Protein Utilization Efficiencies of Two Species of Lepidopteran Larvae, *Hyalophora cecropia* L. and *Antheraea polyphemus* Cramer.

by

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ABSTRACT

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Protein utilization efficiencies of two species of Lepidoptera larvae, Hyalophora cecropia L. and Antheraea polyphemus Crarner were determined. Larvae reared in a laboratory were fed leaves of the black cherry, Prunus serotina Ehrh. Samples of these leaves and the feces produced during their fourth and fifth larval instars were examined for protein content using standard techniques.

The amount of protein in the leaves ingested (I) was compared to the amount of protein excreted in the feces (F). The apparent protein assimilated (A) was determined by the difference of the ingested and excreted protein (A = I-F). The protein utilization efficiency (PE) was determined by the ratio of protein assimilated to that ingested (PE = A/I*100).

Fourth instar larvae of A. polyphemus assimilated protein at the lowest rate, 60.1%±5.5. Fifth

instar larvae of A. polyphemus and fourth instar larvae of H. cecropia used protein at about the same rates, 73.5%±6.9 and 79.3%±3.9, respectively, while the fifth instar larvae of H. cecropia assimilated protein at the highest rate, 89.2%±2.6.

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LIST OF SYMBOLS

SYMBOL	DEFINITION	UNITS OR REFERENCE
PE	Apparent protein assimilation efficiency	%
I	Ingestion of protein	grams
F	Protein content of feces	grams
R	Respiration of larvae	
A	Apparent protein assimilation (I - F)	grams

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CHAPTER I

INTRODUCTION

Plants have coevolved qualitative and quantitative barriers to protect their energy reserves from herbivores (Feeny 1976). Qualitative barriers are those secondary plant substances that interfere with metabolic processes and are effective, even in small concentrations, against non-adapted insect species. These substances are not effective on adapted insect species that can rapidly detoxify them. Glucosinolates, a primary chemical defense of crucifers, are very effective in small amounts against non-adapted insects (Feeny 1976). Quantitative defenses of plants are plant substances that interfere with digestion and reduce fitness of a wide range of insects that feed on the plants. Substances such as these are not readily susceptible to counteradaptation (Feeny 1976). Quantitative defenses are characteristic of apparent plants and plant tissues, which are easy for herbivores to locate, whereas qualitative defenses are characteristic of unapparent plants and tissues (Rhoades 1979). Plants which are highly apparent in their habitat have developed quantitative barriers, even to insects which naturally feed on their leaves (Feeny 1976). Tannins, resins, silica, and lignins (crude fiber) all reduce the

digestibility of the food presented by apparent plants. Plant secondary substances such as these have been shown either to have a negative effect on herbivore fitness (increased mortality, lowered growth rates or fecundity), or to have a deterrent effect on herbivore grazing activities (Rhoades and Cates 1976).

The chemical defenses of most plant species may be a consequence of "co-evolution" with a variety of predators, parasites, pathogens and competitors. Thus, while the role of plants in shaping chemical counter-adaptation by insects is often clear, the effects of evolution of plant defenses must be evaluated against a broader coevolutionary background (Feeny 1976). Chemical coevolution between plants and phytophagous insects tor herbivores in general) must encompass all groups of organisms that interact with them.

The effects of food quality upon physiology and behavior at the species, population and ecosystem levels are too often neglected. A knowledge of nutritional ecology is essential to properly interpret life history phenomena (e.g. manner of feeding, habitat selection, defense and reproduction) both in ecological and evolutionary time (Scriber and Slansky 1981). Food is of particular importance to immature insects. Herbivorous insect fitness depends on the insect's ability to efficiently and/or rapidly ingest, digest and convert plant material into insect material (Scriber 1981). For

most herbivorous insects there is abundant food quantity, however food quality may be inadequate. Protein content is a primary determlnant of food quality. The digestible protein content determines the amount of nitrogen available to the herbivorous insects (Davis 1974a). There is a lot of nitrogen in the world, but most is in a form that is unavailable to animals. It is not the amount of energy in the ecosystem that limits the abundance of organisms, it is the amount of nitrogen readily available for lncorporatian into body tissues. Availability of protein in plants plays a major role in limiting organisms at all trophic levels above them (White 1978).

Much research has been done on the quality of food and its relationship to insect larval growth (Davis 1974b, Feeny 1970, Lincoln 1985, Rauscher 1981, Scriber and Slansky 1981), behavior (Wlllams 1983) and survival (White 1974). Still more research has been aimed at the determination of the efficiency of the larvae to assimilate its ingested and digested food into body tissue (Pandian 1973, Pandian and Delvl 1973, Schroeder 1971, 1976a, 1976b, 1977a, 1977b, 1978, Schroeder and Dunlap 1970, Schroeder and Malmer 1980, Soo Hoo and Fraenkel 1966).

In order to obtain the protein necessary for proper growth and maintenance, the insect must not only ingest food but must be able to digest the proteins. Insects that are placed on diets that regulate the protein

available to them show changes in growth rates (Ito 1967, Davis 1974a, Schroeder 1986b, White 1978). Tannins reduce the digestibility of soluble proteins in plants and lignins make plants less palatable (Swain 1979).

The intent of this study was to determine the amount of protein digested from leaves fed to two species of Lepidopteran larvae, Hyalophora cecropia L. and Antheraea polyphemus Cramer. Because over 90% of food ingestion occurs during the fourth and fifth instars, these instars were selected for study (Mattson 1980). The feeding rate determined as mass of dry food/ mass live weight/ day actually decreases in some species (Pandian 1973). The larger size of the larvae and the larger total food intake make it much easier to work with the later instars rather than with earlier ones. While this study was concerned with food quality as determined by soluble and insoluble proteins, it was realized that the other determinants of food quality cannot be ignored. One factor cannot be isolated and adequately studied, as all affect the nutritional aspect of the organism. Digestibility (Bhandari et al. 1979, Soo Hoo and Fraenkel 1966), Ingestibility (Feeny 1970, Rausher 1981), temperature (Merkel 1977), water content (Schroeder 1986a, Scriber 1978, Scriber and Slansky 1981), nutrients (Feeny 1970, Ito 1967, Schroeder 1986a), chemical composition (Feeny 1970, Lincoln 1985, Robinson 1979, Walker 1976),

availability (Schroeder 1976b, White 1974), suitability (Scriber 1984) and apparency (Feeny 1976, Rhoades 1979, Rhoades and Cates 1976) are extremely important and must be considered as a whole.

The ecological success of an organism is dependent on a variety of factors including food quantity and quality. Density, competition, humidity, light, temperature, parasites and pathogens are all extremely important in the overall understanding of the role of the organism within the ecosystem (Merkel 1977, Scriber and Slansky 1981, Rausher 1981).

CHAPTER II

METHODS

Larvae of the cecropia moth, Hyalophora cecropia L., were obtained from a mass culture reared in a laboratory at Youngstown State University by Dr. Lauren Schroeder. These larvae were the progeny of wild female moths that had been captured and allowed to lay eggs in the laboratory. Six late third or newly molted fourth instar larvae were obtained from the mass culture on June 18, 1986, and placed on individual leaves of black cherry, Prunus serotina Ehrh. that same day.

A mature polyphemus (Antheraea polyphemus Cramer) larva was found in the fall of 1985, pupated and the cocoon kept in an unheated garage over the winter so that it would be exposed to near normal temperatures to assure proper development. Upon emergence in the spring of 1986, the female moth was placed outside in a wire mesh cage to allow mating. Approximately thirty eggs were collected and kept in a glass vial until they hatched, on June 15, 1986. Larvae were collected as they hatched and were placed on freshly cut individual black cherry leaves and reared to the fourth instar. Ten newly molted fourth instar larvae were selected from the mass culture for the experiment.

Individual larvae were placed In cardboard cones that had been fashioned from 7" X 9" pieces of posterboard and set in 400 ml beakers for support. A leaf petiole was inserted through a small cut in the side of the cone so that the cut end of the petiole was immersed into a small vial of water taped to the side of the beaker. The feces were allowed to drop into the beaker through an opening in the apex of the cone. The top of the cone was covered with cheesecloth held in place by a rubberband. This allowed good light penetration and air circulation. The setup was placed near a north facing window to allow the larvae to be exposed to normal diurnal light and ambient temperature cycles. The window was open most of the time to allow for air circulation.

The leaves were changed at approximately the same time each day. Fresh leaf stalks were cut from the tree and the cut ends immediately immersed in water to be transported to the lab. This was to preserve their freshness and diminish water loss during transportation. Two different trees were used during the research but to minimize variance in food protein content all larvae of each species were fed from the same tree. Samples of the leaves fed to each larvae were taken by punching several disks from each leaf with a #6 cork borer having a diameter of eleven millimeters. The leaf samples were immediately frozen for future protein analysis. The daily

samples were pooled by individual larva and instar. Feces were collected in the morning and evening each day and immediately frozen to reduce any bacterial action that might further break down protein excreted with the feces. The feces from each individual larva were also pooled by instar. At the end of the fifth instar the larvae were allowed to pupate.

Samples were kept frozen at -25° C. until it was time to determine the amount of leaf and fecal protein. The leaf material was prepared for analysis by haphazardly selecting four leaf disks from each of the frozen samples. These disks were ground two at a time in a glass mortar and pestle with a small amount of fine white grinding sand. The material from each grinding of two leaf disks was then extracted in 4 ml of 1.0 M NaOH for 6 hours. The extract was then centrifuged for 15 mlnutes at maximum speed in an Adams Dynac Centrifuge.

Six fecal pellets from each frozen sample were selected for protein determination. These were ground three at a time in a glass mortar and pestle with a small amount of fine white grinding sand. The ground material was extracted in 1.0 M NaOH for six hours. Due to the much larger size of the fifth instar H. cecropia fecal pellets, four pellets, two per ground sample, were used for protein determination. This number was reduced because a larger sample size would be close to the upper limits of the testing procedure.

The Bradford method (Bradford 1976) of protein analysis was used to determine protein content. Two 100 ul aliquots of each sample were transfered to 9.5 ml test tubes using a Chempette Series 7857 micropipette, combined with 4.9 ml of Coomassie brilliant blue G-250 reagent, and mixed by inverting the tubes twice. Blank tests were prepared in the same manner by using 100 ul of the 1.0 M NaOH. The absorption was read at 595 nm after 15 minutes at room temperature on a Shimadzu UV-Recording Spectrophotometer Model UV-260.

The samples were tested against standards that were prepared from bovine gamma globulin purchased from Sigma Chemical Company. The standards were diluted from purchased stock solution concentration of 80 mg/ml to concentrations similar to those expected in the leaves and feces. Concentrations of 16 ug/100 ul, 32 ug/100 ul, 48 ug/ 100 ul and 64 ug/ 100 ul were achieved by placing 20, 40, 60 and 80 ul of the stock solution in 10 ml volumetric flasks and bringing the volumes of each to 10 ml by adding 1.0 M NaOH. Two 100 ul aliquots of each standard and two blanks prepared with 100 ul of NaOH were tested by the Bradford method.

A Tris buffer of pH = 9 was tried as an extraction solution, because the gut pH of most Lepidoptera is in this range (Berenbaum 1980). The Tris buffer extracted only 3% or 4% of the expected leaf protein. An extraction

solution using 0.1 M potassium phosphate buffer (Robinson 1979) did not produce any better results. NaOH extracted a much higher amount of the expected protein (Ahmad and Saleemudin 1981).

Optimum concentration of NaOH for maximum protein extraction of leaves and feces was determined by using a range of NaOH concentrations and extracting over 24 hours. Triplicate samples of 12 mg of freeze-dried, ground black cherry leaves and 0.2 g of freeze-dried, ground fifth instar cecropia fecal material were extracted with a NaOH concentration range of 0.1, 0.2, 0.5, 1.0, 1.5, and 2.0 M. The 1.0 M NaOH extract had the highest concentration of protein for both leaves (Fig. 1) and feces (Fig. 2) over 24 hours.

The samples were analyzed for protein again after 72 hours to determine if the NaOH hydrolyzed the protein that had been extracted (Fig. 1). There was a marked reduction in protein with all concentrations of NaOH after 72 hours of extraction. The higher the concentration of NaOH the greater the loss of extracted protein. The optimum NaOH concentration was 1.0 M for obtaining the highest protein extraction with the least amount of hydrolysis in leaves (Fig. 1) and feces (Fig. 2).

The optimum extraction time was determined using 1.0 M NaOH. 120 mg of leaf material in 30 ml of 1.0 M NaOH and 2.0 g of fecal material in 40 ml of 1.0 M NaOH were extracted for 30 hours (Figs. 3 and 4). Samples (1.5 ml)

FIGURE 1

Extraction of Freeze-dried Black Cherry (*P. serotina* Ehrh.) Leaf Material Over 24 hours and 72 hours Using Different Concentrations of NaOH (vertical lines through circles represent standard deviations)

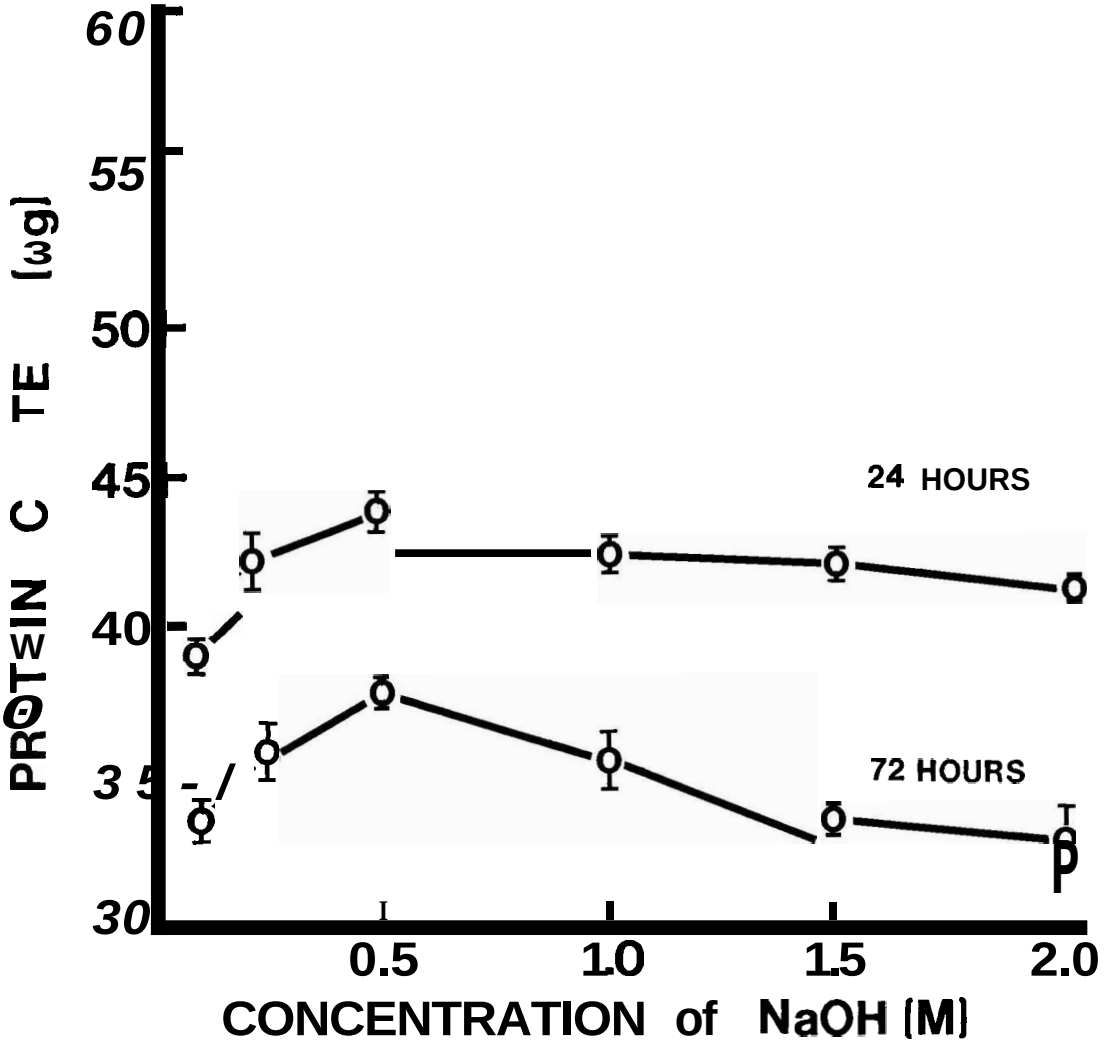

60
55
50
45
40
35
30
PROTEIN CONTENT (ωg)
24 HOURS
72 HOURS
0.5
1.0
1.5
2.0
CONCENTRATION of NaOH (M)

FIGURE 2

Extraction of Freeze-dried Fifth Instar *H. cecropia* Fecal Material Using Different Concentrations of NaOH Over 24 Hours (vertical lines through the circles represent standard deviations)

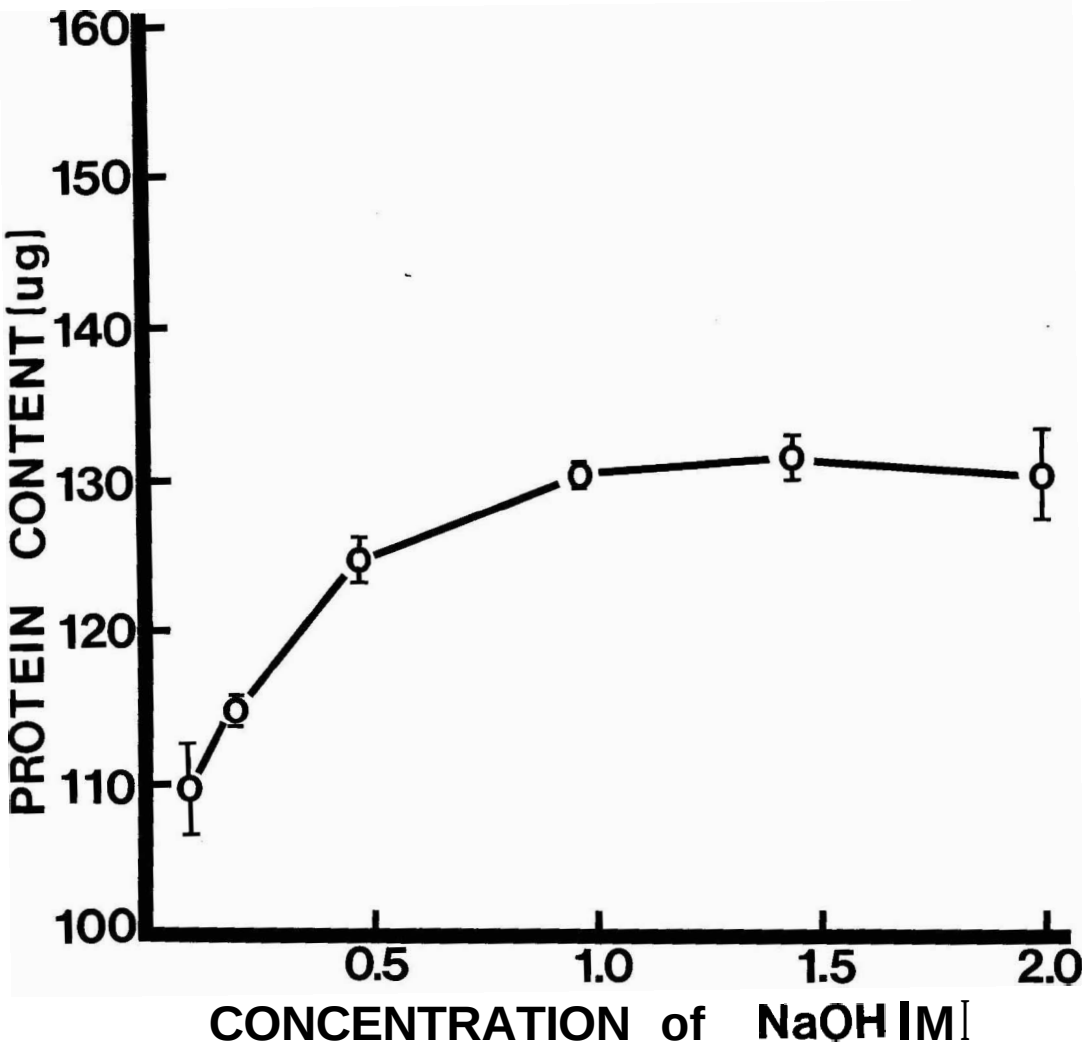

160
150
140
130
120
110
100
PROTEIN CONTENT (ug)
0.5
1.0
1.5
2.0
CONCENTRATION of NaOH (M)

FIGURE 3

**Timed Extraction of
Freeze-dried Black Cherry (*P. gerotina* Ehrh.)
Leaf Material
Using 1.0 M NaOH Over 30 Hours
(vertical lines through the circles
represent standard deviations)**

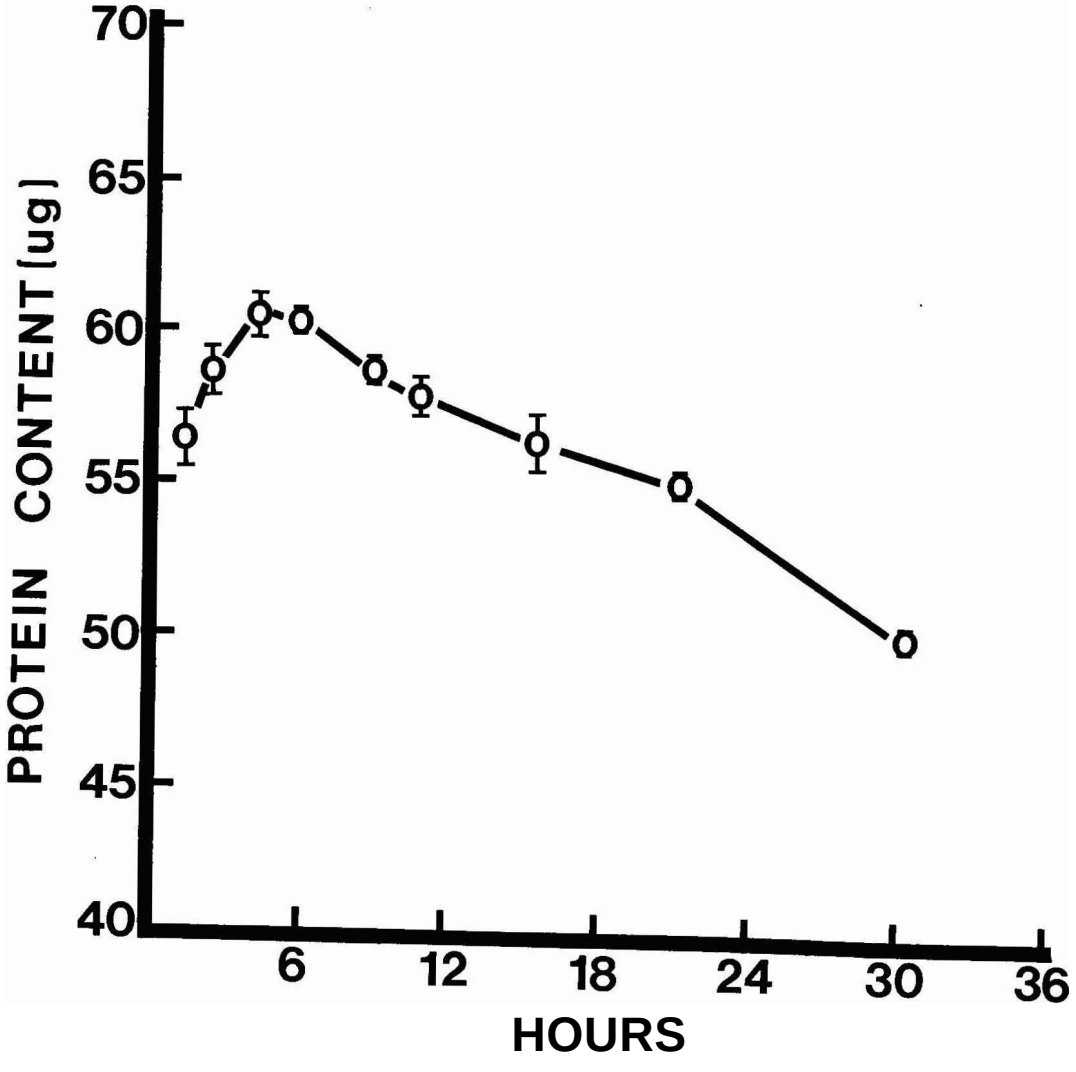
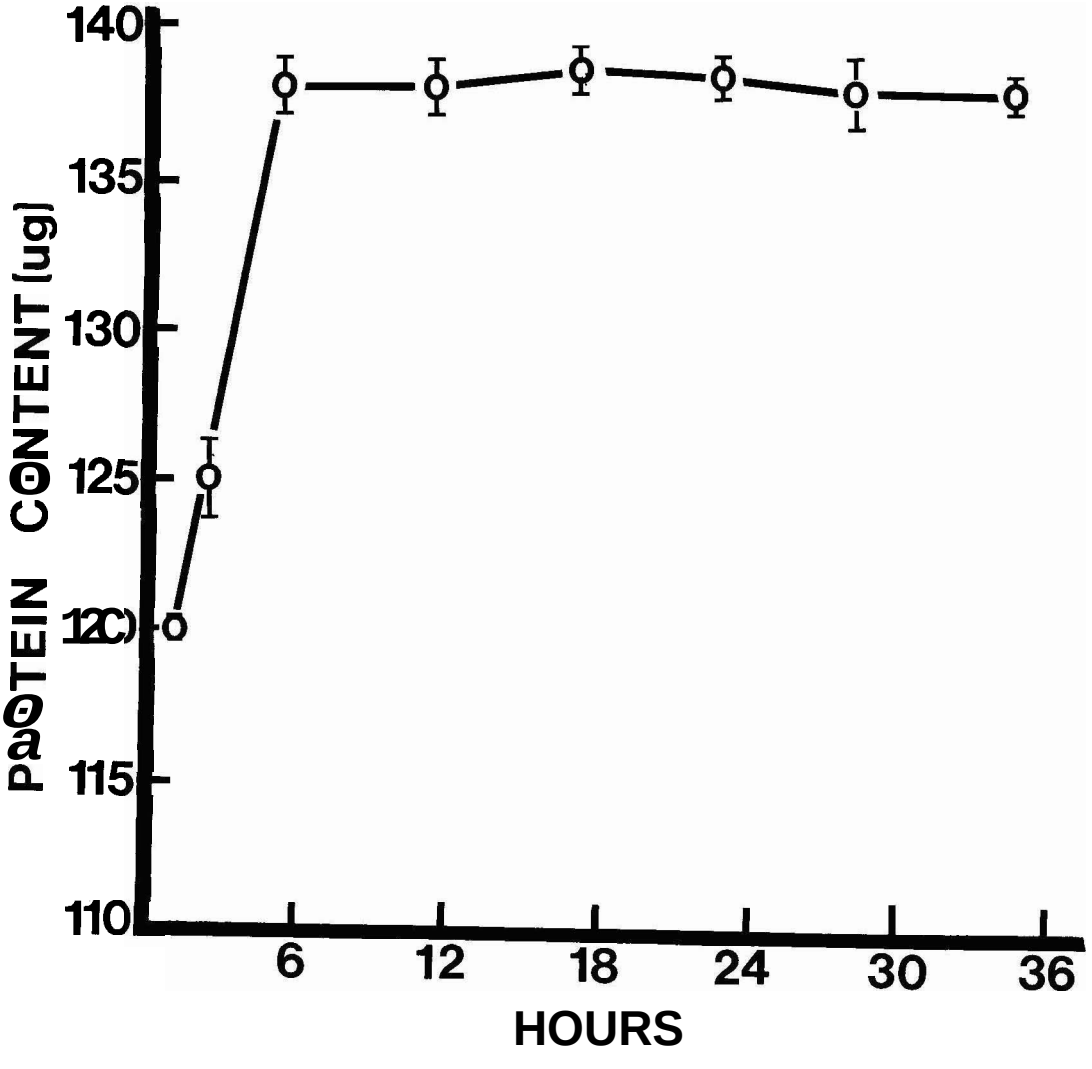

70
65
60
55
50
45
40
PROTEIN CONTENT (ug)
6
12
18
24
30
36
HOURS

FIGURE 4

Timed Extraction of Freeze-dried Fifth Instar *H. cecropia* Fecal Material Using 1.0 M NaOH Over 30 Hours (vertical lines through the circles represent standard deviations)


140
135
130
125
120
115
110
PROTEIN CONTENT (ug)
6
12
18
24
30
36
HOURS

of the extract were removed at regular time intervals and protein content determined (Table 1). This stock solution was kept uniform by vortexing it on a magnetic stirrer as each aliquot was removed and immediately tested for protein with the procedure described above. Three replications of each sample were taken.

Dry matter content of the leaves was determined by haphazardly selecting two leaf disks from each individual sample and drying them for 48 hours at 90° C. in a convection oven. The dried leaf disks were then weighed to the nearest 0.001 mg on a Cahn 29 Automatic Electrobalance. The mean dry weight of the sample disks was used to express protein content on a dry weight basis. Fecal pellets were selected and treated in the same manner, weighed to the nearest 0.01 mg on a Sartorius single pan balance, and the mean dry weights used in the calculations.

The Pierce BCA protein assay reagent was rejected as a method for determination of protein content of the extract from leaves and feces (Pierce bulletin no. 23225, 1984). The method resulted in slightly higher absorbance than the same extract tested with Coomassie blue by the Bradford method. However, for feces, the BCA method resulted in absorbances that were more than ten times the expected levels. The high absorbance of the BCA reagent

TABLE 1

Extraction of Freeze-dried Black Cherry (*P. serotina* Ehrh.) Leaf Material and Freeze-dried Fifth Instar H. cecropia Fecal Material Using Different Concentrations of NaOH Over 24 Hours

NaOH Molarity	Mean Protein Content (ug)	Protein in Dry Matter (%)
	leaf material	
0.1	40.0 ±1.3	13.3
0.2	40.2 ±0.8	13.4
0.5	44.0 ±0.5	14.7
1.0	44.3 ±0.8	14.8
1.5	42.4 ±0.8	14.1
2.0	45.8 ±0.9	15.3
	fecal material	
0.1	108.8 ±3.1	2.2
0.2	113.6 ±0.4	2.3
0.5	125.9 ±1.3	2.5
1.0	133.9 ±0.5	2.7
1.5	138.8 ±1.4	2.8
2.0	137.5 ±3.2	2.8

N = 4

with feces was due to binding with uric acid and giving a false reading.

Protein utilization efficiency (PE) of the larvae was determined by dividing the apparent protein assimilated (A) by the protein ingested (I).

$$PE = A/I \times 100 \quad (1)$$

Using the equation for determination of dry matter budgets,

$$I = G + R + F \quad (2)$$

where I = ingestion, G = growth, R = respiration and F = feces produced (Schroeder 1986a) and values for gross growth efficiency = G/I and net growth efficiency = G/(I-F) a calculated value for the amount of fecal protein (F) was determined. These values were multiplied by the fraction of protein content of the feces to determine the amount of protein excreted in the feces.

Flnal calculations of PE were made assuming 1 gram of ingested leaf material. This was multiplied by the percent protein content of the leaves to get I. F was determined by multiplying the calculated value for the dry weight of feces by the percent of protein in the feces. The apparent assimilation (A) was determined to be the difference between I and F (A = I - F). The PE was calculated by dividing the apparent assimilation by the ingestion (A/I) and multiplying by 100.

CHAPTER III

Results

Dry matter content of the leaf disks fed to H. cecropia was **6.5** mg and that fed to A. polyphemus was **8.0** mg (Table 2). The difference in dry weights was due partly to different trees used for each species and partly due to the decreasing quality of the leaves as the feeding period continued.

Leaves fed to IV and V instar H. cecropia larvae increased in mean protein content from **10.7%** ±1.8 to **12.9%** ±0.6. Feces of IV and V instar H. cecropia larvae decreased in protein content from 3.1% **f0.3** to **2.0%** ±0.3 (Table 3). Leaves fed to IV and V instar A. polyphemus larvae showed a protein content increase from **8.6%** ±1.6 to **10.4%** ±1.4. Feces of A. polyphemus decreased in protein content from **3.9%** ±0.5 to **3.1%** ±0.5 (Table 3). Mean leaf protein content fed to all larvae resulted in a one-way analysis of variance (ANOVA) F value of **11.4,** $p < 0.0001$ (Table 3). Mean protein content of all larval feces resulted in a one-way ANOVA F value of **23.7,** $p < 0.0001$ (Table 3).

Using equation (2) for determination of dry matter budgets, along with a G/I = **0.16** and a G/(I-F) = **0.53** for the H. cecropia (Schroeder 1986a) a dry matter value of the feces was calculated to be **0.69.** Using a

TABLE 2

Mean Dry Weights of
Black Cherry (*P. serotina* Ehrh.)
Leaf Disks and Fecal Pellets
of Fourth and Fifth Instar
H. cecropia and *A. polyphemus*
(mg)

H. cecropia		*A. polyphemus*	
IV Instar	V Instar	IV Instar	V Instar
leaf disks			
6.3 f0.8	6.6 ±0.8	8.0 ±0.8	7.9 ±1.2
fecal pellets			
11 ±2.6	70 ±12	6.0 f1.3	26 ±4.6

N = 30 for all means

TABLE 3

Protein Content of
Black Cherry (*P. serotina* Ehrh.)
Leaves and Feces of Fourth and Fifth Instar
H. cecropia and *A. polyphemus*

Species	Number of Larvae	Protein Content of Leaves (%)	Protein Content of Feces (%)
A. polyphemus			
IV instar [a]	10	8.6 ±1.6	3.9 ±0.5
V instar [b]	10	10.4 ±1.4	3.1 ±0.5
H. cecropia			
IV instar [b]	6	10.7 ±1.8	3.1 ±0.3
V instar [c]	6	12.9 ±0.6	2.0 ±0.3
ANOVA F score		11.4	23.7
probability		<0.0001	<0.0001

Differences among means at $p < 0.05$ are indicated by different superscript letters.

G/I = 0.08 and a G/(I-F) = 0.69 for the A. polyphemus (Scriber and Feeny 1979) a dry matter value of the feces was calculated to be 0.88. These values were multiplied by the fraction of protein content of the feces to determine the amount of protein excreted in the feces. The protein utilization efficiencies (PE) of all larvae were determined by using equation (1), dividing the apparent protein assimilated (A) by the protein ingested (I). The PE of H. cecropia for the fourth lnstar was 79% ±4 and in the fifth was 89% ±3 (Table 4). Fourth instar A. polyphemus larvae had a PE of 60% ±5 and 73% ±7 in the fifth instar(Table 4). PE means were judged to be significantly different by a one way ANOVA (Gustafson 1984). PE mean values resulted in an F value of 39.63, $p < 0.0001$. A Keuls multiple range test (Woolf 1968) determined that the mean PE of the fifth instar A. polyphemus and the fourth instar of H. cecropia were the same. The fourth instar of A. polyphemus was different from all others as was the fifth instar of H. cecropia (Table 4).

TABLE 4

Protein Efficiency of Fourth and Fifth Instar *H. cecropia* and *A. polyphemus*
I=Ingested Protein, F=Feces Protein, A=Apparent Assimilation and PE=Protein Efficiency(A/I)

Species	Number of Larvae	I	F	A (I-F)	PE (A/I) X100
A. polyphemus					
IV instar [a]	10	0.086 ±0.016	0.034 ±0.005	0.053 ±0.013	60.1 ±5.5
V instar [b]	10	0.104 ±0.014	0.027 ±0.005	0.077 ±0.017	73.5 ±6.9
H. cecropia					
IV instar [b]	6	0.106 ±0.019	0.021 ±0.001	0.085 ±0.018	79.3 ±3.9
V instar [c]	6	0.129 ±0.006	0.013 ±0.002	0.116 ±0.009	89.2 ±2.6
ANOVA F score		11.0	35.0	23.4	39.6
probability		<0.0001	<0.0001	<0.0001	<0.0001

Differences among means at $p < 0.05$ are indicated by different superscript letters.

Chapter IV

Discussion

Protein utilization efficiencies (PE) of both species of Lepidoptera larvae increased from the fourth to the fifth instars. The PE for A. polyphemus larvae increased 21.6% and for H. cecropia larvae it increased 12.7%. Net growth efficiency (G/A) usually increases at each successive developmental molt (Mattson 1980). Since the G/A is directly dependent upon the nitrogen assimilated and this nitrogen is found in the ingested protein, it follows that these larvae should show an increased PE in the final instar. This increased PE and subsequent increased G/A, is important to supply the protein needed in the metamorphosis from the larval stage to the adult moth.

Macrolepidoptera larvae, e.g. H. cecropia and A. polyphemus, feed not only in early summer (June and early July) but have phases that feed in the latter part of the growing season (late July, August and September) when food leaves are declining in quality and increasing in toughness (Mattson 1980, Schroeder 1986a). It is believed that their larger size allows adaptations that permit them to make more efficient use of the low quality food. Larger size allows easier movement and provides a mechanical advantage while feeding, giving the

larvae access to a greater quantity of food (Mattson 1980). The larger size also permits larger mouth parts that make it easier for the larvae to chew the tough, fibrous leaves encountered as the growing season progresses (Mattson 1980). Internal gut adaptations of larger larvae allow better digestion of low quality ingested food (Berenbaum 1980, Mattson 19801.

As the larval stages progressed it was noted that the fourth instar A. polyphemus was the smallest and the fifth instar H. cecropia was noticably the largest. The fourth instar H. cecropia and fifth instar A. polyphemus were approximately the same size. This also correlates with the PE of each larval instar.

The larvae are well adapted to make efficient use of the small amount of protein available to them in low quality leaves. The black cherry fed to both species fits the definition of an apparent plant and has evolved quantitative barriers (tannins and lignins) to feeding by polyphagous larvae such as H. cecropia and A. polyphemus (Feeny 1976, Rhoades and Cates 1976). The leaves used in this study increased in protein content through the feeding periods. The trees were rather small and were undoubtedly stressed by removing the large volume of leaves needed to feed the larvae. This stress may have mobilized proteins that would not normally be available to larvae (Mattson 1980, White 1984). The

larvae, particularly H. cecropia, had high protein efficiencies. This may have been due to their use of the mobilized proteins in the stressed trees.

Herbivores are limited from below by lower trophic levels and not from above by higher trophic levels (White 1978). The high PE indicates that H. cecropia and A. polyphemus are well adapted to use low quality mature leaves. Like most Macrolepidoptera these two species feed well into late summer and early fall (Mattson 1980). Their efficient use of protein is an adaptation to quantitative barriers present in maturing food leaves that allows better use of the tough, fibrous, low quality leaves.

CHAPTER V

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