes or fat body cells. The tubes were sealed with Parafilm and frozen rapidly in a freezer (-15 °C). Frozen hemolymph, kept as such to a maximum of 4 weeks, was thawed in cold water before use. In

some cases, freshly collected hemolymph was used for analytical purposes. Rapid freezing minimized any physiological alteration.

3.3 Hemolymph Proteins

Hemolymph was pooled from about 30 larvae or 25 pupae for each experimental conditions and rapidly frozen for later use.

3.3.1 Hemolymph Protein Concentration

Hemolymph protein concentration was determined by the Biuret Assay (Gornall et al., 1949). 0.25 ml of fresh or thawed hemolymph was mixed with 0.75 ml of distilled water. To this, 4.0 ml of the Biuret reagent was added and briefly mixed on a vortex mixer. The reaction mixture was allowed to stand at room temperature (24-25 °C) for 30 min to allow color development. A blank consisted of 1.0 ml of distilled water and 4.0 ml of the Biuret reagent. After 30 min, 1.5 ml of the mixture was taken in spectrophotometer cuvettes and the absorbance measured against the blank in a Pye-Unicam SP-100 uv spectrophotometer at the wavelength of 550 nm. Protein levels in the hemolymph were determined by comparing the absorbance values with a standard curve made from known amounts of bovine serum albumin.

3.3.2 Electrophoresis

The sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique described by Laemmli (1970) was modified to analyze hemolymph proteins. Sample preparation was done as follows : 200 μ l of fresh or thawed hemolymph were mixed with 20 μ l of 20% (v/v) Triton X-100 on ice for 1 hour. Then, 100 μ l of 10% (w/v) sodium dodecylsulphate, 10 μ l of 2-mercaptoethanol, 200 μ l of 50% (v/v) glycerol and 10 μ l of 0.002% (w/v) bromophenol blue were added and briefly vortexed. Final volume (1.0 ml) was made by adding sample buffer (1.5 M Tris-HCl buffer, pH 8.8, 25 °C). Following this, the samples were heated in a boiling water bath for 3 min to ensure denaturation of all proteins. Samples were allowed to cool to room temperature (25 °C). Prior to loading for electrophoresis, the samples were centrifuged at 7000 Xg for 2 min at 25 °C to get all the solution to the bottom of the microcentrifuge tube. 40 μ g of protein were loaded in each lane on the gel.

Vertical slab gel electrophoresis was done using a Hoefer SE400 apparatus (Hoefer Scientific Instruments, San Francisco, California). A 15% resolving and 5% stacking gel was used. The reagents used for electrophoresis are listed in Appendix B. The gel was transferred to a glass trough and the proteins stained and fixed with 0.5% (w/v) Coomassie blue R-250 in 7% (v/v) acetic acid and 40% (v/v) methanol for 30 min (Hames and Rickwood, 1981). Destaining was done

with a solution containing 5% (v/v) methanol and 7.5% (v/v) acetic acid to clear the gel of excess stain. A series of changes with fresh destaining solution were given initially at hourly intervals until the gel was clear enough to facilitate protein identification. Both staining and destaining were done at room temperature on a slow shaker.

Destained gels were transferred to a glass plate for safe handling and to avoid breakage. Gels were wrapped in saran wrap and stored in the refrigerator at 5 °C. Destained gels were photographed using an orange filter and then scanned on a Beckman DU-8 spectrophotometer with gel scanning system (Beckman Instruments, Inc. Berkeley, California). Scanning was done at 550 nm wavelength. Relative mobility (R_f = ratio of distance migrated by the protein to distance migrated by the tracking dye) was calculated and molecular weights were determined by running a series of protein standards, obtained from Sigma Chemical Co. (St-Louis, MO). A typical molecular weight standard curve is given in Figure 2. A hemolymph protein profile is shown in Figure 3. For simplicity, the term "protein" rather than "polypeptide" has been used with the understanding that not all proteins reported are necessarily the functional form.

S

Figure 2: Semilog plot of molecular weight standards against relative mobility in a 15% polyacrylamide gel. Standards were obtained from Sigma Chemical Co., St. Louis, Missouri. The standards are :

- 1) Bovine Serum Albumin (M.W. 66,000)
- 2) Egg Albumin (M.W. 45,000)
- 3) G-3-P Dehydrogenase (M.W. 36,000)
- 4) Carbonic Anhydrase (M.W. 29,000)
- 5) Trypsinogen (M.W. 24,000)
- 6) Trypsin Inhibitor (M.W. 20,100)

a

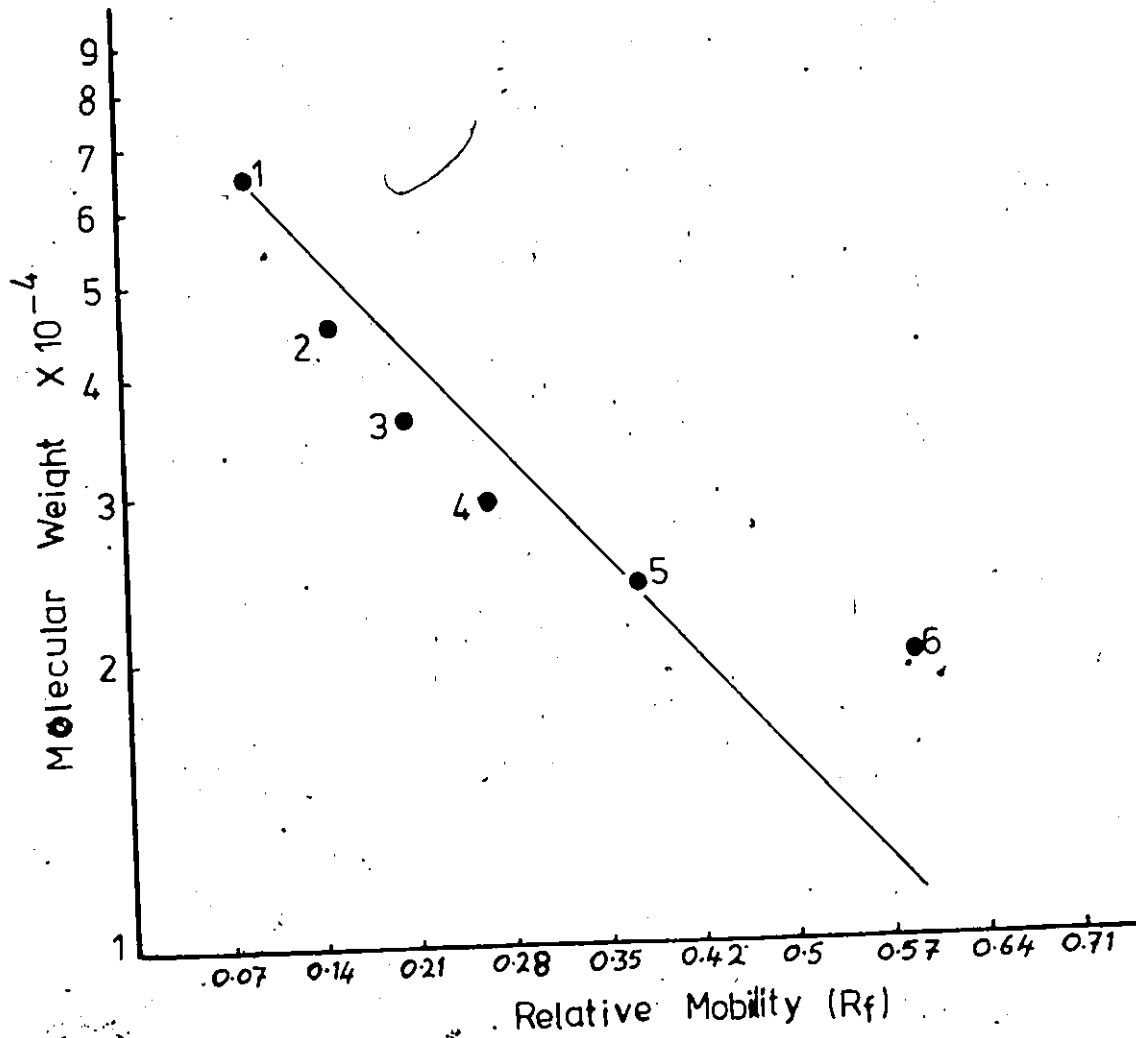


Figure 3: SDS-Polyacrylamide gel electrophoresis of proteins in the hemolymph of 5th instar Manduca sexta larvae.

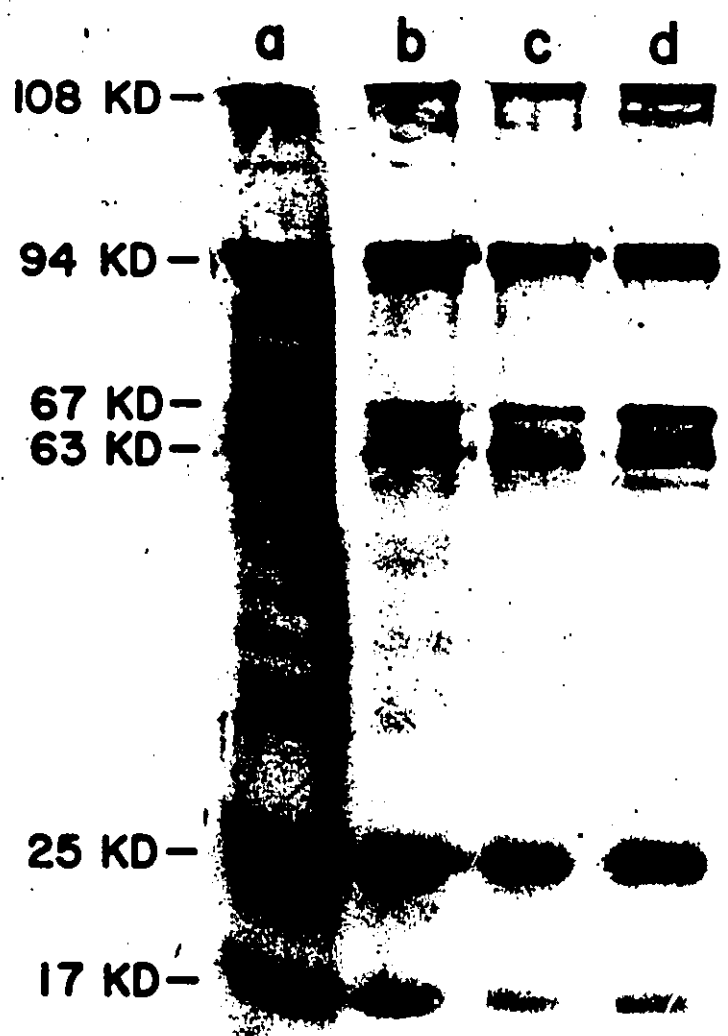
A 5% stacking and 15% resolving gel was used to separate proteins. Molecular weights are given in kilodaltons (kD).

a: Long day-Indorsun

b: Long day-Blacklight blue

c: Short day-Indorsun

d: Short day-Blacklight blue



3.4 Hemolymph Free Amino Acids

Fresh hemolymph was pooled from about 25 larvae or 25-30 pupae for each experimental condition to determine free amino acids. This was followed by centrifugation at 360 Xg for 2 min at room temperature (25 °C) to precipitate hemocytes. Microscopic examination of the hemolymph revealed that it was cell-free. Norleucine was added as an internal standard and the amino acid recoveries corrected accordingly. Sample preparation was done as follows : 400 µl hemolymph was deproteinized by adding 3.0 ml of cold 5% aqueous trichloroacetic acid and briefly mixing on a Vortex (Morgan and Ghippendale, 1983).

Amino acid analysis of the extracts was achieved with a Beckman Automatic Amino Acid Analyzer, Model 121 MB with Model 126 data system (Beckman Instruments, Inc. Berkeley, California). A three buffer, single column method for the analysis of physiological fluids was used (Benson and Patterson, 1971). Extracts were transferred to 20 µl sample loops which were then loaded on the autoinjector of the analyzer. A Beckman 121 MB instrument control program for physiological fluid analysis was used. Amino acids were eluted from a 2.8 mm X 100 mm column packed with Durham resin (DC 4A, size 9 µ ±0.5 µ) with a series of three buffers. Lithium citrate buffer (pH 2.83, 0.2 N; 3.7, 0.2 N; and 3.75, 1.0 N) was used as the eluting agent at a flow rate of 10.0 ml/h and the column temperature was raised from 38 °C

to 67 °C over 66 min from injection. Ninhydrin was used as the detection agent at a flow rate of 5 ml/h and Lithium hydroxide (0.3 N) was used as the regenerating agent at a flow rate of 10 ml/h.

Ninhydrin, a powerful oxidizing agent, causes oxidative decarboxylation of amino acids, producing carbon dioxide, ammonia and an aldehyde with one less carbon atom than the parent amino acid. The reduced ninhydrin then reacts with the liberated ammonia, forming a blue-purple complex which maximally absorbs at 570 nm. Whereas most of the amino acids give purple colors, certain imino acids such as proline and hydroxyproline give yellow colors with ninhydrin maximally absorbing at 440 nm (Harper *et al.*, 1977). Hence, colorimetric determination was done at a dual wavelength of 440 nm and 570 nm. A representative chromatogram is shown in Figure 4. Commercial amino acid standards (Hamilton, U.S.A.) were run after every six injections and the amino acid quantification was done using a Beckman System AA Computing Integrator.

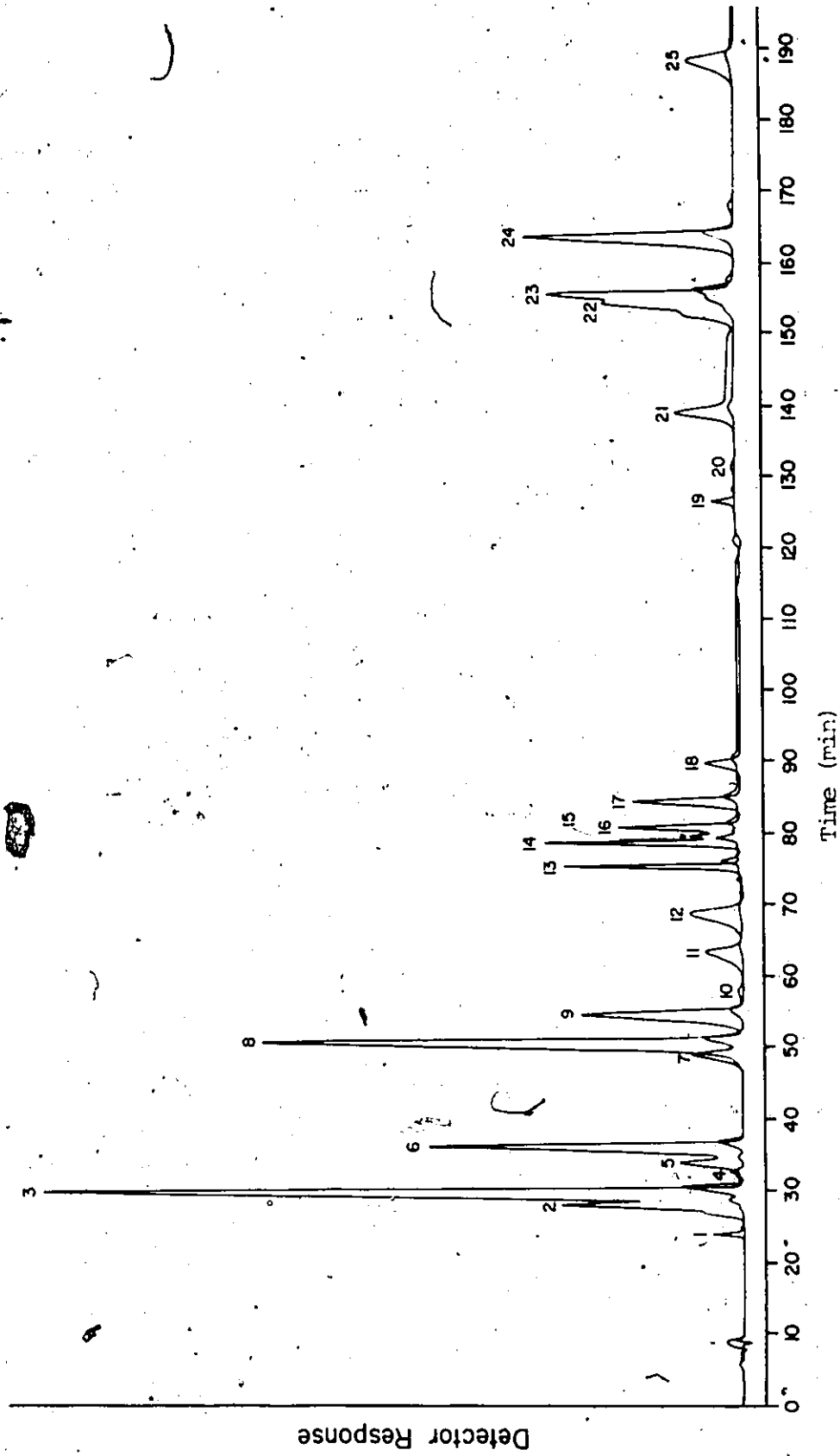
3.5 Hemolymph Sugars

Hemolymph was pooled from about 15-20 larvae or 15 pupae for each experimental condition. The methods used by Baust and Edwards (1979) were modified for sugar extraction from the hemolymph. Fresh hemolymph was diluted with distilled water in a proportion of 1 : 1 (v/v) and mixed briefly on a

Figure 4: Chromatogram of free amino acids present in the hemolymph of Manduca sexta. Extracts were analyzed in a Beckman Automatic Amino acid Analyzer with the 121 MB instrument control program for physiological fluid analysis.

The amino acids are:

- | | |
|------------------------|-------------------|
| 1) Aspartic acid | 14) Cystathionine |
| 2) Threonine | 15) Isoleucine |
| 3) Serine | 16) Leucine |
| 4) Asparagine | 17) Tyrosine |
| 5) Glutamic acid | 18) Phenylalanine |
| 6) Glutamine | 19) GABA |
| 7) Proline | 20) Tryptophan |
| 8) Glycine | 21) Ammonia |
| 9) Alanine | 22) Ornithine |
| 10) Citrulline | 23) Lysine |
| 11) Alpha butyric acid | 24) Histidine |
| 12) Valine | 25) Arginine |
| 13) Methionine | |



Vortex. A known amount of trehalose was added as an internal standard and the recoveries were estimated by equating with a duplicate sample. The extraction efficiency was over 90%. The mixture was partitioned against an equal volume of chloroform : methanol (2 : 1, v/v) in a flask at room temperature (25 °C) and then deproteinized by adding 20 µl/ml aqueous phase each of 15% potassium ferricyanide (w/v) and 30% zinc sulphate (w/v) followed by mixing. The mixture was then centrifuged at 800 Xg for 30 min at room temperature to precipitate the solids. The supernatant extracts were aspirated for 60 min at 80 °C in a rotary flash evaporator (Buchi, Switzerland) to volatalize the organic solvents. This was followed by deionization by adding a few grains (approximately 0.5 mg/ml of extract) of analytical grade Rexyn (H-OH) resin (Fisher Scientific Co., N.J.). The deionized extracts were filtered through a 0.4 µm inert nylon filter (Schleicher and Schuell, Inc., N.H.) and stored in a refrigerator at 5 °C before analysis.

Analysis of sugars in the hemolymph extracts was accomplished by a Beckman High Pressure Liquid Chromatography (HPLC) system (Beckman Instruments, Inc., Berkeley, California). Two Model 110A piston pumps controlled by a Model 420 System Programmer, delivered the solvents over a mixing chamber to the top of the column. Mean column pressure was 250 psi. Injection was performed by a Sample Injection Valve (Model 210, Altex, Berkeley, CA) fitted with a 20 µl

loop. An Ultrasil-Amino column (Altex) (250 mm X 4.6 mm i.d. particle size 10 μ) was used to separate sugars. Sugars were monitored with a Model 165 Variable UV-VIS Detector. The analytical protocol was described by Binder (1980). HPLC grade acetonitrile and water (Fisher Scientific Co., N.J.) in a proportion of 75 : 25 (vol/vol) were pumped at a flow rate of 2.0 ml/min at room temperature (25 °C). Since carbohydrates absorb only at wavelengths lower than 200 nm, very high demands are placed on the purity of the solvent, because the acetonitrile itself begins to absorb very strongly in this region. For all sugars, the absorption maximum lies between 187 and 188 nm and at this wavelength, the influence of the strong absorption of acetonitrile becomes noticeable. Moreover, when decreasing the wavelength, the noise of the detector signal increases rapidly. Therefore, UV detection of sugars was done at 192 nm, the slight loss in sensitivity being offset by the higher concentrations of trehalose and glucose in the extracts.

Commercial standards of trehalose and glucose obtained from Sigma Chemical Co. (St.-Louis, MO) were run under identical analytical conditions and the chromatograms were evaluated with reference to Standard retention time and peak height. A typical chromatogram is shown in Figure. 5.

Figure 5: HPLC chromatogram of trehalose and glucose present in the hemolymph of Manduca sexta.

1) Glucose

2) Trehalose

1.0 AUFS: One Absorbance Unit Full Scale.

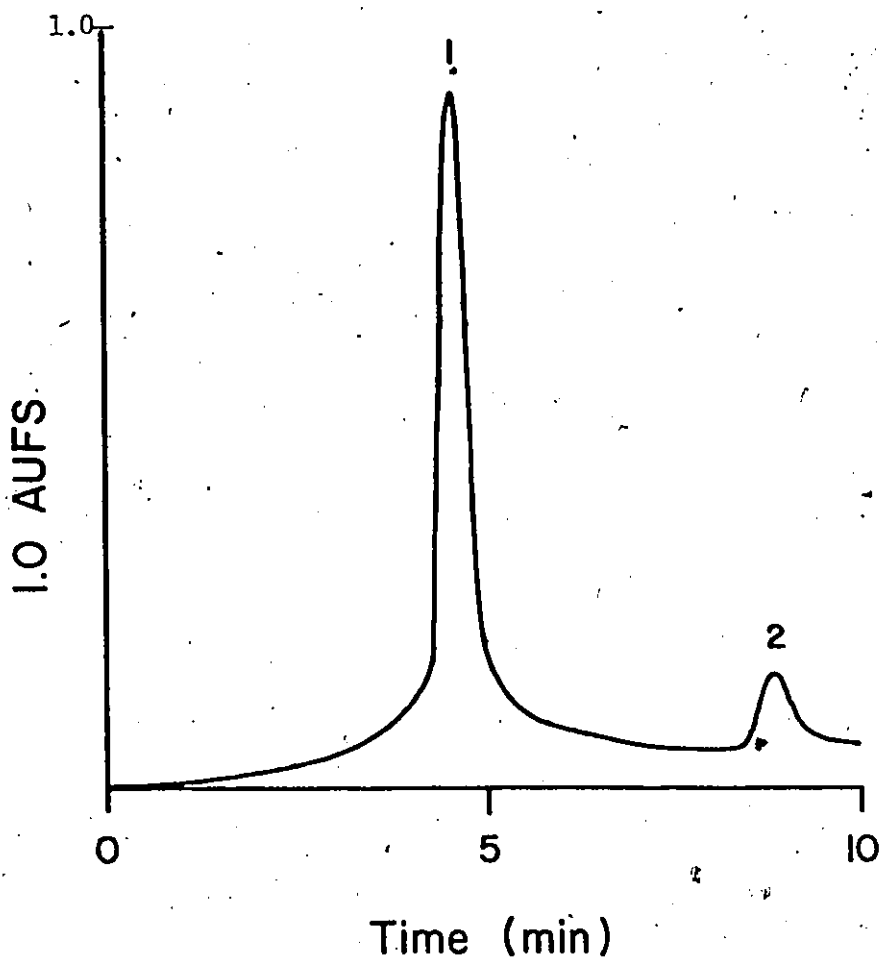
A Beckman HPLC system was used.

Mobile phase: Acetonitrile/Water (75/25).

Flow rate 2.0 ml/min.

Column: Ultrasil-Amino (250 mm X 4.6 mm i.d.)

UV detection at 192 nm wavelength.



3.6 Hemolymph Osmolality

Hemolymph was pooled from about 10 larvae or 10 pupae for each experimental condition and centrifuged at 800 Xg for 15 min at room temperature to sediment the cellular material.

Osmolality is an expression of the total concentration of dissolved particles in a solution without regard for the particle size, density, configuration, or electrical charge. Osmolality can be measured indirectly because of the fact that the addition of solute particles to the solvent changes the free energy of the solvent molecules which results in a modification of the cardinal properties of the solvent such as vapor pressure, freezing point and boiling point. Any changes in the levels of proteins, free amino acids or sugars in the hemolymph should, therefore be partially reflected by changes in the osmolality of the hemolymph.

A Wescor Model 5100C Vapor Pressure Osmometer (Wescor, Inc. Logan, UT) was used to measure hemolymph osmolality. 8 μ l of fresh hemolymph was pipetted onto the sample disc in the sample slide of the osmometer using a Wescor micropipette. Then the sample slide was inserted into the osmometer and after the processing time of 50 seconds, direct readings of the osmolality were obtained for the sample. Prior to the actual analysis, the osmometer was thermally balanced and stabilized at room temperature (25 °C) for 24 hours, followed by standardization with Wescor Osmolality Standards.

3.7 Statistical Analysis of Results

The experimental design used was a factorial in randomized blocks (Photoperiod X Light Quality X Age of Instar). Data collected from experiments were subjected to an analysis of variance. An Amdahl 470/V7A processor running the IBM VM370/CMS system (University of Ottawa) and the Statistical Package for Social Sciences (SPSS) were used to analyze the data. Statistical significances of differences were estimated by Duncan's Multiple Range Test, $P < 0.05$. (Nie et al., 1975).

Chapter IV

RESULTS

4.1 Larval Growth

Larval growth is significantly influenced by photoperiod, light quality and the age of instar ($F=31.38, d.f.=1$; $F=23.98, d.f.=1$; $F=460.20, d.f.=14$; respectively. $P < .05$). Photoperiod/age and light quality/age interactions are also significant ($F=2.98, d.f.=14$; $F=5.37, d.f.=14$; respectively. $P < .05$).

Figure 6 shows the growth curves for larvae reared under combinations of Long day-Indorsun (LD-IND), Long day-Blacklight blue (LD-BLB), Short day-Indorsun (SD-IND) and Short day-Blacklight blue (SD-BLB). The general trend with respect to live weight over the larval life for these four rearing conditions is LD-IND < LD-BLB < SD-IND < SD-BLB. Maximum mean weights attained by the larvae on day 15 under LD-IND, LD-BLB, SD-IND and SD-BLB were 5.01, 6.76, 6.44 and 7.49 g respectively. Statistical significances of differences of means, as estimated by Duncan's Multiple Range Test ($P < 0.05$) are shown in Appendix C.

Figure 6: Development of the tobacco hornworm larvae under different conditions of photoperiod and light.

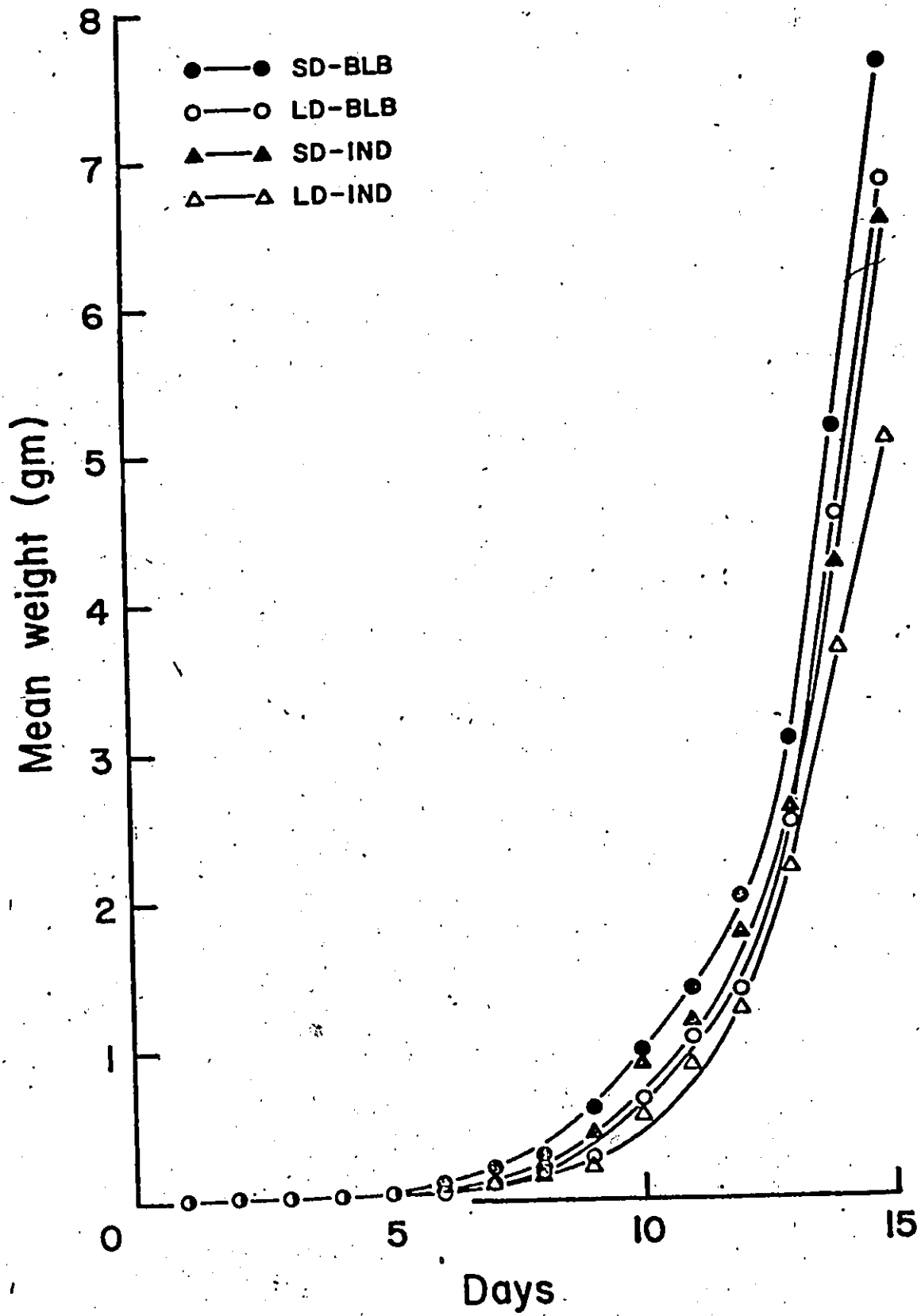
LD-IND: Long day-Indorsun

LD-BLB: Long day-Blacklight blue

SD-IND: Short day-Indorsun

SD-BLB: Short day-Blacklight blue

Standard deviations are given in Appendix C.



4.2 Pupal Weight

Photoperiod alone had a significant effect on pupal weight ($F=5.38, d.f.=1; P < .05$). The variations in pupal weights (day 5) according to the rearing conditions followed a pattern similar to that of the larvae. Pupae reared under LD-IND weigh, on an average, 4.12 g (S.D.=0.98; $n=42$) while those reared under LD-BLB weigh 4.39 g (S.D.=0.56; $n=45$). SD-IND and SD-BLB reared pupae weigh 4.58 g (S.D.=0.75; $n=37$) and 4.90 g (S.D.=0.75; $n=38$), respectively. However the differences between values for LD-BLB and SD-IND pupae, as estimated by Duncan's Test (0.05 level) were not statistically different.

4.3 Hemolymph Protein Concentration

The mean hemolymph protein concentration of M. sexta reared under different conditions is shown in Table 2. A statistical analysis of the data obtained shows that protein concentration of the hemolymph is significantly influenced by the age of instar ($F=44.11, d.f.=6; P < .05$). Interaction between photoperiod/age of instar, light quality/age of instar, and photoperiod/light quality/age of instar are statistically significant ($F=7.81, d.f.=6; F=2.80, d.f.=6; F=2.84, d.f.=6$; respectively. $P < .05$). The mean hemolymph protein concentration of M. sexta larvae (Day 1, 5th instar) reared under LD-IND, LD-BLB, SD-IND and SD-BLB are 16.6, 22.33, 16.67 and 20.33 mg/ml, respectively. A decline on day 2

Table 2: The hemolymph protein concentration of the tobacco hornworm, Manduca sexta reared under different experimental conditions. Hemolymph was pooled from 25-30 animals. Determinations were made by the Biuret Assay with Bovine serum albumin as a standard.

Values are reported as Mean (S.D. of Mean) of three analyses. Values followed by the same letter in the column indicate no significant difference at the 0.05 level (Duncan's Multiple Range Test).

Hemolymph protein concentration (mg/ml) of Manduca sexta reared under different conditions.

Rearing Condition	Age of Instar									
	V d1	V d2	V d3	V d4	V d5	P d5	P d10			
Long day-Indorsun	16.60 ^a (4.01)	14.00 ^{ab} (0.86)	23.83 ^{ab} (0.28)	26.17 ^{ab} (0.76)	36.00 ^b (1.00)	30.83 ^a (2.08)	37.00 ^b (3.77)			
	Long day-Blacklight blue	22.33 ^a (0.28)	11.00 ^a (3.12)	23.67 ^{ab} (1.25)	25.00 ^a (3.00)	31.50 ^a (2.29)	30.73 ^a (0.25)	34.33 ^b (2.08)		
Short day-Indorsun		16.67 ^a (4.31)	22.00 ^b (4.44)	24.50 ^b (1.32)	29.33 ^b (0.76)	34.17 ^{ab} (1.60)	38.33 ^a (12.7)	28.00 ^{ab} (1.32)		
	Short day-Blacklight blue	20.33 ^a (1.25)	21.50 ^b (6.24)	21.83 ^a (1.04)	35.50 ^c (3.04)	38.67 ^{bc} (1.52)	26.33 ^a (1.44)	26.33 ^a (5.50)		

occurred under LD-IND and LD-BLB to 14.0 and 11.0 mg/ml respectively. By contrast, hemolymph protein concentration rose in SD-IND and SD-BLB conditions to 22.0 and 21.5 mg/ml respectively. On day 3, protein levels in the hemolymph rose under LD-IND and LD-BLB conditions whereas they remained stable under SD-IND and SD-BLB. Day 4 is characterized by sharp increases in protein titres under all conditions, particularly under SD-IND and SD-BLB regimen. A further rise in the protein concentration of the hemolymph of M. sexta was seen on day 5, the prepupal instar, under all conditions of rearing. Protein concentration in the hemolymph of early pupae (Day 5, pupal instar) decreased slightly except under SD-IND, when a slight rise was evident. However, it was not statistically significant. 10 day old developing pupae again show a rise in protein concentration of the hemolymph under LD-IND and LD-BLB to 37.0 and 34.3 mg/ml respectively. In contrast, protein concentration fell under SD-IND to 28.0 mg/ml and remained constant under SD-BLB at 26.3 mg/ml (Table 2).

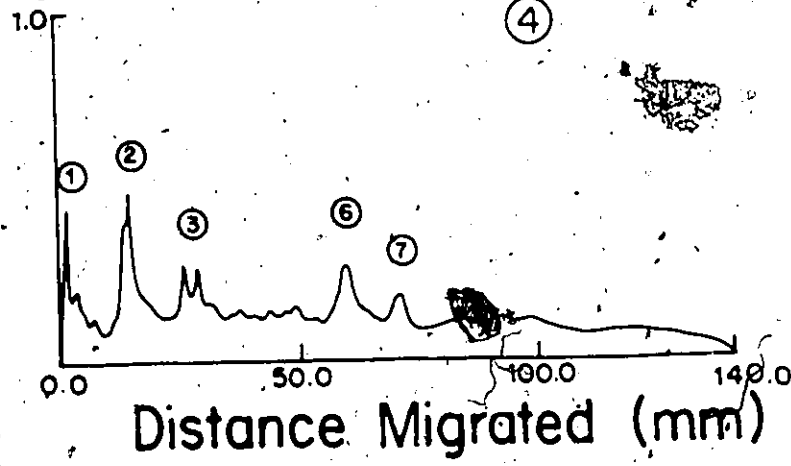
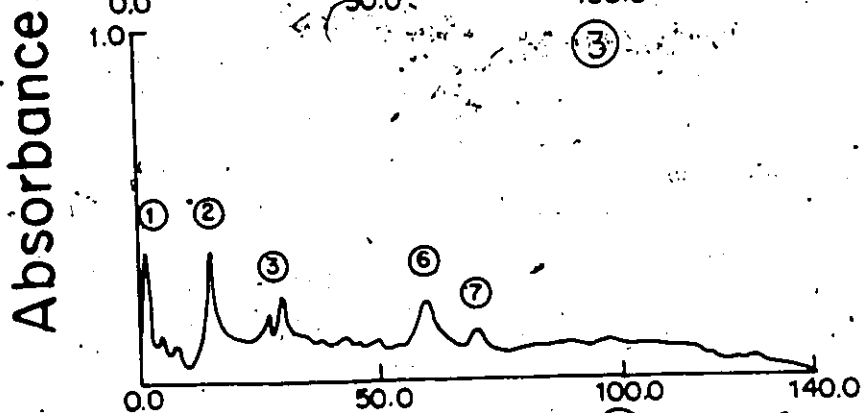
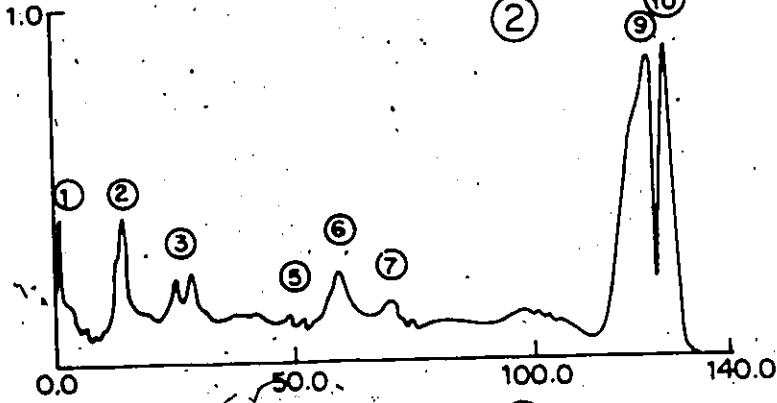
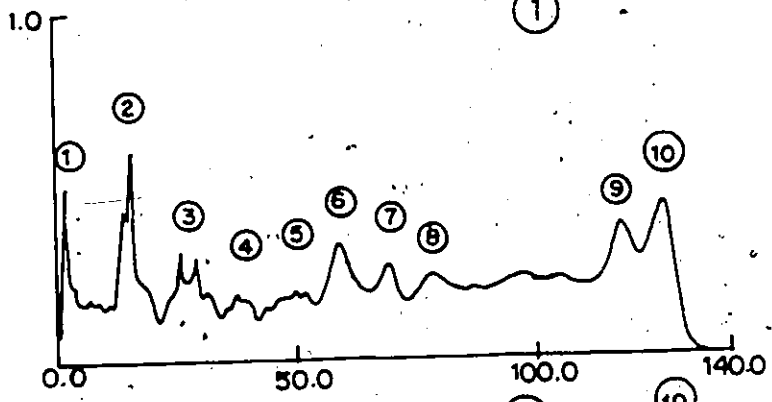
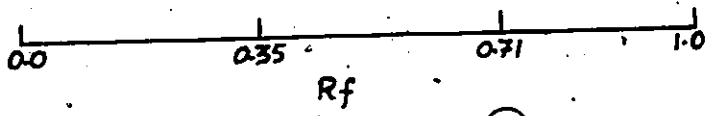
4.3.1 Hemolymph Protein Profile

Figure 7 shows the hemolymph protein profile of 5th instar day 1, M. sexta reared under various conditions. Under LD-IND, several protein bands were detected in the hemolymph. Under LD-BLB conditions, some bands were detected in the hemolymph. Levels of band 4 and 8 are trace in the hemolymph. However, in this population, there is a major

Figure 7: Electrophoregram showing qualitative distribution of proteins in the hemolymph of day 1, 5th instar Manduca sexta under different rearing conditions.

40 μ g of protein was loaded in each lane and all samples were run on the same gel. Destained gels were scanned at 550 nm wavelength on a Beckman DU-8 gel scanner. Hemolymph was pooled from 30 insects.

- 1: LD-IND
- 2: LD-BLB
- 3: SD-IND
- 4: SD-BLB



rise in the concentration of bands 9 and 10, which are low molecular weight proteins. There are some changes in the levels of other proteins. In the diapause programmed SD-IND insects, there are some protein bands detected in the hemolymph, with absence of band 9 and 10. Levels of other proteins show slight change. Under SD-BLB condition, some bands were detected in the hemolymph, and as compared to SD-IND, there are minimal changes in the levels of protein bands in the hemolymph.

Figure 8 shows the hemolymph protein profile of 5th instar day 5 tobacco hornworms reared under different conditions of photoperiod and light. Under all rearing conditions (LD-IND, LD-BLB, SD-IND and SD-BLB), there are several protein bands detected in the hemolymph of *M. sexta*. Notable differences are seen in the concentration of band 2, which could be the storage protein manducin. This is the major hemolymph protein in the tobacco hornworm prepupae, constituting over 80% of the hemolymph protein profile. Under LD-IND, levels of manducin are lower in the hemolymph as compared to other rearing conditions. There is almost a 50% rise in the concentration of this band in LD-BLB population. Under SD-IND and SD-BLB, there is about 40% and 30% rise in the levels of manducin, respectively. Other protein bands show negligible changes.

The hemolymph protein profile of *M. sexta* pupae (day 5) reared under different conditions is shown in Figure 9.

Figure 8: Qualitative distribution of proteins in the hemolymph of day 5, 5th instar Manduca sexta reared under various conditions. 40 μ g of protein was loaded in each lane and all samples were run on the same gel. Destained gels were scanned at 550 nm wavelength to obtain this profile. A Beckman DU-8 scanning system was used. Hemolymph was pooled from 30 larvae.

1: LD-IND

2: LD-BLB

3: SD-IND

4: SD-BLB

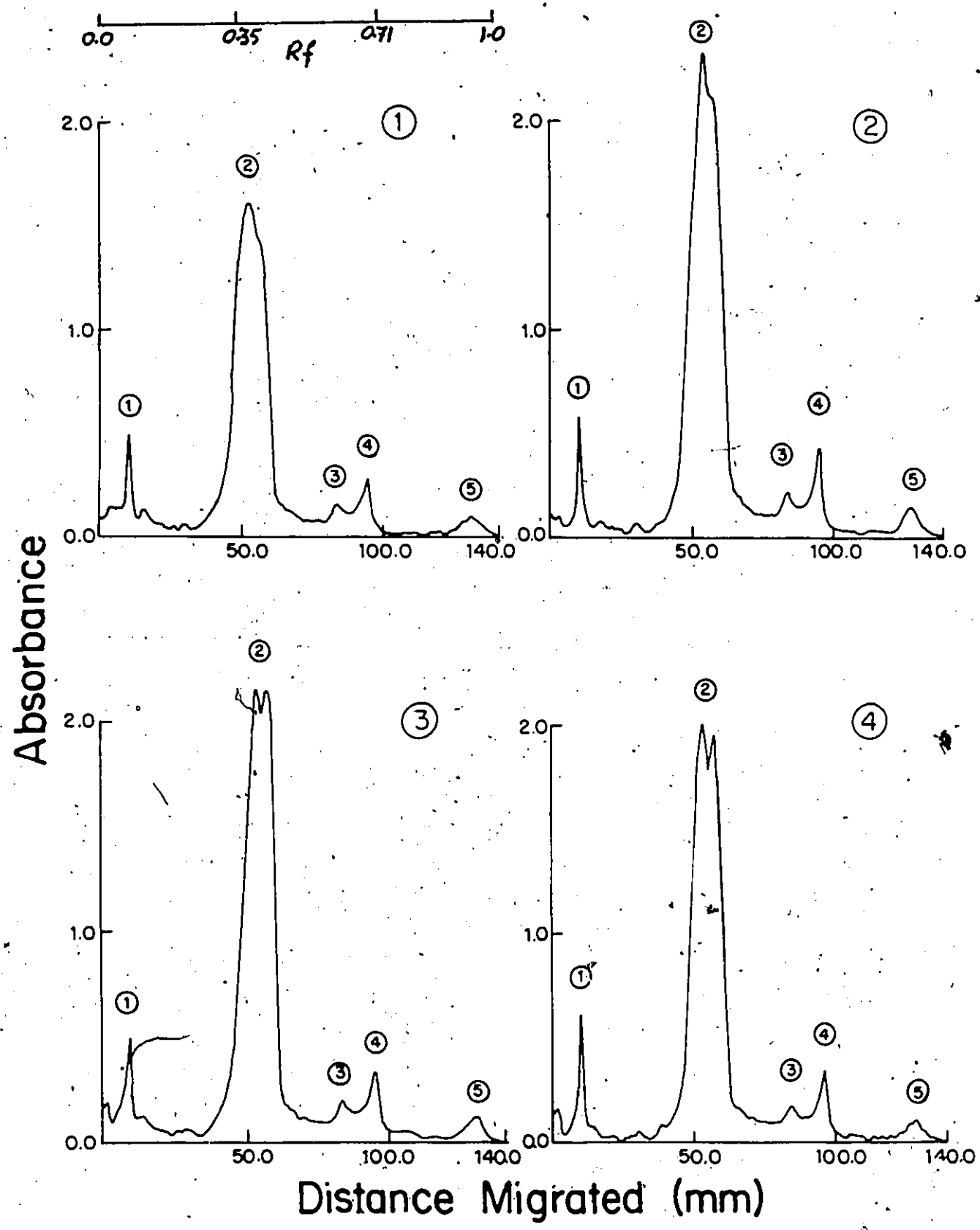


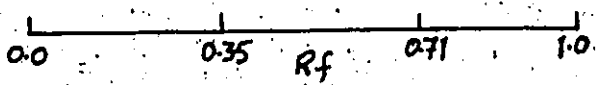
Figure 9: Qualitative distribution of proteins in the hemolymph of day 5 pupae of Manduca sexta reared under different conditions. 40 μ g of protein was loaded in each lane and all samples were run on the same gel. After destaining, gels were scanned at 550 nm on a Beckman DU-8 gel scanning system. Hemolymph was pooled from 25 animals.

1: LD-IND

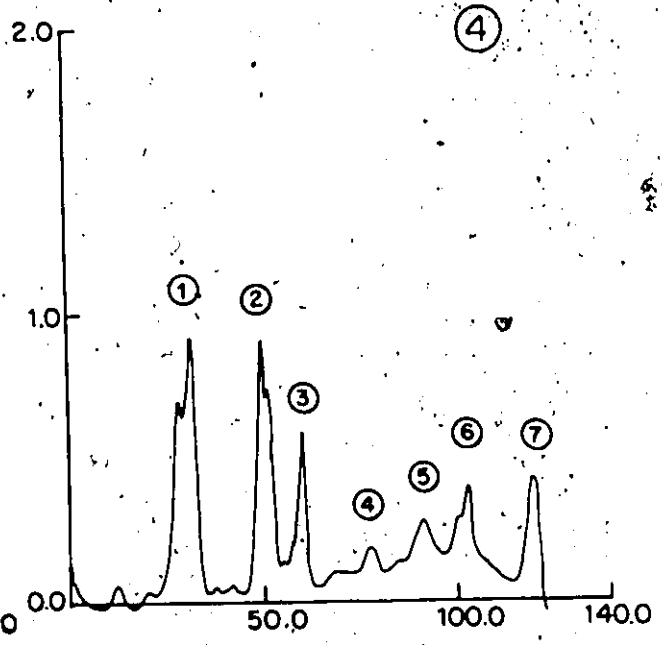
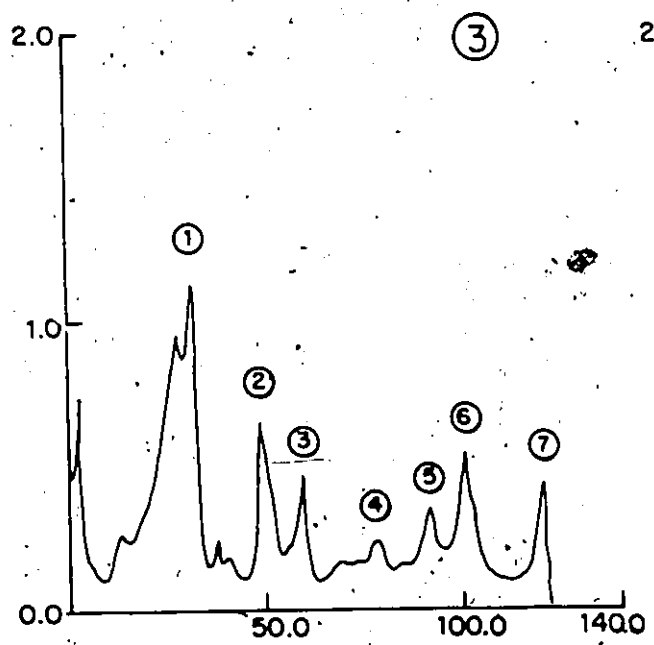
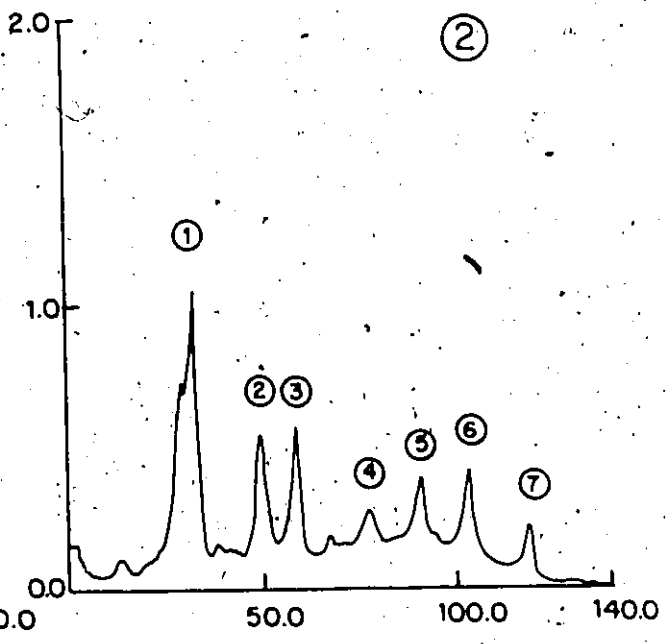
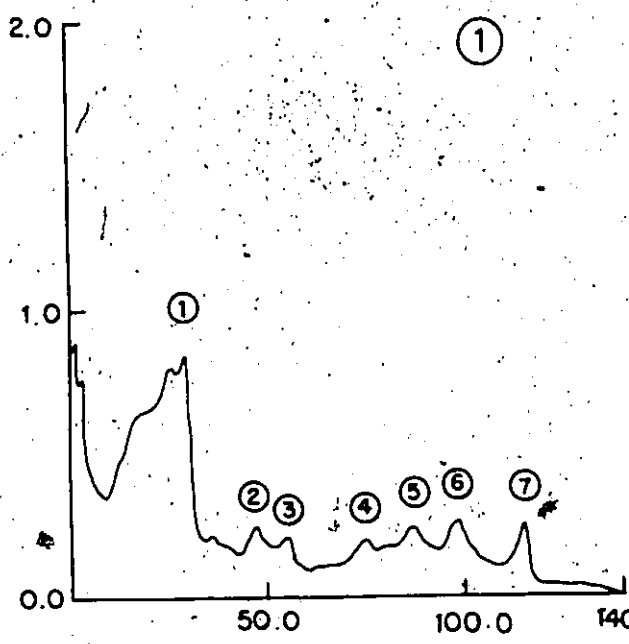
2: LD-BLB

3: SD-IND

4: SD-BLB



Absorbance



Distance Migrated (mm)

There are some differences in the levels of different protein fractions under various rearing conditions. Under LD-IND, band 1 is a major protein band in the hemolymph, while levels of other proteins are lower in the profile. As compared to this, under LD-BLB there is some change in the resolution of band 1 and there is a rise in the levels of band 2, 3, 4, 5 and 6. Under diapause programming SD-IND conditions, there is a rise in the concentration of band 1, 2 and 6, with a slight change in other protein bands in the hemolymph. As compared to this, in SD-BLB population, there is a slight fall in the concentration of band 1 and a rise in the levels of band 2 in the hemolymph.

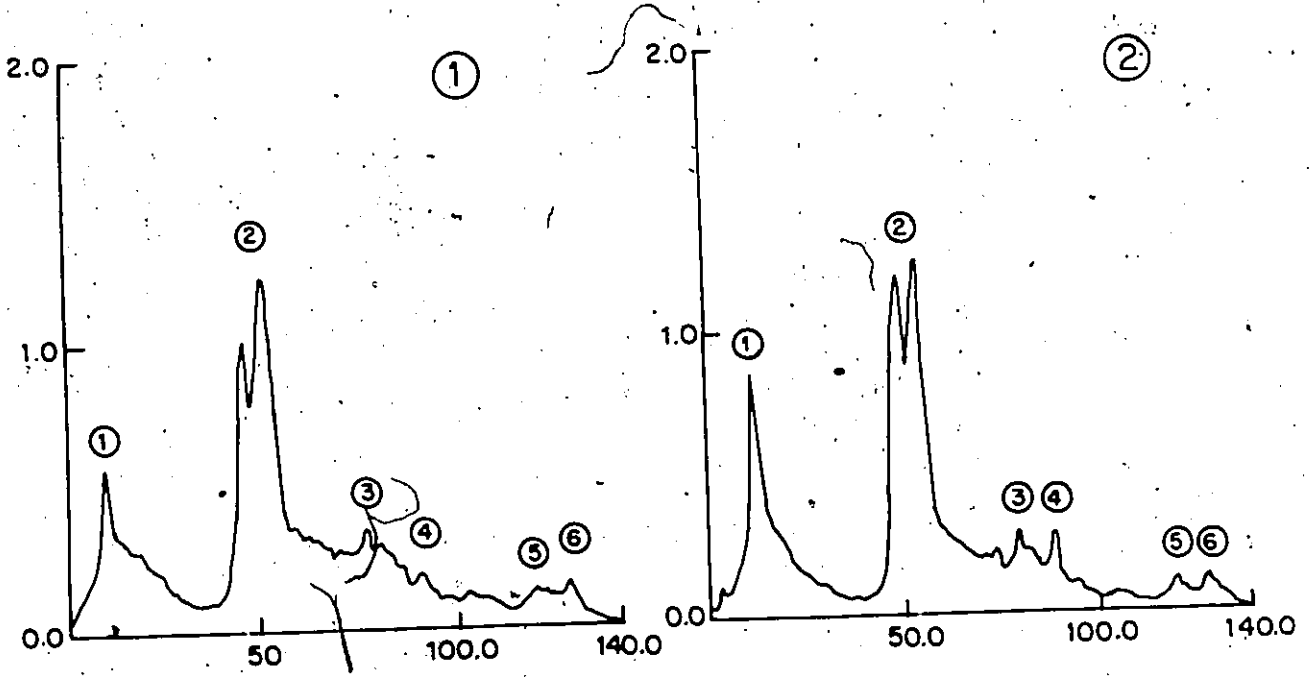
The protein profile of the hemolymph of hornworm pupae (day 10) reared under various conditions of photoperiod and light are shown in Figure 10. There are several protein bands detected in the hemolymph of M. sexta reared under LD-IND, LD-BLB, SD-IND and SD-BLB, with some changes in the concentration of individual protein fractions. There are some differences in the levels of band 2, which could be the storage protein manducin. There are minimal changes in the concentration of other individual protein bands in the hemolymph of insects reared under various conditions.

Figure 10: Qualitative distribution of proteins in the hemolymph of day 10 pupae of Manduca sexta reared under different experimental conditions.

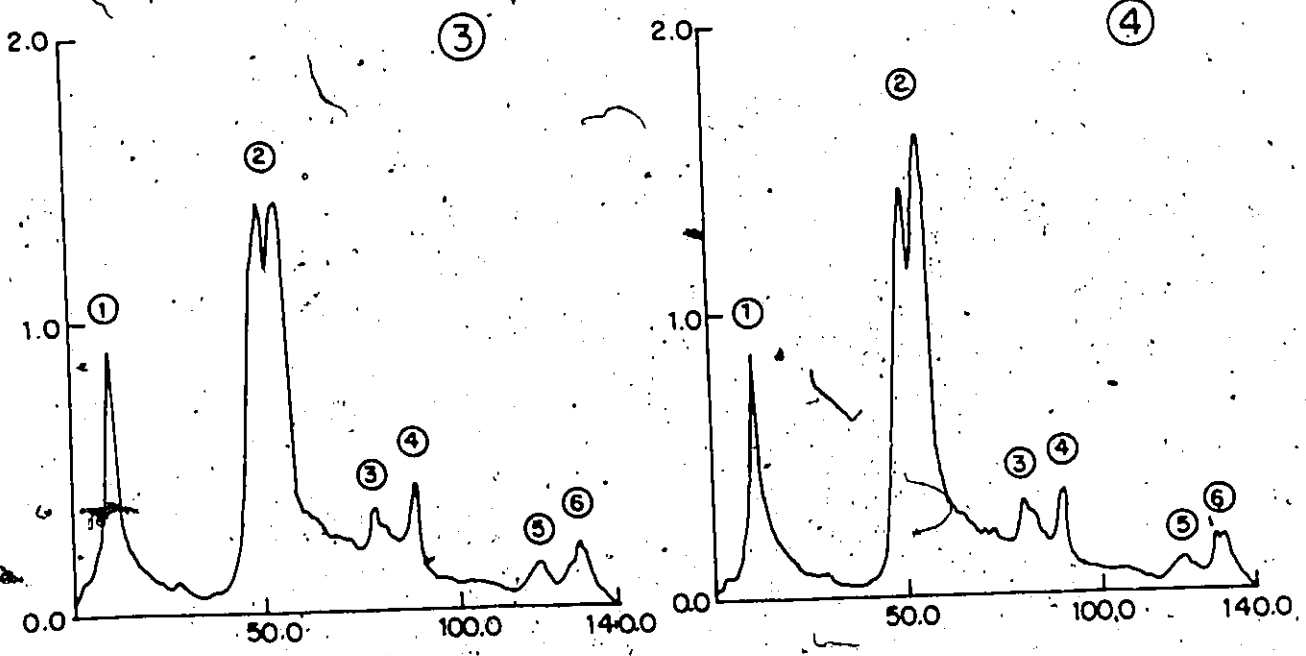
40 μ g of protein was loaded in each lane and all samples were run on the same gel. Destained gels were scanned at 550 nm wavelength on a Beckman DU-8 gel scanner. Hemolymph was pooled from 25 insects.

- 1: LD-IND
- 2: LD-BLB
- 3: SD-IND
- 4: SD-BLB

0.0 0.35 Rf 0.71 1.0



Absorbance



Distance Migrated (mm)

4.4 Hemolymph Free Amino Acids

The titres of free amino acids found in the hemolymph of M. sexta reared under different experimental conditions are shown in Tables 3-6. Statistical analysis of the data obtained shows that photoperiod and the age of instar are the important factors which significantly influence the titres of many free amino acids in the hemolymph of M. sexta. Light quality also has a significant effect on a few amino acids in the hemolymph of the tobacco hornworm. The analysis of variance of the main effects as well as various interactions between the main effects for various free amino acids found in the hemolymph of the tobacco hornworm are given in Appendix D.

In the 5th instar, day 1 larvae reared under LD IND+UV (Table 3), the most abundant free amino acids are serine (24.2 mM), glycine (19.8 mM), proline (18.9 mM) and glutamine (13.7 mM), followed by alanine (8.3 mM), histidine (7.9 mM), threonine (7.3 mM) and lysine (6.9 mM). Insects reared under LD IND-UV do not show any significant difference from IND+UV population. Under diapause-inducing SD IND+UV conditions, the most abundant amino acids are serine (17.6 mM) and glycine (12.5 mM), followed by proline (9.2 mM) and histidine (6.0 mM). Levels of proline are significantly lower than in insects reared under developing conditions. Under SD IND-UV conditions, hornworms have higher levels of serine (21.5 mM), glycine (13.1 mM) and glutamine (8.7 mM).

Table 3: Titres of free amino acids present in the hemolymph of day 1, 5th instar Manduca sexta reared under different conditions. Hemolymph was pooled from 25 animals. Extracts were analyzed on a Beckman Automatic Amino Acid Analyzer. Values are reported as Mean (S.D. of Mean) of three analyses. Values followed by the same letter for each amino acid indicate no significant difference at the 0.05 level (Duncan's Multiple Range Test).

Hemolymph free amino acids (μM) in the 5th instar, day 1 Manduca sexta reared under various conditions.

Amino acid	LD IND+UV	LD IND-UV	SD IND+UV	SD IND-UV
Alanine	8.30 (1.87) ^b	6.38 (0.46) ^{ab}	4.72 (0.28) ^a	7.09 (2.50) ^{ab}
Arginine	3.20 (0.50) ^c	3.01 (0.25) ^{bc}	2.25 (0.50) ^{ab}	2.10 (0.34) ^a
Asparagine	4.16 (1.30) ^a	4.96 (2.50) ^a	2.26 (0.73) ^a	2.45 (0.52) ^a
Aspartic acid	0.46 (0.06) ^b	0.42 (0.01) ^b	0.28 (0.03) ^a	0.28 (0.02) ^a
Citrulline	0.26	0.13	*	0.80 (0.50)
Cystathionine	2.62 (0.67) ^b	1.75 (0.28) ^a	1.26 (0.15) ^a	1.46 (0.44) ^a
Glutamic acid	2.35 (0.37) ^b	1.37 (0.76) ^{ab}	1.12 (0.54) ^a	1.44 (0.62) ^{ab}
Glutamine	13.77 (6.30) ^a	8.81 (3.38) ^a	6.01 (2.94) ^a	8.71 (1.27) ^a
Glycine	19.80 (6.70) ^a	14.63 (0.60) ^a	12.51 (1.36) ^a	13.11 (2.77) ^a
Histidine	7.91 (1.81) ^b	6.70 (0.54) ^{ab}	6.01 (0.61) ^{ab}	5.61 (0.21) ^a
Isoleucine	1.22 (0.13) ^b	1.49 (0.02) ^b	0.83 (0.27) ^a	0.83 (0.18) ^a
Leucine	3.01 (0.36) ^a	2.95 (0.25) ^a	2.37 (0.64) ^a	2.17 (0.83) ^a
Lysine	6.90 (0.91) ^a	5.77 (1.22) ^a	5.36 (0.60) ^a	6.03 (1.13) ^a
Methionine	2.44 (0.53) ^a	2.05 (0.28) ^a	1.83 (0.07) ^a	2.11 (0.51) ^a
Ornithine	3.65 (4.15)	2.50 (2.05)	1.75 (2.21)	4.67
Phenylalanine	1.10 (0.28) ^b	1.07 (0.22) ^b	0.47 (0.16) ^a	0.56 (0.05) ^a
Proline	18.97 (3.76) ^b	18.18 (6.92) ^b	9.22 (3.85) ^a	7.93 (1.02) ^a
Serine	24.21 (1.51) ^a	20.53 (6.42) ^a	17.60 (2.42) ^a	21.54 (4.45) ^a
Threonine	7.36 (2.02) ^b	4.83 (1.45) ^{ab}	3.03 (0.81) ^a	4.09 (0.66) ^a
Tyrosine	2.67 (0.47) ^a	2.49 (1.04) ^a	2.13 (0.77) ^a	1.87 (0.52) ^a
Valine	5.03 (0.16) ^b	5.24 (0.37) ^b	3.22 (0.57) ^a	3.35 (0.78) ^a
Total	139.24 (14.42) ^f	115.24 (6.10) ^b	84.28 (9.30) ^f	95.14 (4.51) ^a

* indicates 'trace'.

followed by proline (7.9 mM), alanine (7.0 mM) and lysine (6.0 mM), although not statistically different from those reared under SD IND+UV.

In the prepupae (5th instar, day 5) (Table 4) reared under LD IND+UV, the most abundant amino acids in the hemolymph are histidine (12.8 mM), glycine (12.5 mM) and serine (11.8 mM). Levels of proline, serine and glutamine are very low as compared to the early 5th instar insects. Under LD IND-UV, titres of histidine (11.4 mM), serine (9.4 mM) and glycine (9.1 mM) are lower, although not statistically significant from those reared under IND+UV. In the prepupae reared under SD IND+UV, levels of serine (10.5 mM) and proline (0.8 mM) rise slightly, while levels of histidine, glycine and glutamine decline slightly in the hemolymph. Under SD IND-UV conditions, titres of serine (11.2 mM), histidine (11.7 mM), glycine (3.9 mM) and proline (0.9 mM) rise in the hemolymph. However, the changes are not statistically significant. Other free amino acids in the hemolymph show slight changes.

In the early pupae (Day 5) (Table 5), reared under LD IND+UV, the most abundant amino acids in the hemolymph are serine (16.0 mM), lysine (14.4 mM) and arginine (13.4 mM) followed by histidine (8.7 mM), proline (5.9 mM) and glutamine (5.6 mM). In this stage, levels of arginine are much higher as compared to the larval instar. Insects reared under LD IND-UV, as compared to IND+UV, have higher levels

Table 4: Titres of free amino acids present in the hemolymph of day 5, 5th instar Manduca sexta reared under different conditions. Hemolymph was pooled from 25 insects. Extracts were analyzed on a Beckman Automatic Amino Acid Analyzer.

Values are reported as Mean (S.D. of Mean) of three determinations.

Values followed by the same letter for each amino acid indicate no significant difference at the 0.05 level (Duncan's Multiple Range Test)

Hemolymph free amino acids (mM) in the 5th instar, day 5 Manduca sexta reared under various conditions.

Amino acid	LD IND+UV	LD IND-UV	SD IND+UV	SD IND-UV
Alanine	1.80 (1.10) ^a	1.34 (0.23) ^a	1.29 (0.62) ^a	1.11 (0.35) ^a
Arginine	0.70 (0.83) ^a	1.33 (0.96) ^a	0.94 (0.29) ^a	0.76 (0.65) ^a
Asparagine	0.92 (0.32)	0.96 (0.62)	0.57	1.10 (0.60)
Aspartic acid	0.25 (0.03) ^a	0.22 (0.03) ^a	0.30 (0.25) ^a	0.23 (0.03) ^a
Citrulline	0.30	*	*	*
Cystathionine	0.40 (0.28) ^a	0.51 (0.54) ^a	0.32 (0.10) ^a	0.31 (0.32) ^a
Glutamic acid	2.05 (1.68) ^a	1.41 (0.10) ^a	1.34 (0.95) ^a	1.80 (0.43) ^a
Glutamine	4.44 (0.17) ^a	3.90 (1.46) ^a	3.21 (2.08) ^a	3.95 (0.43) ^a
Glycine	12.57 (9.11) ^a	9.13 (6.57) ^a	9.04 (10.42) ^a	13.52 (10.09) ^a
Histidine	12.84 (1.30) ^a	11.45 (1.80) ^a	10.70 (0.98) ^a	11.71 (2.64) ^a
Isoleucine	0.91 (0.58) ^a	1.17 (0.38) ^a	0.87 (0.69) ^a	1.01 (0.55) ^a
Leucine	0.99 (0.70) ^a	1.43 (0.37) ^a	0.97 (0.84) ^a	0.81 (0.84) ^a
Lysine	1.90 (0.35) ^a	1.64 (0.48) ^a	1.62 (0.72) ^a	1.57 (0.62) ^a
Methionine	1.80 (0.66) ^a	1.13 (0.57) ^a	1.23 (0.53) ^a	1.02 (0.35) ^a
Ornithine	2.19	0.13	2.53	0.41
Phenylalanine	0.72 (0.32) ^a	0.59 (0.49) ^a	0.75 (0.58) ^a	0.71 (0.36) ^a
Proline	0.70 (0.16) ^a	0.72 (0.54) ^a	0.85 (0.22) ^a	0.91 (0.34) ^a
Serine	11.85 (5.02) ^a	9.46 (1.77) ^a	10.55 (3.25) ^a	11.26 (4.83) ^a
Threonine	2.54 (3.46) ^a	0.70 (0.21) ^a	4.26 (6.75) ^a	1.11 (1.12) ^a
Tyrosine	1.26 (0.66) ^a	2.22 (0.16) ^a	1.40 (0.36) ^a	1.47 (0.94) ^a
Valine	2.55 (1.72) ^a	2.44 (1.53) ^a	2.23 (1.58) ^a	1.75 (2.03) ^a
Total	59.33 (12.85) ^a	50.64 (4.58) ^a	52.65 (15.93) ^a	55.60 (10.53) ^a

* indicates 'trace'.

Table 5: Titres of free amino acids present in the hemolymph of day 5, pupae of Manduca sexta reared under different conditions. Hemolymph was pooled from 25 insects. Extracts were analyzed on a Beckman Automatic Amino Acid Analyzer. Values are reported as Mean (S.D. of Mean) of three analyzes. Values followed by the same letter for each amino acid indicate no significant difference at the 0.05 level (Duncan's Multiple Range Test).

Hemolymph free amino acids (mM) in the pupal instar, day 5 of Manduca sexta reared under various conditions.

Amino acid	LD IND+UV	LD IND-UV	SD IND+UV	SD IND-UV
Alanine	1.69 (0.11) ^a	2.37 (0.54) ^a	2.20 (0.86) ^a	2.09 (0.44) ^a
Arginine	13.40 (0.59) ^a	17.74 (2.74) ^b	12.49 (1.87) ^a	12.74 (1.95) ^a
Asparagine	*	*	*	*
Aspartic acid	0.13 (0.02) ^a	0.18 (0.06) ^a	0.15 (0.03) ^a	0.31 (0.05) ^a
Citrulline	*	*	*	*
Cystathionine	0.45 (0.06) ^a	0.67 (0.29) ^a	0.51 (0.36) ^a	0.49 (0.19) ^a
Glutamic acid	2.91 (0.99) ^a	3.91 (1.05) ^a	2.74 (2.29) ^a	4.12 (2.92) ^a
Glutamine	5.69 (0.94) ^a	8.36 (1.64) ^a	6.76 (1.52) ^a	6.64 (1.41) ^a
Glycine	3.94 (0.09) ^a	5.52 (0.72) ^a	4.02 (0.99) ^a	4.64 (1.82) ^a
Histidine	8.70 (0.98) ^a	12.32 (3.00) ^a	8.65 (0.85) ^a	8.85 (1.71) ^a
Isoleucine	1.78 (0.16) ^a	2.53 (0.59) ^a	2.24 (0.53) ^a	2.42 (0.11) ^a
Leucine	1.75 (0.08) ^a	2.38 (0.43) ^a	2.19 (0.70) ^a	2.43 (0.50) ^a
Lysine	14.40 (0.27) ^a	20.58 (3.24) ^b	15.36 (1.84) ^a	16.72 (1.80) ^b
Methionine	0.99 (0.05) ^a	1.22 (0.18) ^a	1.42 (0.28) ^a	1.41 (0.32) ^a
Ornithine	*	*	*	*
Phenylalanine	0.57 (0.01) ^a	0.73 (0.12) ^a	0.63 (0.12) ^a	0.57 (0.13) ^a
Proline	5.98 (0.61) ^a	9.52 (1.50) ^b	6.25 (1.06) ^a	9.23 (2.18) ^b
Serine	16.03 (9.06) ^a	7.67 (4.46) ^a	15.43 (9.05) ^a	17.98 (4.84) ^a
Threonine	3.25 (0.15) ^a	4.39 (0.77) ^a	3.48 (1.57) ^a	3.24 (1.14) ^a
Tyrosine	3.57 (0.90) ^a	4.39 (1.93) ^a	2.27 (1.90) ^a	2.39 (2.08) ^a
Valine	6.09 (0.46) ^a	8.25 (2.52) ^a	7.08 (1.09) ^a	8.01 (0.54) ^a
Total	91.38 (10.04) ^a	112.79 (14.94) ^a	93.86 (12.57) ^a	104.22 (19.60) ^a

* indicates 'trace'.

of lysine (20.5 mM), arginine (17.7 mM) and histidine (12.3 mM), followed by proline (9.5 mM), glutamine (8.3 mM), serine (7.6 mM) and valine (8.2 mM) in their hemolymph. Levels of arginine, proline and lysine are significantly higher in the hemolymph. In SD IND+UV reared pupae, levels of serine are higher, whereas levels of proline, lysine, histidine, glutamine and arginine are lower, as compared to the developing hornworm pupae. Under SD IND-UV conditions, levels of serine (17.9 mM), proline (9.2 mM), lysine (16.7 mM) and arginine (12.7 mM) rise slightly while other free amino acids show minimal change.

In day 10 pupae (Table 6), under LD IND+UV, titres of lysine (15.5 mM), arginine (12.5 mM), histidine (11.5 mM), proline (11.0 mM) and serine (10.3 mM) are abundant, followed by glutamic acid (9.2 mM), valine (6.6 mM) and glutamine (6.8 mM). As compared to this, under LD IND-UV conditions, levels of serine, lysine and arginine rise and levels of proline, histidine and glutamic acid are lower in the hemolymph. Under diapause-inducing SD IND+UV, titres of serine (15.6 mM) rise in the hemolymph and levels of proline, lysine, histidine and arginine are lower as compared to developing pupae. In SD IND-UV population, titres of serine (25.3 mM) are significantly higher in the hemolymph. Levels of proline (6.8 mM), lysine (17.2 mM) and histidine (9.2 mM) are slightly higher in the hemolymph. Titres of other free amino acids show minimal changes in the hemolymph.

Table 6: Titres of free amino acids present in the hemolymph of day 10, pupae of Manduca sexta reared under different conditions. Hemolymph was pooled from 25 animals. Extracts were analyzed on a Beckman Automatic Amino Acid Analyzer.

Values are reported as Mean (S.D. of Mean) of three determinations. Values followed by the same letter for each amino acid indicate no significant difference at the 0.05 level (Duncan's Multiple Range Test).

Hemolymph free amino acids (mM) in the pupal instar, day 10 of Manduca sexta reared under various conditions.

Amino acids	LD IND+UV	LD IND-UV	SD IND+UV	SD IND-UV
Alanine	3.46 (2.34) ^a	2.90 (1.50) ^a	2.28 (1.32) ^a	2.03 (1.72) ^a
Arginine	12.57 (2.07) ^a	14.93 (2.00) ^a	13.38 (3.05) ^a	13.14 (0.36) ^a
Asparagine	*	*	*	*
Aspartic acid	0.32 (0.13) ^b	0.21 (0.04) ^{ab}	0.13 (0.04) ^a	0.17 (0.10) ^{ab}
Citrulline	1.62	*	*	*
Cystathionine	0.70 (0.24) ^a	0.45 (0.18) ^a	0.56 (0.30) ^a	0.43 (0.08) ^a
Glutamic acid	9.22 (3.85) ^b	8.06 (0.18) ^b	2.71 (1.61) ^a	1.80 (1.22) ^a
Glutamine	6.83 (1.37) ^{ab}	7.44 (0.81) ^b	5.55 (2.38) ^{ab}	4.20 (0.99) ^a
Glycine	5.74 (0.30) ^b	4.82 (0.94) ^{ab}	4.65 (1.26) ^{ab}	3.97 (0.48) ^a
Histidine	11.56 (2.86) ^a	9.87 (1.70) ^a	8.50 (2.73) ^a	9.24 (1.31) ^a
Isoleucine	1.71 (0.18) ^a	1.54 (0.35) ^a	1.74 (0.43) ^a	2.03 (0.20) ^a
Leucine	2.17 (0.78) ^a	1.94 (0.83) ^a	1.63 (0.45) ^a	1.76 (0.06) ^a
Lysine	15.56 (1.71) ^a	17.52 (2.47) ^a	15.58 (3.71) ^a	17.23 (1.25) ^a
Methionine	0.77 (0.22) ^a	0.57 (0.11) ^a	1.35 (0.06) ^b	1.58 (0.20) ^b
Ornithine	0.63 (0.67)	0.60	0.08	*
Phenylalanine	0.89 (0.36) ^a	0.68 (0.05) ^a	0.47 (0.15) ^a	0.56 (0.13) ^a
Proline	11.06 (3.93) ^a	8.56 (3.45) ^a	6.03 (2.55) ^a	6.80 (1.38) ^a
Serine	10.35 (1.80) ^a	13.46 (2.05) ^a	15.64 (3.37) ^a	25.36 (7.64) ^b
Threonine	4.60 (2.21) ^a	4.34 (1.90) ^a	2.93 (1.15) ^a	2.74 (0.96) ^a
Tyrosine	3.20 (2.35) ^a	3.24 (2.65) ^a	2.34 (2.03) ^a	2.50 (1.48) ^a
Valine	6.65 (2.75) ^a	4.92 (1.28) ^a	6.61 (2.26) ^a	8.04 (0.77) ^a
Total	108.36 (4.86) ^a	105.69 (12.86) ^a	92.15 (20.46) ^a	103.61 (9.01) ^a

* indicates 'trace'.

4.5 Hemolymph Trehalose and Glucose

The hemolymph trehalose and glucose concentration of M. sexta at different times in the 5th larval and pupal instars are shown in Table 7. Statistical analysis of the data shows that trehalose levels in the hemolymph of M. sexta are significantly influenced by photoperiod and the age of instar ($F=18.1$, $d.f.=1$; $F=16.97$, $d.f.=3$; respectively. $P < .05$). Light quality alone does not show any statistical significance in affecting hemolymph trehalose levels. However, interaction between light quality/age of instar is significant ($F=3.09$, $d.f.=3$; $P < .05$). Glucose levels in the hemolymph of M. sexta are influenced significantly by the age of instar alone ($F=3.08$, $d.f.=3$; $P < .05$).

On the 1st day of the final larval instar reared under LD-IND, trehalose and glucose levels are 3.38 $\mu\text{g/ml}$ and 2.94 $\mu\text{g/ml}$, respectively (Table 7). In the LD-BLB reared insects, the respective levels are 4.15 $\mu\text{g/ml}$ and 4.67 $\mu\text{g/ml}$, indicating a rise under BLB conditions. In the SD-IND reared larvae, the titres of trehalose and glucose are 5.47 $\mu\text{g/ml}$ and 2.22 $\mu\text{g/ml}$, respectively. Under SD-BLB conditions, the levels of trehalose and glucose are 6.90 $\mu\text{g/ml}$ and 3.47 $\mu\text{g/ml}$, respectively. Particularly, in these insects, levels of trehalose are higher as compared to insects reared under other conditions.

In the hornworm prepupae (5th instar, day 5) reared under LD-IND and LD-BLB, levels of trehalose and glucose are

Table 7: Trehalose and glucose levels in the hemolymph of Manduca sexta reared under different experimental conditions.

Hemolymph was pooled from 15-20 animals. Analysis of the sugar extracts were done on at Beckman HPLC system with an Ultrasil-Amino column.

Values are reported as Mean (S.D. of Mean) of three analyses.

Values followed by the same letter in the column indicate no significant difference at the 0.05 level (Duncan's Multiple Range Test).

Levels of trehalose and glucose ($\mu\text{g/ml}$) in the hemolymph of Manduca sexta reared under different conditions.

Rearing Condition	V d1		V d5		Age of Instar		P d10	
	Treha.	Glucó.	Treha.	Glucó.	Treha.	Glucó.	Treha.	Glucó.
Long day-Indorsun	3.38 ^a	2.94 ^a	2.22 ^a	2.41 ^a	1.90 ^a	2.66 ^a	2.49 ^a	2.02 ^a
	(1.20)	(1.41)	(0.68)	(1.22)	(0.30)	(0.72)	(0.70)	(0.80)
Long day-Blacklight blue	4.15 ^a	4.67 ^a	2.00 ^a	1.80 ^a	2.18 ^a	2.13 ^a	1.64 ^a	1.63 ^a
	(1.02)	(0.88)	(0.54)	(1.42)	(0.27)	(0.35)	(0.33)	(0.98)
Short day-Indorsun	5.47 ^a	2.22 ^a	3.51 ^b	2.36 ^a	2.29 ^a	1.31 ^a	4.66 ^b	1.57 ^a
	(2.71)	(1.22)	(0.78)	(1.64)	(0.14)	(1.08)	(1.77)	(1.33)
Short day-Blacklight blue	6.90 ^b	3.47 ^a	3.11 ^{ab}	2.87 ^a	1.97 ^a	1.98 ^a	2.46 ^a	2.10 ^a
	(1.50)	(2.15)	(0.13)	(2.13)	(0.17)	(0.56)	(0.73)	(1.58)

lower in the hemolymph as compared to the early 5th instar insects. SD-IND reared prepupae particularly have higher levels of trehalose (3.51 $\mu\text{g/ml}$) as compared to other rearing conditions. Levels of glucose are not significantly different. Under SD-BLB condition, hemolymph levels of trehalose and glucose are 3.11 $\mu\text{g/ml}$ and 2.87 $\mu\text{g/ml}$, respectively.

In the early pupae (Day 5), reared under LD-IND, LD-BLB, SD-IND and SD-BLB, there are minimal changes in the titres of trehalose and glucose. These changes are statistically not significant. In the day 10 pupae reared under LD-IND, the levels of trehalose and glucose are 2.49 $\mu\text{g/ml}$ and 2.02 $\mu\text{g/ml}$, respectively while under LD-BLB, the respective levels are 1.64 $\mu\text{g/ml}$ and 1.63 $\mu\text{g/ml}$. Under diapause-inducing SD-IND conditions, titres of trehalose and glucose are 4.66 $\mu\text{g/ml}$ and 1.57 $\mu\text{g/ml}$ respectively. In SD-BLB population, there are minimal changes in the levels of trehalose and glucose in the hemolymph. The statistical analysis shows that values for hemolymph glucose are statistically similar. The levels of trehalose and glucose, as a function of photoperiod, are shown in Figure 11 and 12, respectively.

4.6 Hemolymph Osmolality

The variations in the hemolymph osmolality of M. sexta reared under different conditions at various times in the 5th larval and pupal instars are given in Table 8.

Figure 11: Levels of trehalose in the hemolymph of the tobacco hornworm, as a function of photoperiod.

Hemolymph was pooled from 20 animals. Analysis of the extracts was accomplished by a Beckman HPLC system on an Ultrasil-Amino column. Values for light quality were combined.

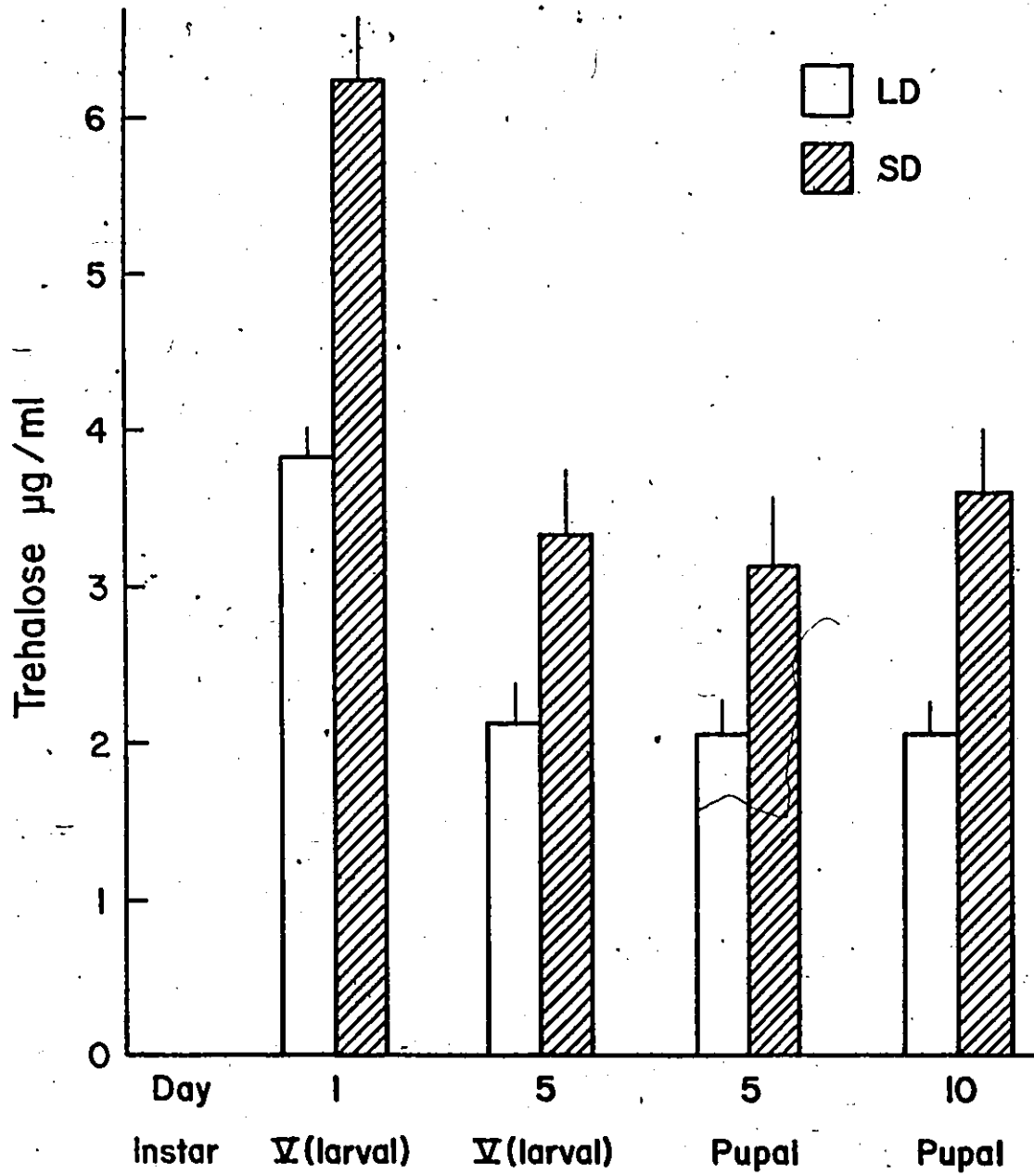
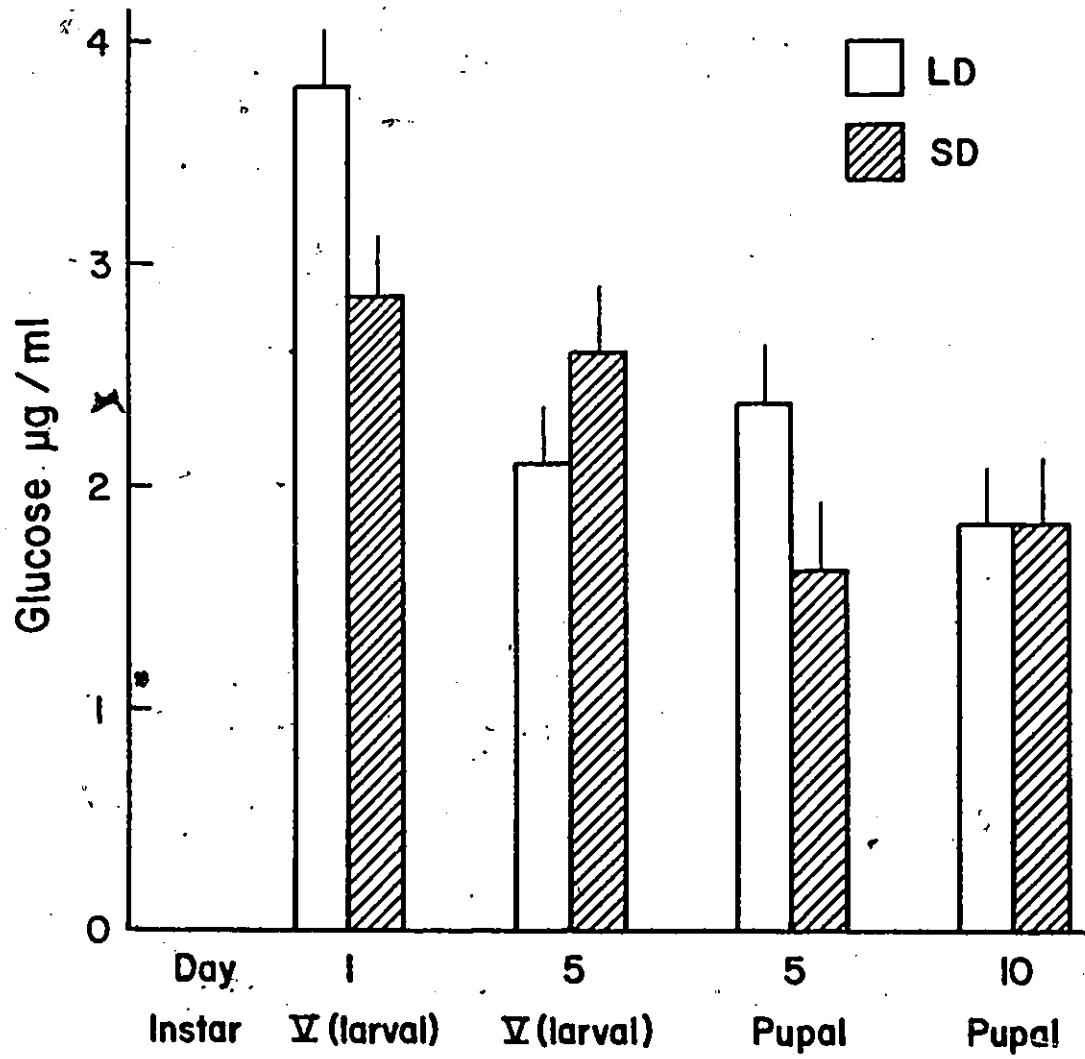


Figure 12: Levels of glucose in the hemolymph of the tobacco hornworm, as a function of photoperiod.

Hemolymph was pooled from 20 insects. Analysis of the sugar extracts was done by a Beckman HPLC system with an Ultrasil-Amino column. Values for light quality were combined.



Statistical treatment of the data shows that osmolality of the hemolymph of M. sexta is influenced by the age of instar alone ($F=4.99$, $d.f.=3$; $P < .05$) and the interaction between photoperiod/light quality and photoperiod/age of instar are also significant ($F=7.60$, $d.f.=1$; $F=5.05$, $d.f.=3$; respectively. $P < .05$).

On day 1 of 5th larval instar, mean hemolymph osmolality values under LD-IND, LD-BLB, SD-IND and SD-BLB are 428, 356, 435 and 450 mMol/kg respectively. By the end of the 5th larval instar, osmolality values are 400, 332, 292 and 369 mMol/kg respectively and show some differences between the groups. In the early pupal (Day 5) hemolymph, osmolality of the hemolymph show some variability, but the values between the groups are statistically not different. In day 10 pupae, hemolymph osmolality under LD-IND, LD-BLB, SD-IND and SD-BLB are 460, 375, 326 and 335 mMol/kg respectively and show statistical variability between different experimental groups.

Table 8: Hemolymph osmolality of Manduca sexta reared under different experimental conditions.

Hemolymph was pooled from 10 animals. Osmolality determinations were made on a Wescor Vapor Pressure Osmometer.

Values are reported as Mean (S.D. of Mean) of three analyses. Values followed by the same letter in the column indicate no significant difference at the 0.05 level (Duncan's Multiple Range Test).

Hemolymph osmolality (mOsm/kg) of Manuca sexta reared under different conditions.

Rearing Condition	V dl	Age of Instar V, d5	P d5	P dl0
Long day-Indorsun	428.00 ^a	400.00 ^b	374.00 ^a	460.00 ^c
	(191.00)	(43.00)	(27.00)	(3.00)
Long day-Blacklight blue	356.00 ^a	332.00 ^{ab}	397.00 ^a	375.00 ^b
	(80.00)	(29.00)	(52.00)	(34.00)
Short day-Indorsun	435.00 ^a	292.00 ^a	388.00 ^a	326.00 ^a
	(80.00)	(61.00)	(7.00)	(5.00)
Short day-Blacklight blue	450.00 ^a	369.00 ^{ab}	366.00 ^a	335.00 ^a
	(22.00)	(28.00)	(25.00)	(7.00)

Chapter V

DISCUSSION

This study has demonstrated that photoperiod and quality of light are two important aspects of the light requirements of M. sexta. Larval growth, pupal weight, hemolymph protein concentration, hemolymph protein profile, some free amino acids, hemolymph trehalose and glucose, and hemolymph osmolality are variously influenced by photoperiod, light quality and the age of instar. These are discussed below.

5.1 Larval Growth and Pupal Weight of *Manduca sexta*

The results presented here show that larval growth, measured as live weight, is significantly influenced by photoperiod and light quality. Although larvae reared under the experimental photoperiod-light quality regimes showed similar trends of growth, there were significant differences among different groups. Little is currently known of the specific physiological mechanisms by which photoperiodic stimuli may influence either growth rates or pathways of differentiation, but some generalizations have been drawn by Beck (1980). There may be rate-controlling effects on hormone synthesis and release, as have been postulated in cases where photoperiod appears to influence the neuroendocrine

system. Photoperiodic responses could also be mediated through the influence of photoperiodic signals on the temporal relationships such as hormone-controlled processes (Beck, 1980). The higher growth of M. sexta larvae reared under UV conditions could be due to changes in hormonal milieu, particularly those hormones which regulate metabolism and growth.

Larvae, reared under non-diapause inducing photoperiod and ultraviolet light (LD-BLB) exhibit more growth as compared with those reared under the same photoperiod but Indorsun fluorescent light which has a "complete" spectral distribution as natural light. The effect of spectral quality is thus clearly evident in modifying larval growth in M. sexta.

Spectral sensitivity of the photoperiodic response has been studied for a number of insects by different authors (see Saunders, 1982). These studies show that most species are optimally sensitive to light in the blue-green region of the spectrum and largely insensitive to red. However, there is a considerable diversity among different species studied so far. More direct evidence of hormonal modification in insects by spectral quality, if any, is still lacking. Light quality has been shown to influence larval development time for Pieris brassicae (Vuillaume et al., 1972) although larval growth, as gain in live weight, was not determined. These authors reported a shortened developmental time at 20

°C in larvae reared under red rather than white light during the photophase. Claret (1972) modified the spectral quality regimes to rear P. brassicae using filters to eliminate any effects of temperature due to radiant heating and found no differences in developmental time. However, studies done by Allot and Weinzaepfel (1976) confirmed the results obtained by Vuillaume et al., (1972). Here again, no attempts were made to characterize any changes in larval growth. Thoms and Philogène (1979) reported that under a 15/9 (L/D) photoperiod, the developmental rate of P. rapae larvae varied under different light sources used, but did not attempt to directly address the question of rate of larval growth.

It seems conceivable that in M. sexta, ultraviolet light (Blacklight blue) may stimulate growth by bringing about changes in its metabolism through hormonal modification, although the nature of these changes, if any, needs to be studied. As will be discussed later in this chapter, light quality does have an effect on biomolecules such as hemolymph proteins and some amino acids.

M. sexta larvae reared under diapause inducing photoperiod and Indorsun (SD-IND), again, exhibit enhanced growth (as weight gain) as compared to those raised under LD-IND. The higher growth rate under SD-IND is obviously the result of a diapause inducing photoperiod. Photoperiodic induction of diapause is a very well studied aspect of insect development. Diapause in M. sexta is induced by short-day photo-

periods of less than 13.5 hours (Bell et al., 1975). Under SD conditions, M. sexta larvae could be accumulating more nutrient reserves of proteins, amino acids and carbohydrates for the metabolic requirements of diapause maintenance and possibly also for later use during post-diapause development. The exact mechanism(s) by which this may occur remains to be researched. Higher levels of hemolymph proteins, some free amino acids and trehalose were detected in diapause-programmed M. sexta larvae (discussed later). Chippendale and Kikukawa (1983) reported that under diapause-inducing photoperiod, fully grown larvae of the sunflower moth, Homoeosoma electellum are significantly heavier than the equivalent non-diapause larvae. However, these authors did not provide any physiological basis for such a difference in larval growth. Diapause programmed larvae of Pieris brassicae show an increase in weight over their non-diapause counterparts (Clanet, 1968).

Larvae reared under diapause-inducing photoperiod and Blacklight blue (SD-BLB) are heavier than those reared under all other rearing conditions. In this group of insects, photoperiod as well as light quality seem to play a role in influencing larval growth. The results would suggest that diapause-induction along with long wave UV are complementary in stimulating growth of M. sexta possibly through influence on hormones which play a role in regulation of metabolism and growth. More work needs to be done in characterizing

the changes in hormones, if any, due to photoperiod and spectral quality in M. sexta.

The trend in pupal weights obtained followed the same pattern as that for larvae reared under equivalent conditions of photoperiod and light quality. However, the slight decline in pupal weights on day 5 of pupal life is mainly due to loss of moisture during the prepupal wandering stage. Pupal weight variations do not show any statistical significance suggesting that photoperiod and light quality are only critical during the period of active larval growth.

5.2 Hemolymph Protein Concentration

Hemolymph protein concentration of M. sexta is significantly influenced by the age of instar. A two-way interaction between photoperiod/age of instar, and light quality/age of instar are significant, while a three-way ANOVA between photoperiod/light quality/age of instar is also significant. The biological significance of the statistical interactions are discussed later.

The changes in the concentration of proteins found in the hemolymph of tobacco hornworms reared under LD-IND are consistent with the results of other workers. Gilbert and Schneiderman (1961) reported that the protein concentration of the hemolymph increased during successive larval stages of Lepidoptera. Hudson (1966) found that a cycle of change in the hemolymph protein was repeated over the molting cycle

of the tomato hornworm, M. quinquemaculata larvae and Dahlman (1969) reported a cyclical pattern of hemolymph protein concentration in M. sexta larvae. He reported a step-like increase in protein concentration between instars and linear increases within instars of the tobacco hornworm illustrating that biochemical differentiation was occurring in successive instars.

Under LD-IND conditions, 5th larval and the pupal instars show considerable variability in titres of proteins in the hemolymph. The fluctuations in the protein concentration of the hemolymph of hornworms could be the result of rapid growth of various tissues, especially fat body, during this time. Martin et al. (1971) have reported that fat body cells of late larval instars of Calliphora stygia make up hemolymph proteins. The higher concentration of protein found in 5th instar, day 4 and day 5 Manduca larval hemolymph is related to the upcoming inactive molting period. The increased concentrations of protein could serve as a nutritional source, as a source of amino acids for protein synthesis (Greene and Dahlman, 1973). The large build-up is essential because the energy required during metamorphosis must be derived from endogenous sources (Pant and Agrawal, 1965).

The decrease in hemolymph protein concentration in the early pupae can be explained by events in the preceding prepupal stage during which, after attaining maximum growth,

the larvae stopped feeding and voided their gut contents. The "wandering" activity consumed energy which must come from sources in the hemolymph or from some other food depot (Wyatt, 1961). Moreover, some additional selective absorption of hemolymph proteins by the tissues could also have occurred (Wyatt, 1961; Loughton and West, 1965).

The increase in protein concentration in the 10 day old developing pupae raised under LD-IND conditions could be the result of extensive synthesis, a process required for the eventual synthesis of adult tissues in situ (Loughton and West, 1965) or possible direct incorporation into adult tissues (Dahlman, 1974).

In the hornworms reared under non-diapause inducing photoperiod and Blacklight blue (LD-BLB), as compared to LD-IND, the differences observed in the hemolymph protein concentration in the 5th instar larvae and the pupae appear to be related to ultraviolet light in the Blacklight blue fluorescent.

The interaction between light quality and age of instar is significant suggesting that ultraviolet light does have an effect during certain periods of larval and pupal development. The interaction is evident on day 5 of the final larval instar, which is the physiologically critical time in larval life. Day 5 of the 5th instar larvae is characterized by "wandering" and preparation for the metamorphic molt. Manifestation of diapause (in diapause-programmed insects) also occurs shortly after this stage.

Hornworm larvae reared under diapause-inducing photoperiod and Indorsun light (SD-IND), again, do not exhibit a statistically significant change in their hemolymph protein concentration, although a photoperiod/age of instar interaction does show significance. In the 10 day old pupae in diapause, as compared to those which were reared as larvae under LD-IND conditions; titres of protein are lower in the hemolymph. However, the overall levels of protein are higher throughout the 5th larval and early pupal instars, although not statistically significant.

Many physiological studies on insect diapause have focussed on proteins in the hemolymph. Stability in the protein composition of the hemolymph is typical of insect diapause. For example, Hyalophora cecropia pupae (Telfer and Williams, 1953), pupae of Pieris brassicae (Chippendale and Kilby, 1969 Claret, 1969) and Dendrolimus pini (Luzev and Belozarov, 1978). On the contrary, no changes were found in the protein composition of the hemolymph of the European corn borer, Ostrinia nubilalis prepupae in diapause that had been reared under different photoperiodic regimes (Chippendale and Beck, 1966). These authors suggested that in O. nubilalis, there is apparently little metabolic activity in the hemolymph until the start of the post-diapause molting cycle. The results presented here show that a similar situation exists in the tobacco hornworms reared under diapause inducing conditions.

In the hornworms reared under SD-BLB conditions, the hemolymph protein concentration is not significantly different from SD-IND, except on day 3 and day 4 of the 5th larval instar. These differences in protein concentration between groups reared under Indorsun and Blacklight blue are due to differences in the spectral quality, however, the role played by light quality in influencing the hemolymph protein concentration is not understood. Overall analysis of variance of the data is not statistically significant for light quality alone as a main effect. An interaction between light quality/age of instar is significant, thus emphasizing that, ultraviolet light has an effect during certain periods in the larval/pupal instars of M. sexta.

5.3 Hemolymph Protein Profile

5.3.1 Effects of Photoperiod

Studies on M. sexta show that in this insect, no specific diapause associated proteins were detected although there are some changes in the overall hemolymph protein profile as well as the concentration of individual protein bands in the profile with respect to photoperiod. Day 1 of the 5th instar hornworms reared under LD-IND and SD-IND conditions show wide differences in the hemolymph protein profile. Of particular interest is the absence of two low molecular weight protein bands (#9 and 10) of molecular weight below 10 kD in the hemolymph of diapause-programmed (SD-IND)

insects. These bands could be amino acid storage peptides (Chen, 1971) and could be used as metabolic reserves in developing tobacco hornworms. However, evidence for such a function in this insect is still lacking. Alternatively, these bands could be the result of proteolytic breakdown of hemolymph protein(s) during sample preparation and/or analysis.

In the prepupae, on day 5 of the 5th larval instar reared under LD-IND and SD-IND conditions, a somewhat similar protein profile is seen. However, the concentration of band 2 (manducin) is increased sharply in the profile. A dramatic increase in the manducin content in the last larval instar of *M. sexta* has been reported by Kramer et al. (1980). These authors have reported that manducin constitutes at least 80% of the total hemolymph proteins and appears to be a storage form for amino acids sequestered during larval life to be used for construction of the adult animal. Under diapause-inducing condition (SD-IND), the concentration of manducin is about 40% higher than in the hemolymph of developing (LD-IND) hornworm prepupae.

It has been suggested that a specific titre of juvenile hormone (JH) and ecdysone brings about the triggering of mRNA and leads to the biosynthesis of proteins and their subsequent release in the hemolymph. JH has actually been shown to regulate protein synthesis in the fat body (Wyatt and Pan, 1978; Laufer and Borst, 1983).

In the early pupae (day 5), an increase in the number of protein bands was seen in insects reared under LD-IND and SD-IND conditions. There is a slight increase in the concentration of some proteins under diapause-inducing conditions, suggesting that these proteins may be used as an energy reserve for diapause maintenance and/or post-diapause development. The slight increase in the number of protein bands in the early pupae is probably the result of massive reorganization taking place in the early pupal life. Chipendale and Beck (1966) studied changes in the hemolymph proteins during the pupal molting cycle of the European corn borer O. nubilalis and concluded that in this insect, histology had contaminated the hemolymph causing some inconsistency of the electrophoretic pattern.

In the 10-day old pupae under LD-IND and SD-IND conditions, there is no change in the number of bands detected in the hemolymph. However, under diapause-inducing conditions, the concentration of some bands is higher in the hemolymph as compared to LD-IND pupae. This suggests that these proteins may be used as metabolic reserves for future use. However, this has to be viewed together with the modifications in other biomolecules such as amino acids, carbohydrates and lipids. An increase in the concentration of the hemolymph proteins towards the end of prediapausing period was also reported in the pine moth, Dendrolimus pini larvae (Luzev and Belozarov, 1978).

5.3.2 Effects of Light Quality

Light quality has some influence on the protein composition of the hemolymph of M. sexta. There are some differences in the hemolymph protein profile in the hornworms reared under Indorsun, which has a spectral distribution close to the solar spectrum and under Blacklight blue, which has a high portion of long wave ultraviolet (350 nm). On day 1 of the 5th larval instar, under LD-IND and LD-BLB, it appears that light quality has an influence on the hemolymph protein profile. There is a drastic rise in the two low molecular weight bands (#9 and 10) under BLB condition. This increase in these protein bands is, at the present time, unexplainable. It is necessary to characterize these bands, and to elucidate their role in metabolism of developing insects, before any conclusion can be drawn about the effects of uv light on the hemolymph protein profile of M. sexta. It is interesting to note that these two bands are absent in diapause-programmed insects. Alternatively, as pointed out earlier, these bands could be the result of proteolysis during sample preparation and/or analysis.

In the prepupae (day 5, 5th instar), there are no differences in the number of proteins detected in the hemolymph of hornworms reared under all rearing conditions. However under LD-BLB, as compared to LD-IND, there is almost a 50% rise in the concentration of band 2 (manducin) in the profile. Ultraviolet light, under developing conditions, does

seem to influence the concentration of band 2 in the hemolymph. The exact mechanism(s) by which this may occur remains to be researched. Under diapause-inducing conditions (SD-IND and SD-BLB) there is a negligible change in the profile.

In the Day 5 and Day 10 pupae reared under LD-IND, LD-BLB, SD-IND and SD-BLB conditions, there are minimal changes in the hemolymph protein profile of M. sexta. This suggests that light quality has an effect during the period of active larval growth.

It, therefore, appears that light quality has an appreciable effect on the physiology of the hemolymph of M. sexta. Spectral sensitivity for the photoperiodic response has been reported in many insects (Saunders, 1982) and Chapman (1971) has suggested that only the short wavelengths are involved in the photoperiodic reaction in most insects.

Since hormones such as juvenile hormone, ecdysone and certain neurosecretions from the median neurosecretory cells of the brain (Thomsen and Moller, 1963; Hill, 1962) have been implicated in the regulation of protein synthesis/uptake in insects, characterization of such hormones in response to different photoperiod and light conditions should yield valuable information on the role played by light in the physiology of insects.

The SDS-PAGE system used in this study allowed separation of several proteins in the hemolymph of M. sexta reared under different experimental conditions. However, there are certain limitations to the system which should also be discussed here (Houseman, personal communication). It is important that the proteolytic breakdown of hemolymph proteins should be minimized by use of low temperatures during sample preparation and analysis. Proteolysis in the sample may contribute to inconsistent separation on the gels and/or result in low molecular weight bands in the profile. It is also possible that direct comparison between protein separations on two different gels may not be accurate because of the differences between the gels used. Factors such as reagent purity, period of storage, degassing and temperature can influence the gel structure and may result in differences in mobility of the protein bands in the sample. Consistency in the staining and destaining procedure is also important. The porosity of the polyacrylamide gel can influence the separation of proteins of a wide range of molecular weights. High density (low porosity) gels may exclude some high molecular weight proteins. Obviously, these factors should be carefully considered when using SDS-PAGE for analyzing hemolymph proteins.

5.4 Hemolymph Free Amino Acids

5.4.1 Effects of Photoperiod

The results show that high titres of serine (20-24 mM) are present in the early 5th instar developing hornworms under both IND+UV and IND-UV conditions. By the end of the 5th larval instar, the titres dropped sharply and increased slightly in the early (Day 5) and late (Day 10) pupal hemolymph, with some variation under IND+UV and IND-UV conditions. In the early 5th instar diapause-programmed hornworms (SD) reared under IND+UV and IND-UV, the titres of serine were not significantly different than those reared under developing (diapause-averting) conditions. However, unlike developing insects, the titres of serine did not drop sharply but slightly by the end of the 5th larval instar (Day 5) and in the early pupae rose to early 5th instar levels with variations under the two light regimes used. In the 10 day old pupae in diapause, the levels of serine rose even higher compared to the developing insects. The most significant rise was observed in pupae under IND-UV conditions. However, in some cases, the differences observed were not statistically significant, mainly because of higher variation in the data. This shows that titres of serine in the hemolymph of M. sexta are dependent on the developmental program of the hornworm, whether diapause or non-diapause, and may play an important role in the physiology of diapause in this insect. High titres of serine have also been

reported in the hemolymph of pink bollworms, Pectinophora gossypiella in diapause and diapause pupae of Antheraea pernyi and Heliothis armigera. (Rostom et al., 1972 Mansingh, 1967; Boctor, 1981). A very high titre of serine (65 mM) in the hemolymph of Chilo suppressalis larvae in diapause and high levels of serine (30 mM) in the hemolymph of post-diapause Mamestra brassicae pupae have been reported (Mitsuhashi, 1978). High levels of serine have also been reported in overwintering Diatraea grandiosella and Ostrinia nubilalis in diapause (Morgan and Chippendale, 1983). Morgan and Chippendale (1983) have suggested that large amounts of serine in overwintering larvae or pupae of these insects may be used during post-diapause development for synthesis of uric acid and purines (Tojo and Hirano, 1966, 1968). The results suggest that a similar situation probably exists in M. sexta in diapause.

Moderate titres of glutamine and low levels of aspartic acid are detected in the hemolymph of M. sexta with considerable variation during the age of instar as well as under developing and diapause-inducing photoperiod and light regimes. Levels of glutamine have been reported to change significantly in diapause eggs of the cricket, Teleogryllus commodus and in non-diapause eggs of the silkworm, Bombyx mori (MacFarlane and Hogan, 1966 Suzuki et al., 1984). Morgan and Chippendale (1983) have reported high titres of glutamine and aspartic acid in the field-collected, overwin-

tering O. nubilalis and concluded that the increase in glutamine may have resulted from the action of glutamine synthetase on glutamate and ammonia, thus indicating a higher rate of ammonia production in O. nubilalis in diapause. It has been proposed that glutamic acid and glutamine may be utilized for energy production in insects (Sacktor, 1961).

The levels of proline were higher in the early 5th instar Manduca larvae under both photoperiods and lights and then remained moderate throughout the rest of larval and pupal life under study and exceeded 15 mM at certain times in the hemolymph. The titres of proline reported here are in general agreement with those in the hemolymph of overwintering larvae of O. nubilalis (Morgan and Chippendale, 1983). Cold-hardened O. nubilalis in diapause can survive freezing (Hanec and Beck, 1960). In the freeze tolerant gallfly Eurosta solidaginis larvae, Storey et al. (1981) reported an accumulation of proline and alanine. These amino acids also accumulated in the hemolymph of cold-exposed Anthraea pernyi and Galleria melonella pupae in diapause (Marek, 1979 Mansingh, 1967). Titres of proline were moderate in the hemolymph of M. sexta and did not show any accumulation. This is because these insects were not exposed to low rearing temperatures.

The abundance of some of the amino acids (serine, lysine, histidine, arginine and glutamine) in the hemolymph of diapause-programmed hornworms may help them to survive

the cold temperatures that normally prevail during diapause. Titres of amino acids in the hemolymph can be influenced by changes in the levels of carbohydrates and their metabolic intermediates and derivatives (Somme, 1967; Chino, 1958). Glucose and its metabolic intermediates can provide carbon skeleton for the synthesis of amino acids in insects (Man-singh, 1964).

The changes in alanine and proline under diapause-inducing and developing conditions suggests the presence of a proline- alanine pathway as in the diapause eggs of B. mori (Suzuki et al., 1984) and in flight muscle and fat body of the tsetse fly, Glossina morsitans (Bursell, 1977 Bursell and Slack, 1976; Hoek et al., 1976 Olembo and Pearson, 1982). It is one of the important pathways in energy production via the oxidative degradation of amino acids (Lehninger, 1975). Similarly, the presence of ornithine and citrulline in M. sexta hemolymph suggests the existance of an ornithine-citrulline path in this species similar to that suggested in Heliothis armigera (Boctor, 1981). This pathway is of significance in urea synthesis via the urea cycle (Lehninger, 1975). However, evidence for such a pathway in insects is limited (Inokuchi, et al., 1969 Chen, 1971) and direct evidence for the existance of proline-alanine and ornithine-citrulline pathways in M. sexta is still lacking.

It seems conceivable from the foregoing discussion that, in M. sexta, amino acids such as serine, glycine, glutamine,

alanine, proline, ornithine and citrulline show changes in response to photoperiodic induction of diapause and that, in some cases, there is considerable variation according to the light quality. Following this, when winter conditions ensue, further biochemical changes must take place to ensure survival and successful post-diapause adult emergence. More work needs to be done on this aspect of diapause in M. sexta and should provide useful insight into such mechanisms in insects in general.

5.4.2 Effects of Light Quality

The results provide some explanation of the role played by light quality on hemolymph free amino acid titres. Until now no studies had focussed on this aspect of insect photoperiodism and diapause physiology. There is significant variation in some hemolymph free amino acids in hornworms reared under Indorsun+UV, which has a spectral distribution close to that of natural light and Indorsun-UV, in which the ultraviolet component of light is filtered off at wavelengths above 400 nm. Light quality and intensity act at several levels which remain to be properly identified. The likely sites are the simple eyes (ocelli and stemmata) (Singleton-Smith, 1980), the compound eyes (Bernard, 1979; Wolken, 1975) and more importantly extraretinal photoreceptors such as the neurosecretory cells of the brain, the suboesophageal ganglion and the abdominal ganglia (Saunders, 1982). In M. sexta, the neurosecretory system coupled

with a photosensitive pigment receptor has been suggested as a site of photoreception (Bell et al., 1975). Pigments such as carotenoids (Rothschild et al., 1975), caroteno-proteins (Veerman, 1980), flavins (Ninneman, 1980) and polycyclic quinones (Banks and Cameron, 1973) have been implicated. Another level at which light quality and intensity could act is the cuticle which has pigments such as biliverdin that absorb ultraviolet over the entire body surface. Long wave ultraviolet has been reported to induce melanization in the cabbage worm, Pieris rapae (Thoms and Philogène, 1979), a process in which tyrosine plays a role via DOPA polyphenol oxidase and decarboxylase (Wigglesworth, 1972; Hori et al., 1984). An intermediate in tryptophan catabolism, 3-Hydroxykynurenine, is utilized by some insects as a precursor of pigments known as ommochromes (Lehninger, 1975). It is also possible that exposure to ultraviolet light could induce stress in the hornworms and thus changes in hemolymph free amino acids could have occurred because of their role in energy metabolism in some insects.

5.5 Hemolymph Trehalose and Glucose

5.5.1 Effects of Photoperiod

The results presented here demonstrate that photoperiod and age of instar are the most important factors influencing trehalose and to a lesser extent glucose levels in the hemolymph. Under LD photoperiod, trehalose levels in the hemo-

lymph are consistently lower than from those under SD photoperiod (Figure 11). This shows that trehalose levels are regulated, at least partly, by photoperiod. The diapause-inducing photoperiod must program the levels of trehalose which, along with glycogen, is the storage form of carbohydrate fuel in many insects (Wyatt, 1961). Trehalose is readily broken down by the enzyme trehalase into glucose, which is the metabolizable source of energy required by insects. High levels of trehalose in diapause-programmed or in insects in diapause have been reported in a number of insect species: Trichiocampus populi (Asahina and Tanno, 1964), Popilius disjunctus (Rains and Dimock, 1978), Phylosamia cynthia ricini and pryeri (Hayakawa and Chino, 1981), Pieris brassicae (Chippendale, 1978; Moreau et al., 1981), Belgica antarctica (Baust and Edwards, 1979), Eurosta solidaginis (Baust and Lee, 1981; Storey et al., 1981) and Leguminivora glycinivorella (Shimada et al., 1984).

Higher levels of blood trehalose found in diapause-programmed M. sexta suggest that this disaccharide may serve as a reserve source of energy for diapause maintenance and post-diapause development. In diapausing insects, triglycerides, glycogen and trehalose and to a lesser extent proteins and amino acids accumulate in the fat body and hemolymph during the active feeding period which precedes the onset of diapause (Chippendale, 1978). Glycogen and treha-

lose reserves are utilized preferentially during the early stages of diapause to supply glucose to meet the limited energy demands and serve as precursors for the synthesis of polyhydric alcohols which contribute to increased coldhardiness (Chippendale, 1978). However, Wyatt (1961) has stated that in instances such as Bombyx mori eggs and Hyalophora cecropia pupae, accumulation of trehalose is generally not well correlated with diapause.

The role of trehalose as a cryoprotectant has been clearly shown in the overwintering prepupal sawfly, T. populi and other insects. Direct evidence of such a function in M. sexta is still lacking. The present results raise the question of the role of photoperiod as an environmental cue to bring about changes in trehalose levels in diapause or non-diapause insects.

Hemolymph glucose levels, however, do not show any corresponding trend in response to photoperiod (Figure 12). Levels of glucose are higher in the early 5th instar developing larvae and fall substantially by the end of the instar. This could be because of active glucose metabolism in the developing larvae. Glucose could be actively used for synthesis of trehalose. Immediately after the molt, M. sexta larvae undergo a phase of physiological adjustments which need an immediate energy source. In the early developing pupae, levels of glucose rise again slightly and decline in 10 day old pupae. This is most likely the result

of metabolic adjustments after the larval-pupal molt and the subsequent use of glucose for trehalose synthesis as well as metabolism.

Blood glucose levels of diapause-programmed M. sexta show an interestingly reverse trend with respect to their titres. In the early 5th instar (Day 1), glucose levels are lower than in those which are continuously developing. It is possible that under diapause-inducing conditions, less of glucose-derived energy is required. Alternatively, glucose could be used in synthesis of trehalose as a metabolic reserve for later use. At the end of the instar (Day 5), glucose levels in the hemolymph fall slightly, but remain higher than in developing insects. In the early diapause pupae, levels of glucose drop sharply to a level lower than in early developing pupae. This would indicate that more and more of glucose is being used in trehalose synthesis. In the 10 day old pupae in diapause, plasma glucose levels rise slightly to the level found in the 10 day old developing pupae.

The patterns of hemolymph trehalose and glucose levels reported in this study are in general agreement with data obtained for other insects (Wyatt, 1961; Chippendale, 1978; Tate and Wimer, 1974). However, the titres reported here are lower than those reported for developing, diet-fed M. sexta (Dahlman, 1975). The probable reason for this could be one or more of the following: 1) The trehalose/

glucose levels are at least partially dependent upon the physiological milieu of the insect being studied. The hornworms used in this study were raised on a different photoperiodic regime and light quality than what was used by Dahlman (1975). Thus, although the sugar content of the artificial diet used in this study (Yamamoto, 1969; Stewart, 1981) was quite similar to the one used by Dahlman (1975), the physiological state of the hornworms must be different. 2) The extraction procedure used (Baust and Edwards, 1979) was different from that used by Dahlman (1975). The solvent extraction procedure used here has been widely used (Nettles et al., 1971) with different degrees of modifications. Nettles et al. (1971) reported that extraction with chloroform-methanol and ethanol followed by immediate deionization reduced the conversion of trehalose to glucose. However, these authors were unable to determine whether extraction with chloroform-methanol, or immediate deionization, or a combination of both were responsible for minimizing the conversion of trehalose to glucose. Obviously, this factor should be carefully considered during carbohydrate extraction from biological samples. 3) The analytical procedure used by Dahlman (1975) was enzymatic, thus allowing more specificity. Kramer et al. (1978) have reported a quantitative spectrophotometric method for trehalose determination using gel-permeation chromatography and the anthrone colorimetric procedure. However, in this method,

enzymatic (trehalase) and non-enzymatic components of the hemolymph such as monosaccharides, disaccharides and nucleotides may interfere in trehalose determination. Recently, quantitative gas-liquid chromatography and gas chromatography have been used for separating and quantifying carbohydrates from insect material (Lequellec et al., 1982 Moreau et al., 1982, 1984 Binder et al., 1984). This study has taken the new approach of using HPLC which provides for rapid separation of sugars, which are also easy to quantitate. This system is suitable for routine analysis, because, except for the separation of solids contained in the extracts, no further treatment of the sample is necessary (Binder, 1980). The relatively higher titres reported for glucose may be due to some conversion of trehalose to glucose during sample preparation and/or analysis.

One aspect which needs to be studied in precise details is the possible regulation of trehalose by photoperiod. Two facets of this which should be considered is the synthesis of trehalose and the degradation of trehalose in the insect body. Enzymatic regulation of carbohydrate metabolism via metabolites, ions, and hormones control the rate of interconversion of glycogen, trehalose and glucose (Chippendale, 1978). The hormonal aspects of trehalose synthesis seems to be a possibility in the regulation of trehalose levels by photoperiod. The corpora cardiaca of the neuroendocrine system of some insects have been shown to have a

hyperglycemic hormone (Steele, 1961; 1976). Saline extracts of the corpora cardiaca of the American cockroach, Periplaneta americana caused an increase in the titre of hemolymph trehalose and a concomitant decrease in fat body glycogen after it was injected into adult males. The hyperglycemic hormone has been reported in several other insect species such as Blaberus discoidalis, Phormia regina, Calliphora erythrocephala, C. morosus and Leucophaea maderae (Steele, 1983). The hormone is synthesized in the intrinsic neurosecretory cells of the corpora cardiaca or in the cerebral neurosecretory cells which are directly connected to the corpora cardiaca. The hormone appears to act through cAMP to activate glycogen phosphorylase (Chippendale, 1978). Of particular interest is the report by Tager et al. (1976) that aqueous extracts of corpus cardiacum-corpora allatum complexes of the adult M. sexta produce both hyperglycemia and hypoglycemia when injected into the larval form of the same species. The corpora cardiaca are dependent for their activity on the photosensitive neurosecretory cells of the insect brain (Philogène and Massalski, 1976). In the blowfly, release of the hyperglycemic hormone from the corpora cardiaca is under nervous control (Mordue et al., 1980). Our understanding of photoregulation in insects most likely depends on understanding the trigger mechanisms of hormonal/neurosecretory centres.

There have been no studies done on regulation, if any, of trehalose catabolism in the insect system. The activity of trehalase in vivo is poorly understood. The median neurosecretory cells in the brain of C. erythrocephala have been shown to have a factor with hypotrehalosemic activity and have been shown to reduce the levels of trehalose in the hemolymph (Chen and Friedman, 1977; Steele, 1983). Investigation of corpora cardiaca activity with respect to photoperiod to ascertain the role of the latter in trehalose regulation would seem to be a promising avenue to follow.

5.5.2. Effects of Light Quality

Although, light quality does not seem to have a statistically significant effect on hemolymph trehalose and glucose levels, the overall levels of trehalose may be a reflection of metabolic stress which may be induced by continuous exposure to ultraviolet. An interaction between light quality and the age of instar is statistically significant for hemolymph trehalose suggesting the influence of light quality only during some critical periods in the life of M. sexta. However, the significance of such changes in trehalose levels in M. sexta needs to be researched further.

5.6 Hemolymph Osmolality

In M. sexta, osmolality of the hemolymph is significantly influenced by age of instar. Photoperiod/light quality and photoperiod/age of instar are particularly significant (2-way ANOVA, $P < 0.05$). Hemolymph osmolality is an expression of the total concentration of anions and cations in the plasma without regard to particle size, density or configuration. Insect hemolymph largely contains inorganic components and organic complexes of metallic elements. The organic portion of the hemolymph is made up of free amino acids, non-protein nitrogenous compounds, organic acids, carbohydrates, and related substances, lipids, proteins and enzymes, pigments and various insect hormones (Wyatt, 1961). The extent to which solute concentration varies in the hemolymph is reflected in the wide range of hemolymph osmolality values in insects. Since proteins, amino acids, carbohydrates and lipids form the major constituents of hemolymph, changes in these biomolecules should reflect in osmolality of the hemolymph. For example, in the Japanese caterpillar, Monema flavescens, the fall in water content of the hemolymph from 90 per cent in the feeding larvae to 75 per cent in the overwintering prepupae has been attributed to changes in the production of some solute such as glycerol (Asahina et al., 1954).

In M. sexta hemolymph, changes in osmolality do not reflect the various changes in proteins, free amino acids

and sugars contrary to what was expected. Only the age of instar seems to be a significant factor influencing hemolymph osmolality in the hornworms and may be the common link with changes in the various components studied. Significant interactions between photoperiod/light quality and photoperiod/age of instar further complicate the situation suggesting that some form of regulation of the osmolality, directly or passively, may be taking place in the hemolymph of M. sexta. However, changes in the other components of the hemolymph such as lipids, free carbohydrate related substances (for instance glycerol), organic acids, pigments and other inorganic constituents and their metabolites were not monitored and it is likely that changes in these might have also contributed to variability in the hemolymph osmolality of M. sexta. Changes in some biomolecules such as proteins, amino acids and sugars could be compensated by changes in other biomolecules. Moreover, possible changes in the hemolymph volume in insects can also influence the overall levels of various biomolecules in the hemolymph. Hemolymph is a dynamic tissue such that the capacity for delivery in the open circulatory system of insects compares favorably with the closed circulatory system of higher animals (Jungreis, 1980). It would be of interest to monitor and characterize all the hemolymph components of M. sexta reared under different photoperiodic and light regimes before any conclusions can be drawn about regulation of

hemolymph osmolality and its role in bringing about metabolic homeostasis. Moreover, consideration should also be given to metabolic turnover of various biological molecules in the hemolymph of M. sexta.

Chapter VI

SUMMARY AND CONCLUSIONS

This study has focussed on the question of whether non-diapause and diapause inducing photoperiod and spectral quality of fluorescent light have an effect on the growth and crucial biomolecules namely proteins, free amino acids and sugars in the tobacco hornworm, *M. sexta* and if so, what is the physiological significance of these changes. Several distinct differences, and a few areas of no apparent difference, were seen under different experimental conditions :

1) Larval Growth: Tobacco hornworms attained maximum growth, expressed as live weight, under Shortday-Blacklight blue (SD-BLB) regimen, followed by Shortday-Indorsun (SD-IND) and Longday-Blacklight blue (LD-BLB) as compared to Longday-Indorsun (LD-IND) reared insects. Further research is required on the influence of light quality on larval growth.

2) Pupal Weight: Pupal weight is significantly influenced by photoperiod. Although not statistically different, pupae in diapause are heavier than their non-diapause counterparts.

3) Hemolymph Protein Concentration: The age of instar is the determining factor in protein concentration of the

hemolymph. Interactions between photoperiod/age of instar, light quality/age of instar and photoperiod/light quality/age of instar are also of significance. Protein concentration of the hemolymph generally rises throughout the progression of the 5th larval instar, falls at molting and early pupal life followed by a rise under non-diapause conditions and a decline under diapause conditions in M. sexta. However, this pattern is variously modified by light quality.

4) Hemolymph Protein Profile: Qualitative distribution of hemolymph proteins in M. sexta are variously influenced by photoperiod and light quality at different times in its larval and pupal life. This is evidenced in terms of the concentration of individual protein bands in the hemolymph.

5) Hemolymph Free Amino Acids: Photoperiod, light quality and age of instar significantly influence various free amino acids in the hemolymph of M. sexta. Particularly, amino acids such as serine, glycine, glutamine, alanine, proline, ornithine and citrulline show changes in response to photoperiodic diapause induction and appear to be physiologically important. Arginine and lysine are particularly influenced by light quality.

6) Hemolymph Trehalose and Glucose: Levels of trehalose and glucose in the hemolymph are influenced by photoperiod and age of instar. Quality of light has no apparent effect. Generally, trehalose levels in the hemolymph are higher

under diapause-inducing photoperiod but show variations with respect to light quality. Light quality/age of instar interact significantly. Glucose levels in the hemolymph change according to the age of instar. Higher levels of trehalose under diapause-inducing regimen suggest the importance and possible use of this disaccharide as a metabolic energy reserve.

8) Hemolymph Osmolality: Age of instar has a significant effect on the osmolality of the hemolymph. Photoperiod/light quality and photoperiod/age of instar are also of significance. Changes in hemolymph osmolality of M. sexta do not necessarily reflect the changes in the biomolecules characterized in this study in response to various rearing conditions since factors such as metabolites and other components of the hemolymph were not characterized. The dynamics of the various hemolymph constituents should be monitored to study metabolic homeostasis in the tobacco hornworm, M. sexta.

Chapter VII

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Appendix A

The constitution of the wheat germ based meridic diet used to rear laboratory colonies of tobacco hornworms (from Stewart, 1981).

<u>Ingredients</u>	<u>Weight (g)</u>
<u>Premix</u>	
Wheat germ	50.00
Casein	22.50
Sucrose	20.00
Brewers' yeast	15.00
Wesson's salt mixture	7.50
Ascorbic acid	2.00
Sorbic acid	0.75
Methyl-p-hydroxybenzoate	0.50
Cholesterol	0.25
<u>Vitamins</u> (mg)	
Nicotinic acid	5.00
Calcium pantothenate	5.00
Riboflavin	2.50
Thiamine HCl	1.65
Pyridoxine HCl	1.65
Folic acid	0.10
Biotin	0.10
<u>Others</u>	
Agar	10.00 g
Formalin	10.00 ml
Raw linseed oil	2.00 ml
Distilled water	750.00 ml

Appendix B

Reagents used in preparation of polyacrylamide gels and SDS-PAGE.
(Hames and Rickwood, 1981).

A.	40% Acrylamide monomer solution	
	Acrylamide	39.00 g
	Bis	1.00 g
	Glycerol	20.00 ml
	Distilled water to make	100.00 ml
B.	Resolving gel buffer solution (1.5 M Tris-HCl)	
	Tris	18.30 g
	HCl (12 M)	6.00 ml
	10% Sodium dodecylsulphate	4.00 ml
	Distilled water to make	100.00 ml
	Adjust pH to	8.8
C.	Stacking gel buffer solution (0.25 M Tris-HCl)	
	Tris	3.00 g
	10% Sodium dodecylsulphate	4.00 ml
	HCl (12 M)	2.00 ml
	Distilled water to make	100.00 ml
	Adjust pH to	6.8
D.	Electrode buffer	
	Tris	3.00 g
	Glycine	14.40 g
	10% Sodium dodecylsulphate	4.00 ml
	Distilled water to make	100.00 ml
	Adjust pH to	8.8

Appendix B

To make 15% Resolving gel for SDS-PAGE :

Reagent A	14.00 ml
Reagent B	12.00 ml
TEMED	11.50 μ l
Ammonium persulphate	0.02 g
Distilled water	20.00 ml

To make 5% Stacking gel for SDS-PAGE :

Reagent A	4.00 ml
Reagent C	10.00 ml
TEMED	100.00 μ l
Ammonium persulphate	0.02 g
Distilled water	23.00 ml

All reagents were of 'Electrophoresis Grade' obtained from Bio-Rad Laboratories, Richmond, California.

Appendix C

Duncan's Multiple Range Test at the 0.05 level of data obtained for larval weight of Manduca sexta reared under different conditions. Values are expressed as Mean (S.D. of mean, n) (mg). Values in the column followed by the same letter are not significantly different.

Rearing condition	Day 1	2	3	4	5	6	7
Long day-Indorsun	4.22 ^a (0.96,47)	5.64 ^a (1.46,47)	6.45 ^a (1.36,47)	12.87 ^a (5.60,47)	29.37 ^a (11.46,47)	32.73 ^a (13.41,47)	73.72 ^a (43.77,47)
Long day-Blacklight blue	2.77 ^a (1.17,48)	5.42 ^a (1.55,48)	6.62 ^a (2.42,48)	13.12 ^a (7.27,48)	29.11 ^a (10.30,48)	36.97 ^a (16.11,48)	87.90 ^a (52.71,48)
Short day-Indorsun	3.62 ^b (1.23,41)	7.02 ^b (2.04,41)	10.10 ^b (3.82,41)	24.68 ^b (11.03,41)	36.72 ^b (10.25,41)	70.39 ^b (39.58,41)	154.12 ^b (71.01,41)
Short day-Blacklight blue	3.51 ^b (1.40,42)	7.30 ^b (1.66,42)	10.95 ^b (4.62,42)	26.95 ^b (9.54,42)	39.41 ^b (14.86,42)	91.55 ^c (46.58,42)	183.62 ^c (57.63,42)

Appendix C

Duncan's Multiple Range Test at the 0.05 level of data obtained for larval weight of Manduca sexta reared under different conditions. Values are expressed as Mean (S.D. of mean, n) (g). Values in the column followed by the same letter are not significantly different.

Day	8	9	10	11	12	13	14	15
	0.14 ^a (0.06,47)	0.20 ^a (0.10,47)	0.53 ^a (0.40,47)	0.91 ^a (0.52,47)	1.21 ^a (0.64,47)	2.15 ^a (1.42,47)	3.60 ^a (2.60,47)	5.00 ^a (3.46,42)
	0.15 ^a (0.05,48)	0.23 ^a (0.11,48)	0.64 ^a (0.44,48)	1.07 ^{ab} (0.48,48)	1.37 ^a (0.58,48)	2.50 ^{ab} (1.46,48)	4.52 ^{ab} (2.46,48)	6.75 ^b (3.28,45)
	0.21 ^b (0.07,41)	0.44 ^b (0.28,41)	0.92 ^b (0.52,41)	1.14 ^{bc} (0.43,41)	1.71 ^b (0.72,41)	2.56 ^{ab} (1.48,41)	4.16 ^{ab} (2.56,41)	6.43 ^b (3.17,37)
	0.26 ^c (0.15,42)	0.58 ^c (0.37,42)	0.97 ^b (0.41,42)	1.35 ^c (0.54,42)	1.98 ^b (0.96,42)	3.01 ^b (1.83,42)	5.08 ^b (2.53,42)	7.48 ^b (2.75,38)

Summary of the analysis of variance of the data obtained for titres of free amino acids present in the hemolymph of Manduca sexta reared under different conditions. Values shown are F values ($P < .05$).

A: Main Effects

Amino acid	Photoperiod (d.f.: 1)	Light Quality (d.f.:1)	Age of Instar (d.f.:3)
Alanine	3.46*	0.02*	41.46
Arginine	6.98	4.54	263.28
Asparagine	6.55	0.71*	51.41
Aspartic acid	4.43	0.05*	9.24
Citrulline	0.00*	0.03*	1.98*
Cystathionine	8.68	1.01*	47.56
Glutamic acid	14.50	0.02*	15.33
Glutamine	5.28	0.05*	12.04
Glycine	0.86*	0.06*	12.50
Histidine	8.70	0.04*	17.15
Isoleucine	0.11*	0.96*	26.58
Leucine	1.50*	0.04*	15.32
Lysine	1.47*	6.90	235.26
Methionine	4.03	0.00*	21.40
Ornithine	0.82*	0.71*	6.09
Phenylalanine	6.42	0.02*	0.93*
Proline	17.10	0.20*	42.81
Serine	3.42*	0.22*	8.32
Threonine	1.86*	1.32*	2.82
Tyrosine	3.81*	0.25*	2.54*
Valine	0.06*	0.53*	28.67

* indicates 'Not Significant'.

Appendix D

Summary of the analysis of variance of the data obtained for titres of free amino acids present in the hemolymph of Manduca sexta reared under different conditions. Values shown are F values ($P < .05$).

B: Interactions

Amino acid	Photoperiod X Light Quality (d.f.: 1)	Photoperiod X Age of Instar (d.f.: 3)	Light Quality X Age of Instar (d.f.: 3)	Photoperiod X Light Quality X Age of Instar (d.f.: 3)
Alanine	1.98*	0.88*	0.24*	2.36*
Arginine	4.54	2.05*	1.45*	1.15*
Asparagine	0.00*	5.43	0.27*	0.24*
Aspartic acid	0.87*	2.54*	1.04*	0.46*
Citrulline	5.64	2.57*	2.98	1.58*
Cystathionine	1.20*	3.75	1.20*	2.47*
Glutamic acid	0.65*	10.92	0.98*	0.07*
Glutamine	0.24*	1.83*	0.63*	2.97
Glycine	1.26*	0.54*	0.27*	0.55*
Histidine	0.30*	0.16*	1.43*	1.81*
Isoleucine	0.20*	1.95*	1.09*	0.73*
Leucine	0.04*	1.21*	0.52*	0.18*
Lysine	0.62*	0.38*	3.66	2.05*
Methionine	0.51*	4.66	0.16*	1.24*
Ornithine	0.10*	0.61*	0.17*	0.06*
Phenylalanine	0.21*	3.13	0.18*	0.44*
Proline	0.12*	8.81	1.56*	0.32*
Serine	5.74	2.90	1.85*	0.30*
Threonine	0.03*	1.42*	0.94*	0.80*
Tyrosine	0.21*	0.46*	0.16*	0.08*
Valine	0.17*	2.72*	0.93*	1.22*

* indicates 'Not Significant'.