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Characterization of a haemolymph storage protein and its relationship to insect weight and development in the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera:Pyralidae)

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Abstract

In this study I identified and characterized a storage protein in the European corn borer, *Ostrinia nubilalis*. The relationship between larval weight and larval age on the storage protein levels were examined.

Final instar corn borers were reared on artificial diets in a laboratory. Haemolymph, fat body and intact insects were collected. These samples were processed and analysed using SDS-PAGE. The protein was identified as a storage protein by its native molecular weight of approximately 500 kDa and the observation that it increased in abundance throughout larval development and diminished with adult development. The protein had the characteristics of an arylphorin-like storage protein because it is a heterohexamer and is the most abundant protein in the haemolymph, increasing to the intermoult period with a decline just prior to pupation.

Larval weight accounts for much of the variation in storage protein levels in the fat body (90%) and intact larvae (80%). Similar results were found with larval age and storage protein levels (80% for both fat body and intact larvae). This suggests that larval age and larval weight are both good indicators of storage protein levels and either may be used to predict these levels in the corn borer.

Since nutrition has been proposed as a regulator of storage protein levels, I examined the relationship between storage protein levels, protein quality in the diet and larval weight.

The standard artificial diet was altered by replacing the protein casein with either gluten, zein or haemoglobin. There was no significant effect of protein quality in the diet on storage protein levels after correcting for the weight of the larvae.

Finally, I examined how development was affected by protein quality in the insect's diet. Insects were placed on a diet containing either casein (standard diet), albumin, gluten, zein or haemoglobin, in either 21.5% or 8.0% protein content (for a total of 10 different diets). Differences between males and females were found with female corn borers eating more, remaining in the larval stage longer and having greater pupal weights than males. These results are expected in species with non-feeding adults. Corn borers fed poorer diets could compensate by consuming more and increasing their larval duration, however, they did so at lower pupal weights than those corn borers on the standard diet. Logistic regression analysis showed that pupal weight had a significant effect on survival, suggesting that heavier pupae are more likely to survive to the adult stage than lighter pupae.

Résumé

Dans cette étude, j'ai identifié et caractérisé une protéine de réserve chez la pyrale du maïs, *Ostrinia nubilalis*. J'ai ensuite étudié les relations entre le poids et l'âge des larves et le niveau de cette protéine de réserve.

Des larves de la pyrale du maïs au dernier stade de développement ont été élevées en laboratoire avec une diète artificielle. L'hémolymphe, le corps adipeux et des insectes intacts ont été prélevés. Ces échantillons ont été préparés et analysés en utilisant la technique SDS-PAGE. La protéine a été identifiée comme étant une protéine de réserve par son poids moléculaire d'environ 500 kDa et par l'observation que son abondance augmenta au cours du développement larvaire et diminua avec le développement de l'adulte. La protéine a été caractérisée comme étant une protéine de réserve ressemblant au type 'arylphorin' puisque c'est une protéine hétérohexamère et puisque elle est la protéine la plus abondante de l'hémolymphe, augmentant en abondance pendant le dernier stade de développement larvaire pour ensuite diminuer juste avant la métamorphose.

Le poids des larves explique une proportion importante de la variation du niveau de la protéine de réserve dans le corps adipeux (90%) et les larves intactes (80%). Des résultats similaires ont été obtenus entre l'âge des larves et le niveau de cette protéine de réserve (80% pour le corps adipeux et les larves intactes). Ceci suggère que l'âge et le poids des larves sont tous deux de bons indicateurs du niveau de la protéine de réserve et ainsi l'un ou l'autre peut être utilisé pour prédire le niveau de cette protéine dans la pyrale du maïs.

Puisque la nutrition a été proposée comme étant un régulateur du niveau des protéines de réserve, j'ai examiné la relation entre le niveau de la protéine de réserve, la qualité protéique de la diète et le poids larvaire. La diète standard a été modifiée en remplaçant la protéine caséine par soit du gluten, soit de la zéine ou soit de l'hémoglobine. Aucun effet significatif de la qualité protéique de la diète sur le niveau de la protéine de réserve, après correction pour le poids des larves, n'a été obtenu.

Finalement, j'ai étudié comment le développement était affecté par la qualité protéique de la diète des insectes. Les insectes au dernier stade de développement ont été nourris avec une diète contenant soit de la caséine (diète standard), soit de l'albumine, soit du gluten, soit de la zéine, ou soit de l'hémoglobine, dans des proportions de 21.5% ou 8.0% de teneur en protéines (pour un total de 10 diètes différentes). Des différences dues au sexe ont été obtenues, avec les femelles mangeant plus, demeurant plus longtemps au stade larvaire et ayant un poids au début de la métamorphose plus élevé que les mâles. De tels résultats sont attendus chez des espèces dont les adultes ne se nourrissent pas. Les pyrales du maïs nourries avec une diète pauvre ont été capables de compenser en mangeant plus et en augmentant la durée du stade larvaire. Toutefois, leur poids au début de la métamorphose était plus faible que celui des pyrales nourries avec la diète standard. Une analyse par régression logistique a montré que le poids des pupes avait un effet significatif sur leur survie, ce qui suggère que les pupes plus lourdes ont plus de chance de survivre jusqu'au stade adulte que les pupes plus légères.

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copies and final copies of this work.

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Chapter 1 General Introduction

1.1 Storage Proteins

Insect storage proteins are large (~500 kDa) haemolymph proteins that increase in concentration throughout the larval stages and subsequently decrease during the pupal stages (Kanost *et al.*, 1990). They are synthesized by the fat body, initially released to the haemolymph and later taken back up by the fat body just prior to pupation to be stored as protein granules (Kanost *et al.*, 1990). There are three main classes of storage proteins: 1) methionine-rich storage proteins, which are generally homohexamers (storage proteins that dissociate to six subunits of the same molecular weight) and high in methionine (Kanost *et al.*, 1990); 2) female-specific storage proteins, which are often high in methionine and always more abundant in females than males (Telfer and Kunkel, 1991); and, 3) the arylphorins, which are high in aromatic amino acids, generally heterohexamers (storage proteins that dissociate to six subunits of different molecular weights) and often the most abundant storage protein in insects (Telfer *et al.*, 1983).

Storage proteins are believed to serve as a reservoir for amino acids required during metamorphosis and possibly as an aromatic amino acid reserve for sclerotization (see Kanost *et al.*, 1990; Levenbook, 1985, and Telfer and Kunkel, 1991 for reviews). Both hormonal and nutritional factors are involved in storage protein regulation (Kanost *et al.*, 1990; Webb and Riddiford, 1988a; Tojo *et al.*, 1985). The mechanism for hormonal regulation varies within Lepidoptera. For example, in *B. mori*, juvenile hormone (JH) appears to inhibit fat-body synthesis of an arylphorin-like storage protein (Tojo *et al.*, 1981) but, in *Spodoptera litura*, JH has no effect on the synthesis of the arylphorin-like storage protein (Tojo *et al.*, 1985). Nutritional regulation also differs within this order. Starvation results in a decrease in storage protein concentration in the haemolymph of *M. sexta* (Webb and Riddiford, 1988a) whereas in *B. mori*, the relative storage protein concentration in the haemolymph does not decline with starvation (Nagata and Kobayashi, 1990a).

1.2 Nutritional Ecology

1.2.1 Critical Weights

In many lepidopterans, a threshold, species-specific size or weight must be attained before pupation can occur and produce a viable, reproductive adult (Nijhout, 1975; Ochieng'-Odero, 1990). These thresholds or critical weights, have been identified in a number of insect species (*M. sexta*, Nijhout, 1975; *S. litura*, Morita and Tojo, 1985; *Cnephasia jactatana*, Ochieng'-Odero, 1990), and in some species they differ between the sexes (Ochieng'-Odero, 1990).

Several studies have suggested a direct relationship between insect weight and storage protein concentration. Karpells *et al.* (1990) suggested that weight may be a factor affecting storage protein concentrations in larval haemolymph and intact insects when he found that *Lymantria dispar* females, which have a supernumerary instar, gain more weight and have substantially greater storage protein concentrations than males. Nagata and Kobayashi (1990a) found that *B. mori* fed nutrient poor diets had the lowest levels of storage protein and lowest larval weights while those fed the nutrient rich diets had the highest levels of storage protein and highest weights. Chippendale and Kilby (1969) studied total protein in the fat body of *Pieris brassicae* and found that total protein increased during the last larval instar along with an increase in fat-body dry weight. Since storage proteins are generally the most abundant protein in insects during the last instar (Levenbook, 1985), Chippendale and Kilby's (1969) results also suggest a relationship between larval fat body weight and storage proteins.

1.2.2 Nutritional Studies

Decreasing protein quality and quantity in insect diets adversely affects the insects' growth and development (Broadway and Duffey, 1986; Nagata and Kobayashi, 1990a; Slansky and Wheeler, 1992). Researchers have found that when protein quantity or quality is reduced, insects compensate by consuming more food and remaining in the larval stages longer to obtain sufficient nutrients for growth and development (see Slansky and Scriber, 1985; Simpson and Simpson, 1990; and Slansky, 1993 for reviews). An important ramification of this compensatory behaviour is the potential for increased damage to crops as more food is consumed by larvae over a longer period of time. Both nutritional ecology and physiological studies have used nutritional indices to help explain changes in insect growth rate and development. Two of these indices are approximate digestibility (AD) which estimates the percentage of ingested food that is digested and assimilated and the efficiency of conversion of digested food to biomass (ECD).

1.3 Insect of Study

The European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera:Pyralidae), obtains its adult protein supply as a larva; therefore, proteins ingested during the larval stages should be important to metamorphose and reproduce successfully. As a source of amino acids for metamorphosis, storage proteins should be critical for corn borer development. Although storage proteins have been identified, and nutritional studies involving compensation have been done on other lepidopteran species, these have not been studied in the corn borer. This is possibly due to the corn borer's small size, difficulty in obtaining substantial amounts of haemolymph and identification of the beginning of a moult. The age of an insect is generally identified by head capsule slippage at each moult, however, because the corn borer burrows into its food, the head capsule is difficult to see. The gypsy moth, *L. dispar*, and the tobacco horn worm, *M. sexta*, are often used for the types of studies I have done because they are much larger and easier to handle. However, the corn borer causes a great deal of damage to corn each year and a laboratory colony is relatively easy to grow. Nutritional and physiological studies may help in understanding ways to decrease crop damage by this pest because a basic understanding of the factors affecting feeding, growth and mortality is necessary before attempting to develop an integrated pest management strategy (Slansky and Scriber, 1985). By using an artificial diet and a laboratory colony, we can do many experiments easily and alter one nutrient without affecting other nutrients. Possibly some of the results can be applied to the field.

1.4 Hypothesis and Objectives

My hypothesis is that storage proteins in the European corn borer are an important factor determining the onset of pupation and becoming a viable adult (successful metamorphosis) and that insect weight is an indicator of storage protein levels and thereby the onset of pupation and success of metamorphosis.

My first objective was to identify and characterize the storage protein in the corn borer and follow the normal pattern of storage protein levels throughout the final instar to the adult stage. The normal pattern was defined here as that which occurs on the standard artificial diet. If insect weight is an indicator of storage protein levels, I predicted that as the insect's weight increased there would be an increase in the levels of storage protein in the fat body and intact larvae up to pupation. The storage protein in the haemolymph should show a pattern of increasing to just before pupation with a subsequent decrease brought on by the uptake of the storage protein by the fat body.

My second objective was to examine nutritional effects by altering the protein quality and quantity in the insect's artificial diet in an attempt to affect development events by reducing storage protein levels and growth in the corn borer. Nutritional indices were calculated to determine if the insects compensated for these dietary changes by altering their digestive efficiency. I defined protein quality by each protein's percentage amino acid composition (Appendix I) where protein quantity involved increasing or decreasing one specific protein. I observed the insects throughout the final instar to see how many pupated on the differing diets and how larval duration was affected. I predicted that if levels of storage protein or insect weight affected the onset of pupation or adult eclosion, those insects on diets poorer either in quality or quantity would have longer development times as they attempted to build up sufficient levels of storage protein or attempted to obtain sufficient weights to allow pupation to occur. I predicted that if successful metamorphosis was affected by levels of storage protein or insect weight that fewer insects would pupate and mortality would be higher on the poorer diets than for those insects fed the standard diet and a greater number of heavier pupae should develop to viable adults than lighter ones.

Since these predictions rest on the assumption that storage protein levels are lower when protein quality of the diet is reduced, my third objective was to quantify the levels of storage proteins in insects feeding on the diets differing in protein quality. I predicted that those insects fed the poorer quality diets would have lower levels of storage protein when compared to those insects of similar weights on the standard diet.

Chapter 2 Materials and Methods

2.1 Insects

2.1.1 Colony Maintenance

Insects were reared in a laboratory under a 16 h light:8 h dark photoperiod at 25 °C and 80% relative humidity. Experimental insects were obtained from egg masses, from a stock laboratory colony originally supplied by M. Hudon (Agriculture Canada, St. Jean, Quebec) and G. McLeod (Agriculture Canada, London, Ontario). The standard diet was prepared according to Guthrie *et al.*, 1985: wheat germ 28%, dextrose 21.6%, casein 28.7%, cholesterol 1.7%, Wesson Salts 7.8%, Vander Adk Vitamins 5%, ascorbic acid 6.5%, Aureomycin 1.5% and sorbic acid 4.3%. Agar, Fumidil B, formaldehyde, a propionic acid and phosphoric acid solution and methyl paraben solution were added to the diet. The water content in the diet is 85%. Female adults were distinguished from male adults by the shape of the tip of their abdomen -- the tip of the female's abdomen is rounded while that of the male is pointed.

2.2 Biochemical Techniques

2.2.1 Sample Collection and Preparations

Egg masses were removed from the laboratory colony and placed on a standard artificial diet in small (250 ml) rearing jars. Jar lids were covered with mesh to allow air into the containers. In a separate experiment egg masses were placed in large rearing tubs containing standard artificial diet. Approximately three weeks later, larvae were collected as follows. Larvae from the small jars were collected at weight intervals of 10 mg beginning with 15-25 mg up to 115 mg. Any larvae greater than 115 mg were combined into one size class denoted >115 mg. Larvae between 15 and 22 mg from the large rearing tubs were collected and each placed in an individually labeled 5 ml vial containing standard artificial diet. The vials were plugged with a piece of cotton. Over the next few days the date of head capsule slippage was recorded for each insect. Larvae were collected from day 1 (day of head capsule slippage) to day 10 or 11. Intact pupae (after cuticle hardened - about 48 hours after pupating) and adults from both experiments were also collected and weighed. These experiments were repeated once.

At least 15 individual larvae from each weight interval or day were collected for total protein determination. Pools of insects from each weight interval or day were collected for storage protein determination. Depending on insect size, the number of insects in each pool ranged from 2 to 20 individuals. I weighed the larvae and either placed them intact in 1.5 ml disposable plastic microcentrifuge tubes or kept them on ice for collection of haemolymph and fat body. Intact weighed pupae, adult individuals, and pools of intact pupae and adults were also collected in 1.5 ml disposable plastic microcentrifuge tubes. Samples of hemolymph, fat body with carcass attached and intact larvae, pupae and adults were stored at -20 °C for later protein determination.

2.2.1.1 Haemolymph

Between 1-2 µl (for individuals) or 10-20 µl (for pools) of larval haemolymph was collected by puncturing a proleg and expressing the haemolymph into calibrated microcapillary tubes. The haemolymph was added to 0.15 M NaCl containing a few crystals of 1-phenyl-2-thiourea (PTU) to prevent melanization. It was not possible to collect pupal haemolymph that was not contaminated by fat body. Haemolymph samples were centrifuged at 15,000 g for 10 min. at 4 °C.

2.2.1.2 Fat Body

To determine fat-body protein, I removed the guts and salivary glands of the insects used for haemolymph collection, and rinsed the abdominal cavity with 0.15 M NaCl to eliminate any remaining haemolymph or gut contents. Thawed fat-body samples were homogenized in 0.3 ml of 0.15 M NaCl, rinsed with a further 0.3 ml of 0.15 M NaCl and centrifuged at 15,000 g for 10 min. at 4 °C. For samples from insects that weighed <30 mg (or <day 2) I removed the supernatant and adjusted it to 1 ml. All supernatants were adjusted using 0.15 M NaCl. I rehomogenized and recentrifuged samples >30 mg (or >day 2) as above. Supernatants were pooled, adjusted to 2 ml and vortexed. I removed 1 ml of the supernatant from all samples and centrifuged the supernatants. An aliquot from below the fat layer of the centrifuged supernatant was removed for protein determination.

2.2.1.3 Whole Bodied Insects

Intact larval samples were prepared as for fat body with several changes. A few crystals of PTU were added to these samples when homogenizing. Samples from insects weighing >65 mg (or >day 5) were homogenized and centrifuged a third time. I pooled all resulting supernatants and adjusted to 4 ml samples of insects between 65-80 mg (or day 5 and 6), while those samples from insects >80 mg (or >day 6) were adjusted to 5 ml.

2.2.1.4 Ovary Collection

I removed 4 ovaries from large, female final instar larvae and placed them in 0.3 ml of 0.15 M NaCl. Thawed ovaries were homogenized in the 0.3 ml of 0.15 M NaCl and centrifuged at 15,000 g for 10 min. at 4 °C.

2.2.2 Electrophoresis

Samples for gel electrophoresis were prepared in the same way as for total protein determination (see above). Further dilutions were made where necessary to prevent overloading of samples on the gels. Using the method of Laemmli (1970) I carried out SDS-PAGE with a Bio-Rad Mini-Protean II dual slab electrophoresis apparatus, Bio-Rad reagents and a 4% stacking gel (4% (w/v) acrylamide with 0.125 M Tris, pH 6.8 and 0.1% (w/v) SDS) and a 7.5% separating gel (7.5% (w/v) acrylamide with 0.375 M Tris, pH 8.8 and 0.1% (w/v) SDS). Each lane in the gel contained a constant amount of protein with 2, 3 and 10 µg of total protein for haemolymph, intact insect and ovary, and fat-body samples respectively. Gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250 in 1:4:5 acetic acid:methonal:water and left overnight. Destaining was done using 200 ml of a 10% acetic acid and 40% methanol solution.

2.2.3 Protein Determination

The method of Bramhall *et al.* (1969) was used to determine the amount of total protein, using bovine serum albumin (fraction V; Sigma, St. Louis, MO) as a standard. I determined the efficiency of extracting all soluble protein from the insects using a procedure similar to that for fat body and whole body collection and preparation, however, supernatants were not pooled and each extraction was measured for the amount of total protein.

2.2.4 Densitometry

Densities of putative storage protein bands (protein doublet) were calculated using an LKB Ultrascan XL enhanced laser densitometer. The range of linearity was determined using a gel loaded with high and low standards and various concentrations of bovine serum albumin. All parameters were kept constant for each gel read to eliminate as much as possible any background 'noise' in the samples. Preliminary gels showed a third band, in the region of the protein doublet thought to be storage protein, occurring late in development. Although the third band may be an arylphorin-immunoreactive band (Webb and Riddiford, 1988b) or a homohexamer of another class of storage protein (Webb and Riddiford, 1988a; Tojo and Yoshiga, 1994), it is not consistent throughout the development period studied. Therefore, all analyses related to storage protein levels are based on the two consistent bands only.

2.3 Nutritional Experiments

2.3.1 Sample Preparation

Egg masses were placed in rearing containers containing standard artificial diet (prepared according to Guthrie *et al.*, 1985). Approximately three weeks later, individual late fourth/early fifth instar larvae, weighing between 20 and 25 mg were each placed in a 10 ml vial containing one of 10 different diets. In a second experiment, insects were reared as per section 2.2.1 with egg masses placed on one of four diets differing in protein quality, and insects being collected based on the weight intervals only.

2.3.2 Altering Protein Quality and Quantity in Diets

The artificial diet containing casein was used as the standard diet. To study the effects of protein quality, casein was removed from the standard diet and one of the following proteins: albumin, gluten, zein or denatured haemoglobin was added. Wheat germ in the standard diet contains about 4% protein. Therefore, the protein content of each of these diets was 21.5% by dry weight. A diet containing 8.0% protein by dry weight was then made up containing each of the proteins above (casein, albumin, gluten, zein or haemoglobin). A total of 550 insects was used: 60 insects for each 8.0% diet and 50 insects for each 21.5% diet. This unbalanced design was used to try to adjust for the higher insect mortality on nutrient-poor diets generally found in similar studies. The four diets used for the second experiment were all 21.5% by dry weight and consisted of two animal proteins, casein and haemoglobin and two plant proteins, gluten and zein.

2.3.3 Observations and Collection of Food and Insects

Insects were observed daily and I recorded time to pupation, pupal duration, pupal weight, pupal condition (e.g., larval-pupal intermediate), date of adult emergence, sex of the emerging adult, weight of food in the individual vials at the beginning of the experiment and final weight of food after insects had pupated, weight of frass produced and mortality. Forty-eight hours after pupation, pupae were removed from their vials and placed individually in a clean 10 ml vial. Food and frass remaining in the rearing vial were separated and placed in 5 ml plastic cups. To remove all the frass, frass remaining in the rearing vial was rinsed twice with 0.75 ml of distilled water and added to the 5 ml cup containing the frass. Food and frass were oven dried at 50°C for 48 hours and then weighed. A standard curve was used to estimate the initial dry weight of the food and final pupal weights. The standard curve used to convert all pupal weights was calculated using insects fed the standard artificial diet. For insects that were slow to pupate, fresh diet was added every ten days. A Sartorius MC 1 balance was used to make all gravimetric measurements (accurate to ±0.001 g).

Insects for the second experiment were collected as per section 2.2.1.3 and electrophoresis and protein determination were determined as per sections 2.2.2 and 2.2.3 respectively.

2.4 Statistical Analyses

Where necessary the natural logarithm was used to transform the raw data to meet the assumptions of the particular statistical test used. An alpha of 0.05 was used in all analyses unless otherwise stated below. Only those insects that survived to the adult stage were used in the analyses except in the analysis of mortality rates. I used regression analysis to examine the relationship between total protein and age or weight and protein doublet levels and age or weight. Where the relationships were linear, simple regression analysis was used. Where relationships were non-linear, polynomial regressions were used. In the case of haemolymph total protein, a non-linear model was fit to the data.

T-tests were used to test for sex differences in the amount of food consumed, larval duration and pupal weights. Four two-way ANOVAs (analysis of variance) were performed to determine if protein quality (casein, albumin, gluten and zein) and protein quantity (21.5% or 8.0%) significantly affected dry weight of food consumed, larval duration, dry pupal weights or indices. The indices calculated were approximate digestibility (AD=[food ingested-frass produced]/food ingested) and efficiency of converting digested food to biomass (ECD=final insect weight/food digested). Because three dependent variables were examined using separate ANOVAs in the case of food consumed, larval duration and dry pupal weights, the alpha was adjusted as per Bonferroni (alpha=0.016). The same was done for AD and ECD (alpha=0.02). Initial weights of insects were kept in the range of 20-25 mg, however the initial weights were not quantified further so that only final weight, not total growth, was used to calculate ECD. A two-way ANOVA was run comparing weights of pupae that survived versus those that did not, grouped according to both quantity and quality of the diet. The Scheffé's multiple comparison test was used for all ANOVAs as the post hoc test because it adjusts for unequal sample sizes and allows for comparisons between all diets. To determine if there was a threshold pupal weight, a logistic regression analysis was used. A contingency table analysis using a log-linear model was performed to see if mortality was independent of either protein quality or protein quantity.

ANCOVAs were used to compare the regression lines of either the amount of total protein or protein doublet levels against larval weight for each diet type. The use of ANCOVA allows testing for the effects of diet type (nutrition) on total protein and protein doublet levels after correcting for the weight of the larvae.

Chapter 3 The relationship between larval weight, larval age, total protein and storage protein levels in the European corn borer, *Ostrinia nubilalis* (L.)

3.1 Introduction

Insect storage proteins are large (~500 kDa) haemolymph proteins that attain high concentrations throughout the larval stages (up to 80% of the total protein; Kramer *et al.*, 1980) and subsequently decrease during the pupal stages (Kanost *et al.*, 1990). Storage proteins are synthesized by the fat body, initially released to the haemolymph and later taken back up by the fat body just prior to pupation to be stored as protein granules (Kanost *et al.*, 1990). There are three main classes of storage proteins: 1) methionine-rich storage proteins, which are generally homohexamers and high in methionine (Kanost *et al.*, 1990); 2) female-specific storage proteins, which are often high in methionine and always more abundant in females than males (Telfer and Kunkel, 1991); and, 3) the arylphorins, which are high in aromatic amino acids, generally heterohexamers and often the most abundant storage protein in insects (Telfer *et al.*, 1983).

Storage proteins are believed to serve as a reservoir for amino acids required during metamorphosis and possibly as an aromatic amino acid reserve for sclerotization (see Kanost *et al.*, 1990; Levenbook, 1985, and Telfer and Kunkel, 1991 for reviews). Both hormonal and nutritional factors are involved in storage protein regulation (Kanost *et al.*, 1990; Webb and Riddiford, 1988a; Tojo *et al.*, 1985). The mechanism for hormonal regulation varies within Lepidoptera. For example, in *B. mori*, juvenile hormone (JH) appears to inhibit fat-body synthesis of an arylphorin-like storage protein (Tojo *et al.*, 1981) but, in *S. litura*, JH has no effect on the synthesis of the arylphorin-like storage protein (Tojo *et al.*, 1985). Nutritional regulation also differs within this order. Starvation results in a decrease in storage protein concentration in the haemolymph of *M. sexta* (Webb and Riddiford, 1988a) whereas in *B. mori*, the relative storage protein concentration in the haemolymph does not decline with starvation (Nagata and Kobayashi, 1990a).

In the haemolymph of larval lepidopterans, both total and storage protein levels tend to increase with age and then decrease just before pupation (Levenbook and Bauer, 1980; Chippendale and Kilby, 1969). As larval lepidopterans age, they undergo considerable changes in body size and weight (Reavey, 1993). For example, there is an increase in larval body weight during the last instar of *M. sexta* followed by a decline just prior to pupation (Hayes *et al.*, 1992).

I suspect larval weight may be related to protein levels because of the observed relationships between insect weight and the amount of total protein (Chippendale and Kilby, 1969; Miller and Silhacek, 1982) and insect weight and storage protein concentrations (Karpells *et al.*, 1990). The literature suggests that the relationship between larval age and total protein in the fat body (Chippendale and Kilby, 1969) and intact larvae (Levenbook and Bauer, 1980) is an increasing function.

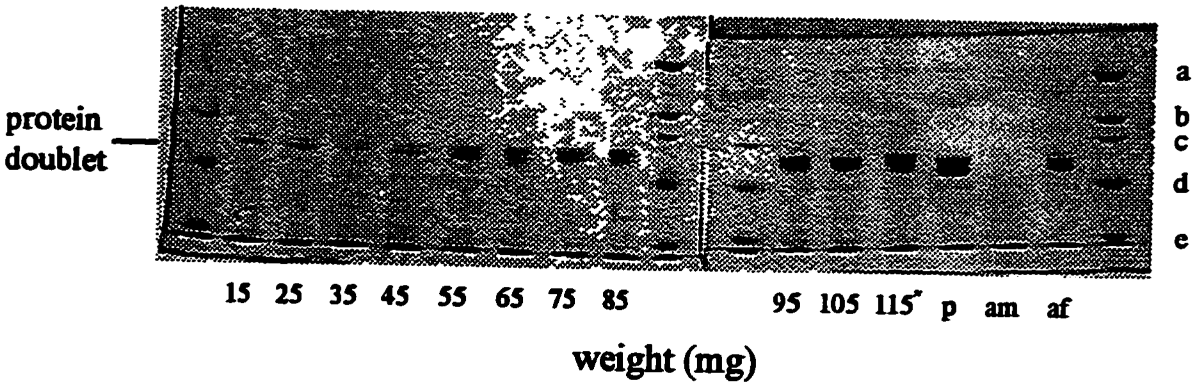
In this study I characterize the major storage protein in the European corn borer, *O. nubilalis*. Both total protein and storage protein levels are usually examined over an insect's age. Identifying the age of a corn borer is problematic because they burrow into their food which makes it difficult to observe head capsule slippage (used as a starting point for age determination). However, it is much easier to determine the weight of an insect than the age. I therefore examine the relationship between larval weight and larval age. This relationship will likely show an increase in weight with age until just prior to pupation where there will be a decrease. I then examine both the relationship between total protein and larval weight and total protein and larval age of this insect. I also examine the relationship between storage protein levels and larval weight and larval age. Although it is unlikely that either larval weight or larval age directly cause changes in either total protein or storage protein levels, I hypothesize that larval weight and larval age will be indicators of both the levels of total protein and storage protein in the corn borer. I predict that larval weight can be used to follow protein levels over a development period in place of age where determining the age of the insect is difficult. Since nutrition has been shown to be related to storage protein levels, I also examine the relationship between dietary nutrition, larval weight and the amount of total protein and storage protein levels. As the quality of the diet decreases, it is expected that larval weight, storage protein levels and amounts of total protein will also decline.

3.2 Results

I extracted on average 94% of the total soluble protein from intact larvae and fat bodies (Appendix II). Column chromatography shows the protein doublet (the putative storage protein) has a native molecular weight of 460 kDa (unpublished results). Using SDS-PAGE, it dissociates into 2 bands with subunit weights of 81 and 87 kDa (Figure 1a). A third band appears late in the final instar in this insect (Figure 1a). Gel staining densities show increasing levels of the protein doublet in intact insects in the last larval instar with a diminishing of the protein doublet with adult development (Figure 1a,b). The 3-day-old adult male shows no existence of the protein doublet, however in the 3-day-old female there appears to be some level of the protein doublet (Figure 1a,b). An egg protein doublet overlaps the protein doublet (the putative storage protein) in female adults (Figure 2). In the larval haemolymph, the protein doublet accounts for up to 80% of the total protein (Figure 3a,b). The maximum levels reached were between the 5th and 7th day after head capsule slippage (Figure 3a,b).

When larval weight was considered as a function of age, a significant 3rd order polynomial regression was found to explain the relationship (Figure 4) and when the amount of total protein is regressed against age (Figure 5a) in intact larvae. A significant positive regression is found when total protein in the fat body is plotted against larval age

Figure 1. (a) SDS-PAGE and (b) plot showing protein doublet profile throughout the final instar in intact European corn borer larvae, pupa and adults. Each lane in (a) has 3 μg of protein. Protein doublet levels for (b) were estimated using densitometric scanning and determined as the area under the curve of the corresponding gels; in this case, the gel read in (a). Molecular weight markers for (a) are: a, myosin (M_r 220,000); b, β-galactosidase (M_r 116,250); c, phosphorylase b (M_r 92,500); d, bovine serum albumin (M_r 66,200); and e, ovalbumin (M_r 45,000). (p=pupa, am=3 day old adult male, af=3 day old adult female). The larval weights shown in (a) are intervals of 10 mg; for example, 15 indicates an interval of 15-25 mg. *115 includes any insects >115 mg.


protein doublet
a
b
c
d
e
15
25
35
45
55
65
75
85
95
105
115*
p
am
af
weight (mg)

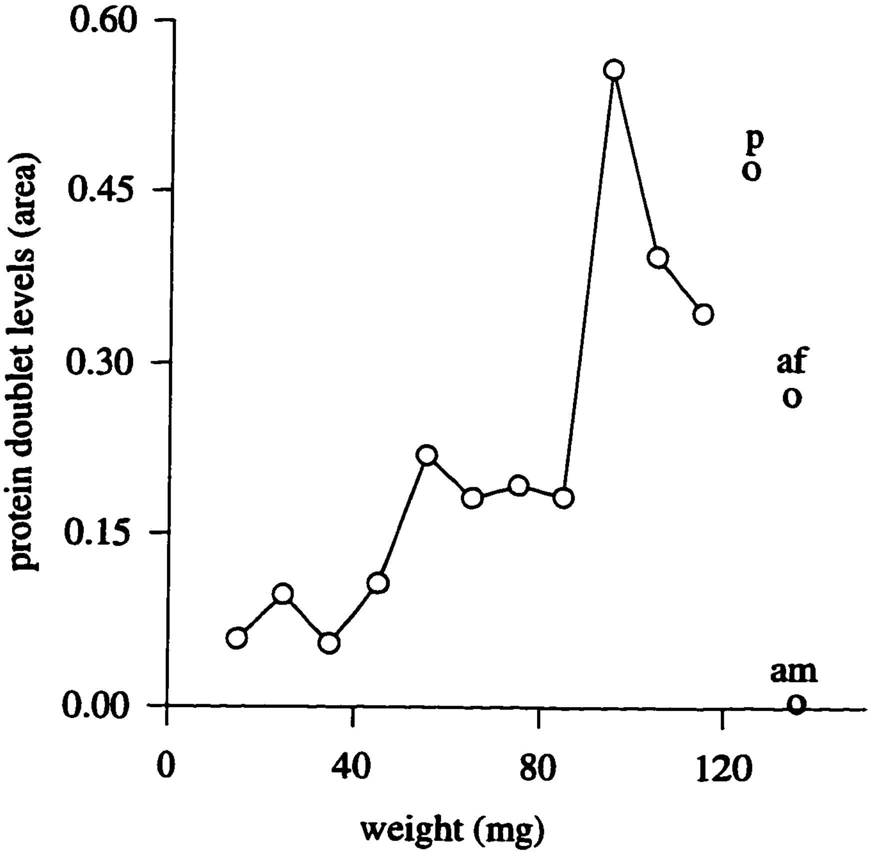

protein doublet levels (area)
0.60
0.45
0.30
0.15
0.00
0
40
80
120
weight (mg)
p
af
am

Figure 2. SDS-PAGE of protein doublet levels for intact last instar larvae (lanes 9, 10) and female adults (lanes 1a - 5a) of the European corn borer. Lane A contains crude egg extract collected from 10 day old female larvae. Each lane contains 3 µg of protein. Note the arrow in lane A indicating an egg protein doublet with a similar molecular weight to that of the protein doublet of intact larvae and female adults. Protein doublet levels for (b) were estimated using densitometric scanning and determined as the area under the curve of the corresponding gels; in this case, the gel read in (a). Molecular weight markers for (a) are: a, myosin (M_r 220,000); b, β-galactosidase (M_r 116,250); c, phosphorylase b (M_r 92,500); d, bovine serum albumin (M_r 66,200); and e, ovalbumin (M_r 45,000). For larvae, the day 1 would be the day of head capsule slippage into the final instar, so day 9 is 9 days after head capsule slippage. For adults, day 1a is the day of adult emergence and day 2a is the day after the day of adult emergence, etc.

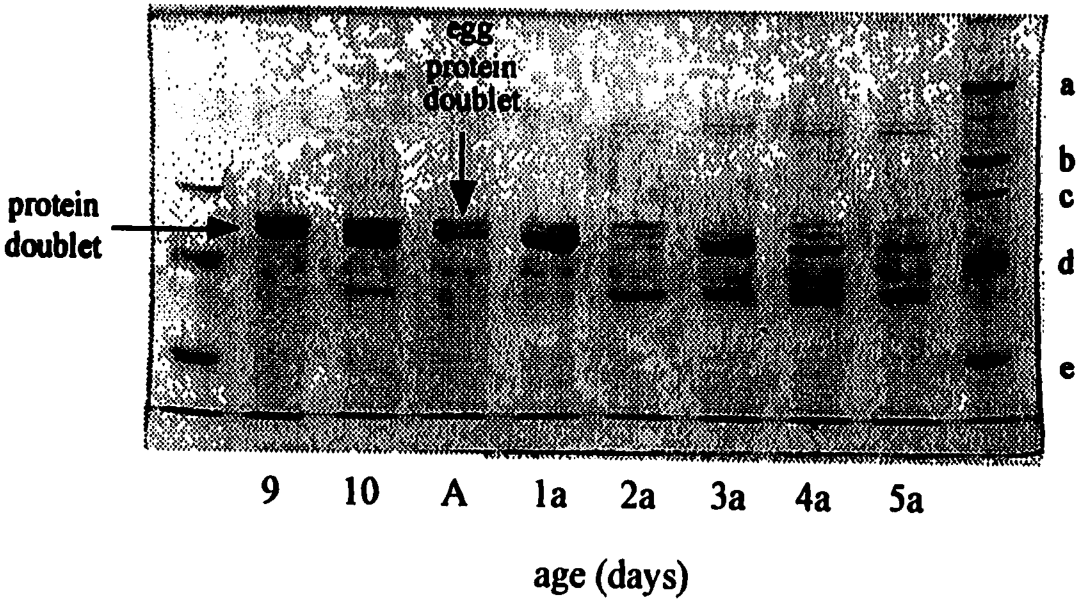
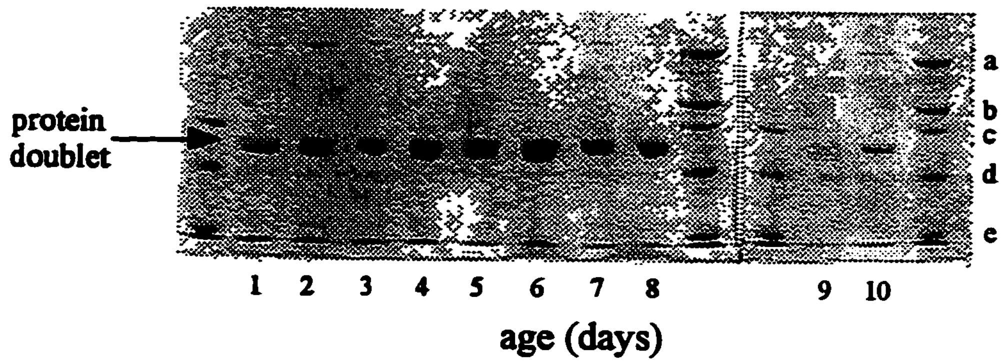

protein doublet
egg protein doublet
a
b
c
d
e
9
10
A
1a
2a
3a
4a
5a
age (days)

Figure 3. (a) SDS-PAGE and (b) plot showing percent of total protein accounted for by the protein doublet in the haemolymph of the European corn borer during the final instar. Each lane in (a) has 2 μg of protein. Protein doublet levels for (b) were estimated using densitometric scanning and determined as the area under the curve of the corresponding gels; in this case, the gel read in (a). Molecular weight markers for (a) are: a, myosin (M_r 220,000); b, β-galactosidase (M_r 116,250); c, phosphorylase b (M_r 92,500); d, bovine serum albumin (M_r 66,200); and e, ovalbumin (M_r 45,000). For larvae, the day 1 would be the day of head capsule slippage into the final instar, so day 2 is the day after head capsule slippage, etc.


protein doublet
a
b
c
d
e
1 2 3 4 5 6 7 8 9 10
age (days)

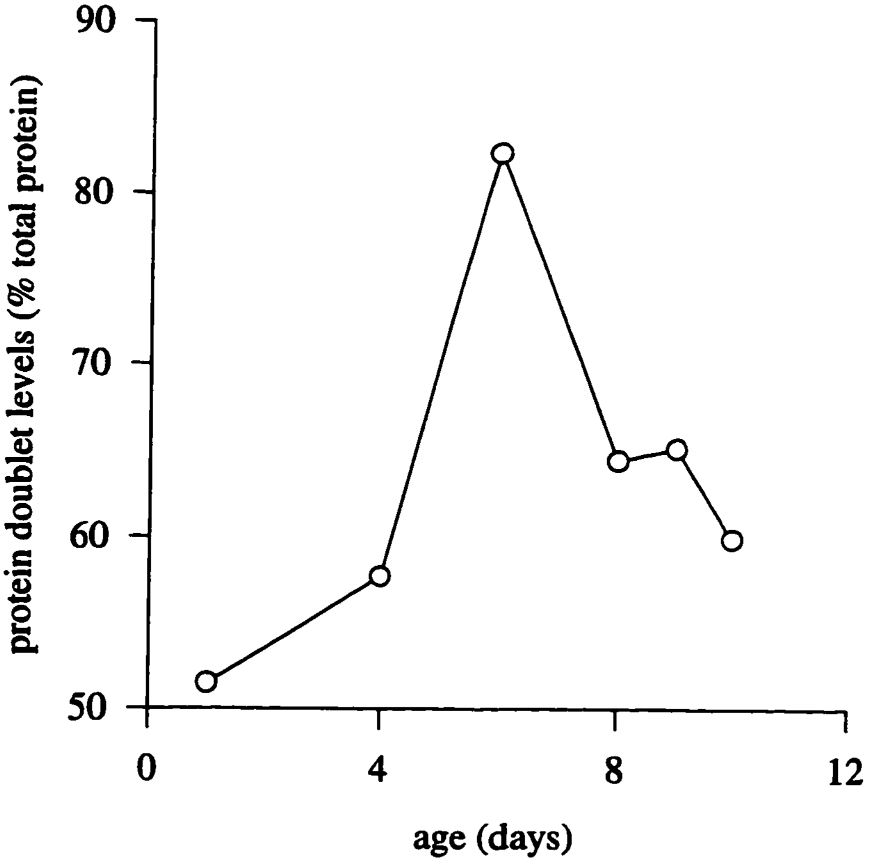

protein doublet levels (% total protein)
90
80
70
60
50
0
4
8
12
age (days)

Figure 4. The relationship between larval weight and larval age in intact European corn borers. The maximum weight occurs on day 8 at a ln(weight) of 4.25 mg. 95% confidence intervals are plotted. (r^2=0.8, F=383, p<0.001, residual mean square error=0.1, n=142)

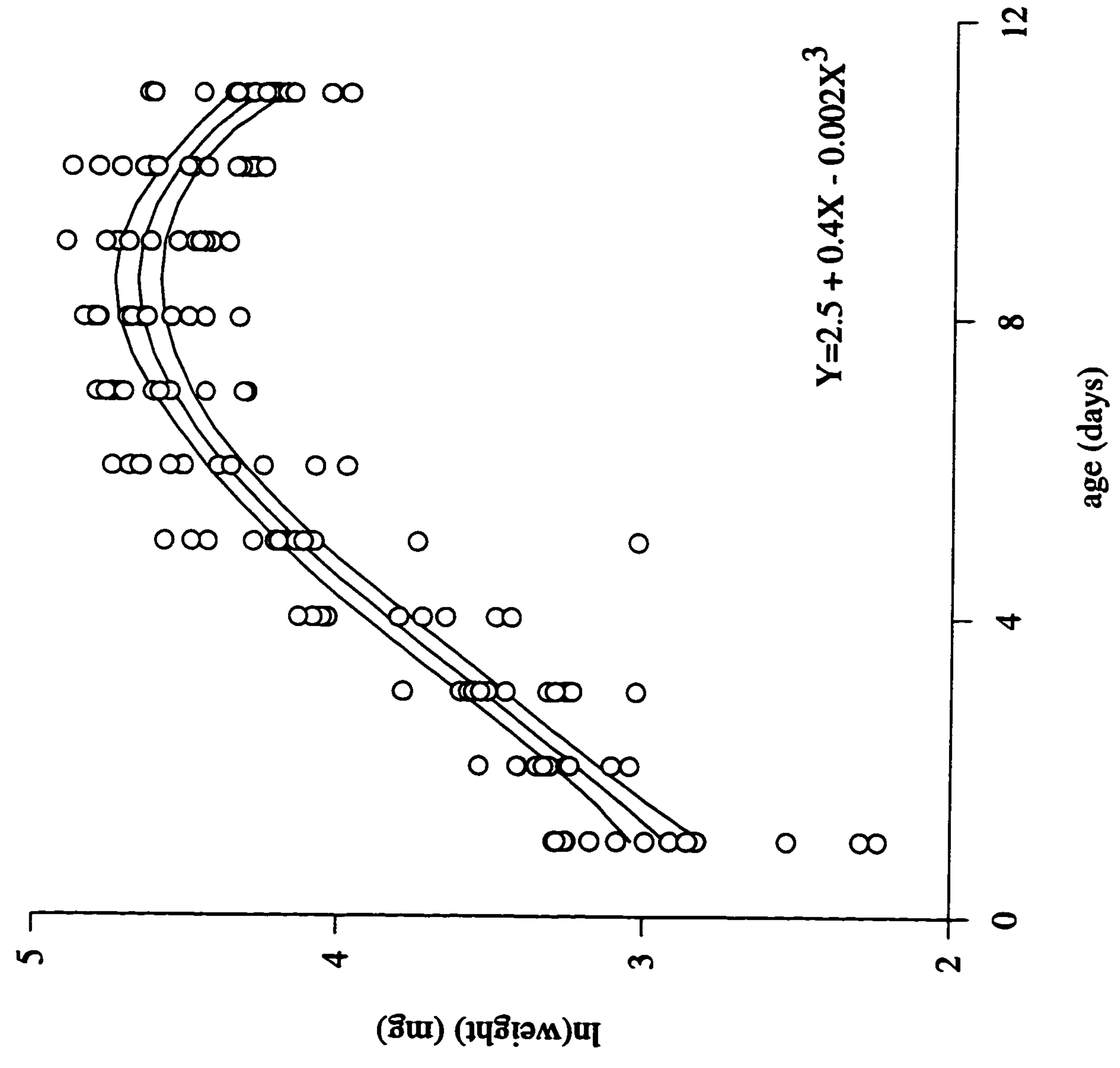
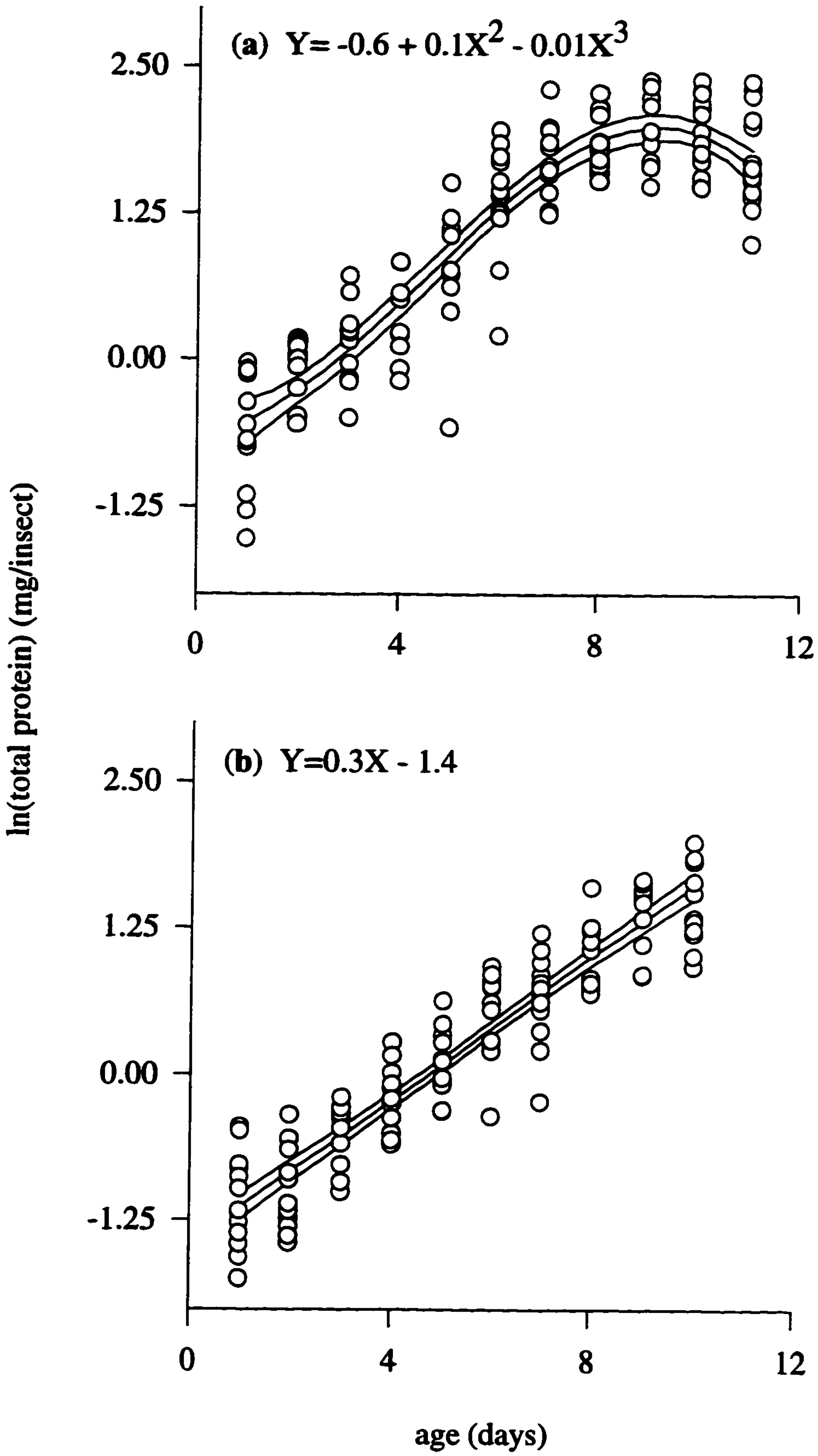

ln(weight) (mg)
2
3
4
5
0
4
8
12
age (days)
Y=2.5 + 0.4X - 0.002X^3

Figure 5. The effect of larval age on the amount of total protein in (a) intact larvae and (b) fat bodies in the European corn borer. In (a) the maximum amount of ln(total protein) is 4.8 mg/insect and occurs on day 9 in (a). 95% confidence intervals are plotted. ($r^2=0.8$, $F=384$, $p<0.001$, residual mean square error=0.1, $n=142$ for intact larvae (a); $r^2=0.9$, $F=829$, $p<0.001$, residual mean square error=0.1, $n=121$ for fat bodies (b))


(a) $Y = -0.6 + 0.1X^2 - 0.01X^3$
2.50
1.25
0.00
-1.25
0
4
8
12
(b) $Y = 0.3X - 1.4$
2.50
1.25
0.00
-1.25
0
4
8
12
ln(total protein) (mg/insect)
age (days)

(Figure 5b). When the amount of total protein is regressed against weight, I find there is a significant positive regression in intact larvae and fat bodies (Figure 6a,b).

Haemolymph total protein concentration shows a significant 3rd order polynomial regression when total protein concentration is plotted against larval age (Figure 7). Visual examination of the plot of total protein concentration in the haemolymph versus larval weight suggests that there are two distinct linear relationships (Figure 8). To objectively determine the larval weight at which these two linear relationships change from one to the other, a piecewise regression (Wilkinson *et al.*, 1992a) was fitted to the data. Based on the piecewise regression, it was determined that there were two separate linear relationships, one in the region of larval weights ^{2}58 mg and the other where larval weights were >58 mg (Figure 8).

When the protein doublet levels are regressed against larval weight, I find there is a significant positive regression in intact larvae and fat bodies (Figure 9a, b). Similar results are found when protein doublet levels are plotted against larval age (Figure 10a, b). The putative storage protein in the haemolymph shows a pattern of increasing with insect weight in the last larval instar to about 75-85 mg with a corresponding decrease just before pupation, however, the second-order regression term is not significant (t= -1.57, p=0.16; Figure 11a,b) and this is likely due to the low values of haemolymph storage protein found at 25-35 mg and 55-65 mg.

Figure 6. The effect of larval weight on the amount of total protein in (a) intact larvae and (b) fat bodies in the European corn borer. 95% confidence intervals are plotted. (r^2=0.9, F=2285, p<0.001, residual mean square error=0.1, n=187 for intact larvae (a); r^2=0.9, F=2115, p<0.001, residual mean square error=0.1, n=165 for fat bodies (b))

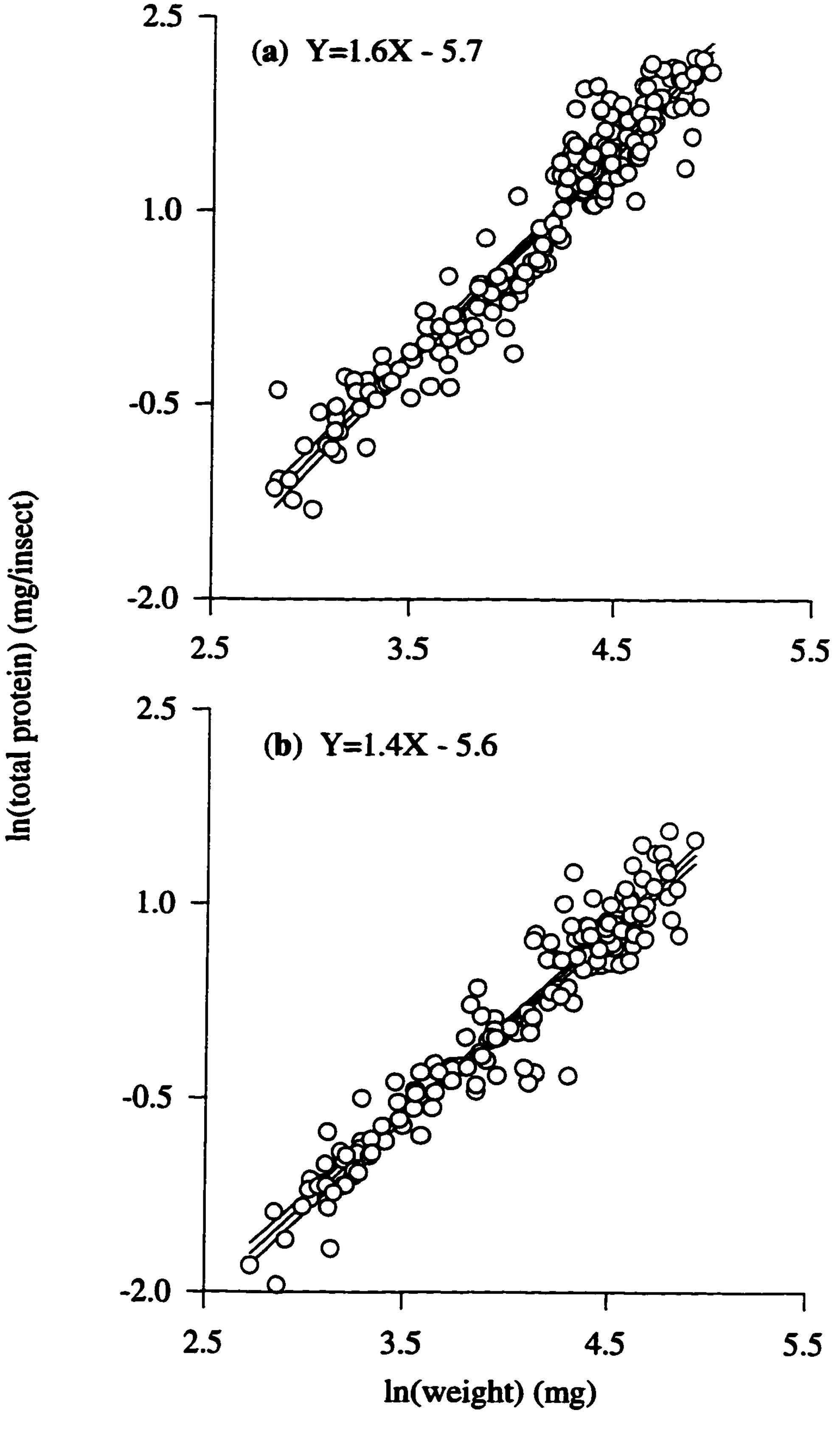

ln(total protein) (mg/insect)
(a) Y=1.6X - 5.7
2.5
1.0
-0.5
-2.0
2.5
3.5
4.5
5.5
(b) Y=1.4X - 5.6
2.5
1.0
-0.5
-2.0
2.5
3.5
4.5
5.5
ln(weight) (mg)

Figure 7. The effect of larval age on total protein concentrations in the haemolymph of the European corn borer. The maximum concentration of ln(total protein concentration) is at 5.0 mg/ml and occurs on day 8.5. 95% confidence intervals are plotted. ($r^2=0.6$, $F=156$, $p<0.001$, residual mean square error=0.4, n=169)

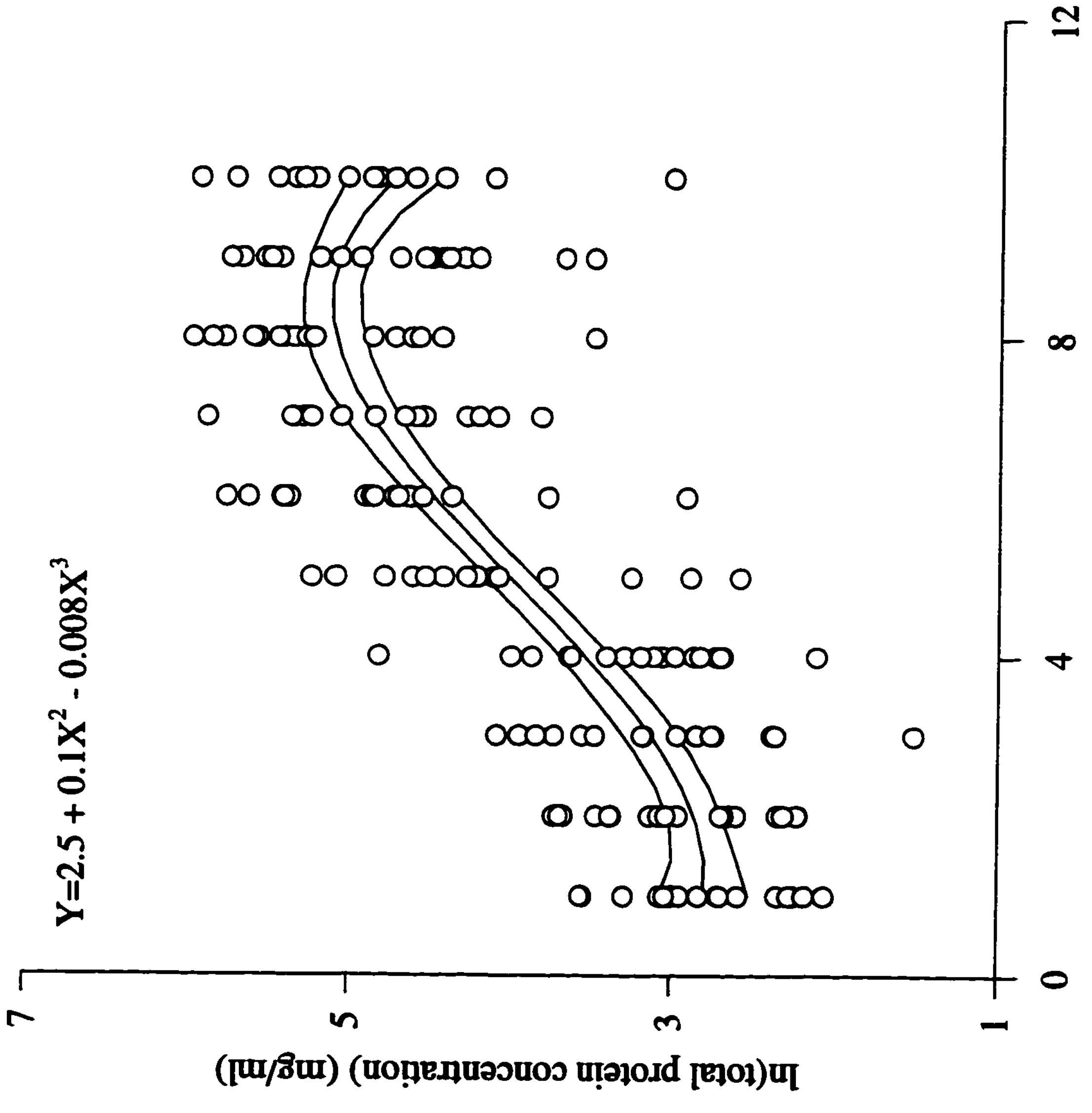

Y=2.5 + 0.1X^2 - 0.008X^3
ln(total protein concentration) (mg/ml)
7
5
3
1
0
4
8
12
age (days)

Figure 8. The effect of larval weight on total protein concentrations in the haemolymph of the European corn borer. A non-linear, piecewise regression model was fit to the data (r^2=0.7).

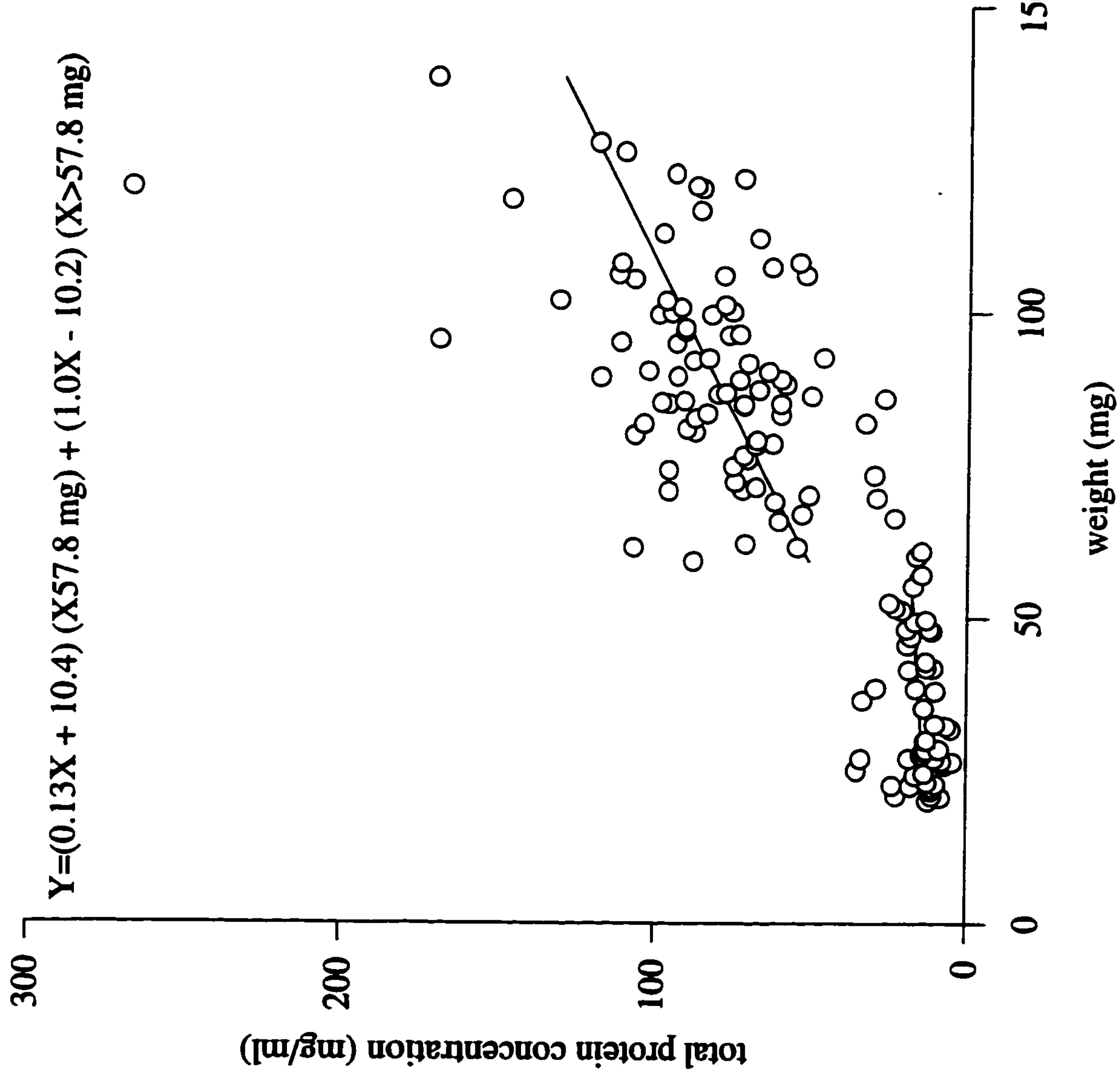

Y=(0.13X + 10.4) (X57.8 mg) + (1.0X - 10.2) (X>57.8 mg)
total protein concentration (mg/ml)
300
200
100
0
0
50
100
150
weight (mg)

Figure 9. The effect of larval weight on protein doublet levels in (a) intact larvae and (b) fat bodies in the European corn borer. 95% confidence intervals are plotted. ($r^2=0.8$, $F=29$, $p<0.001$, residual mean square error=0.2, $n=11$ for intact larvae (a); $r^2=0.9$, $F=60$, $p<0.001$, residual mean square error=0.1, $n=10$ for fat bodies (b))

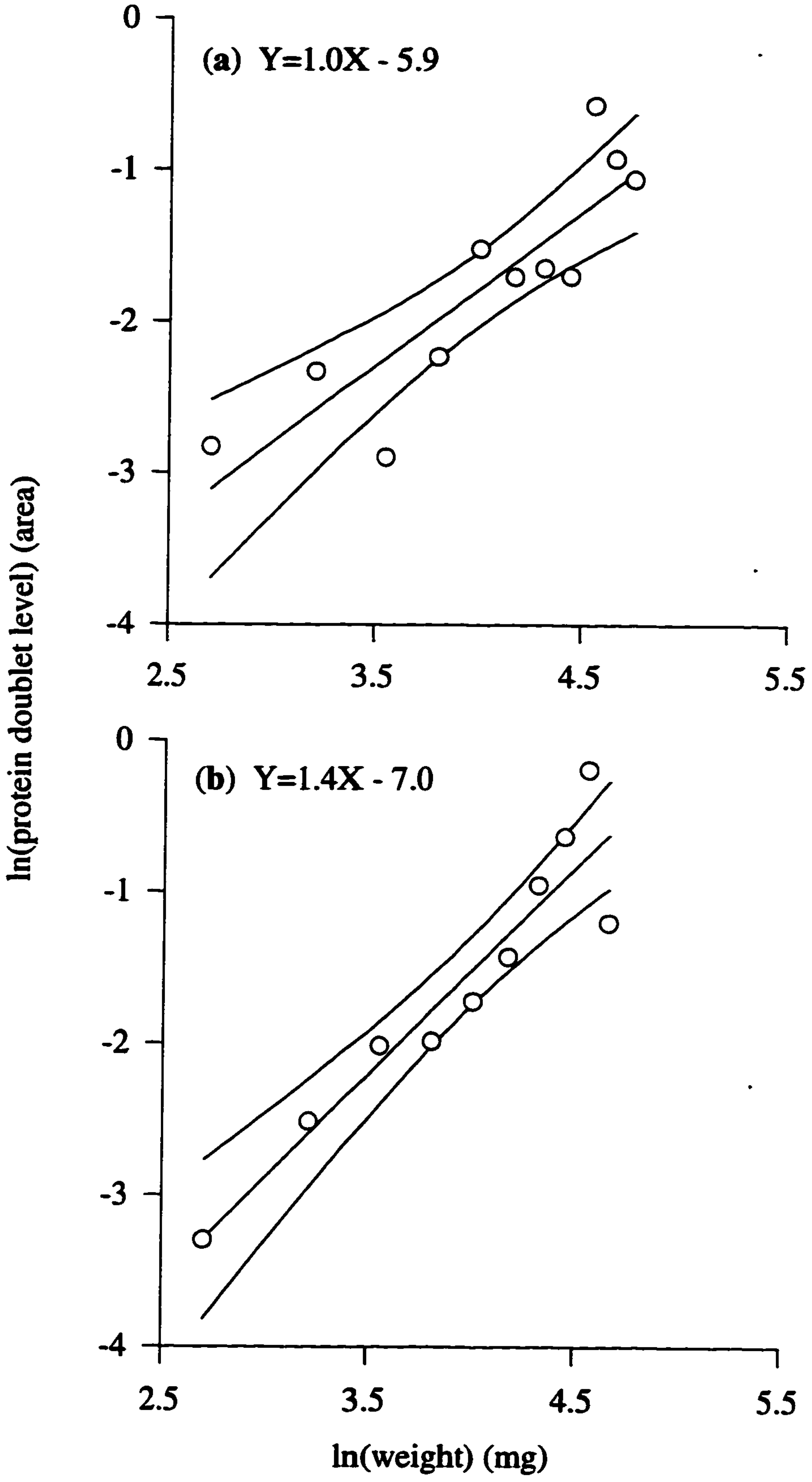
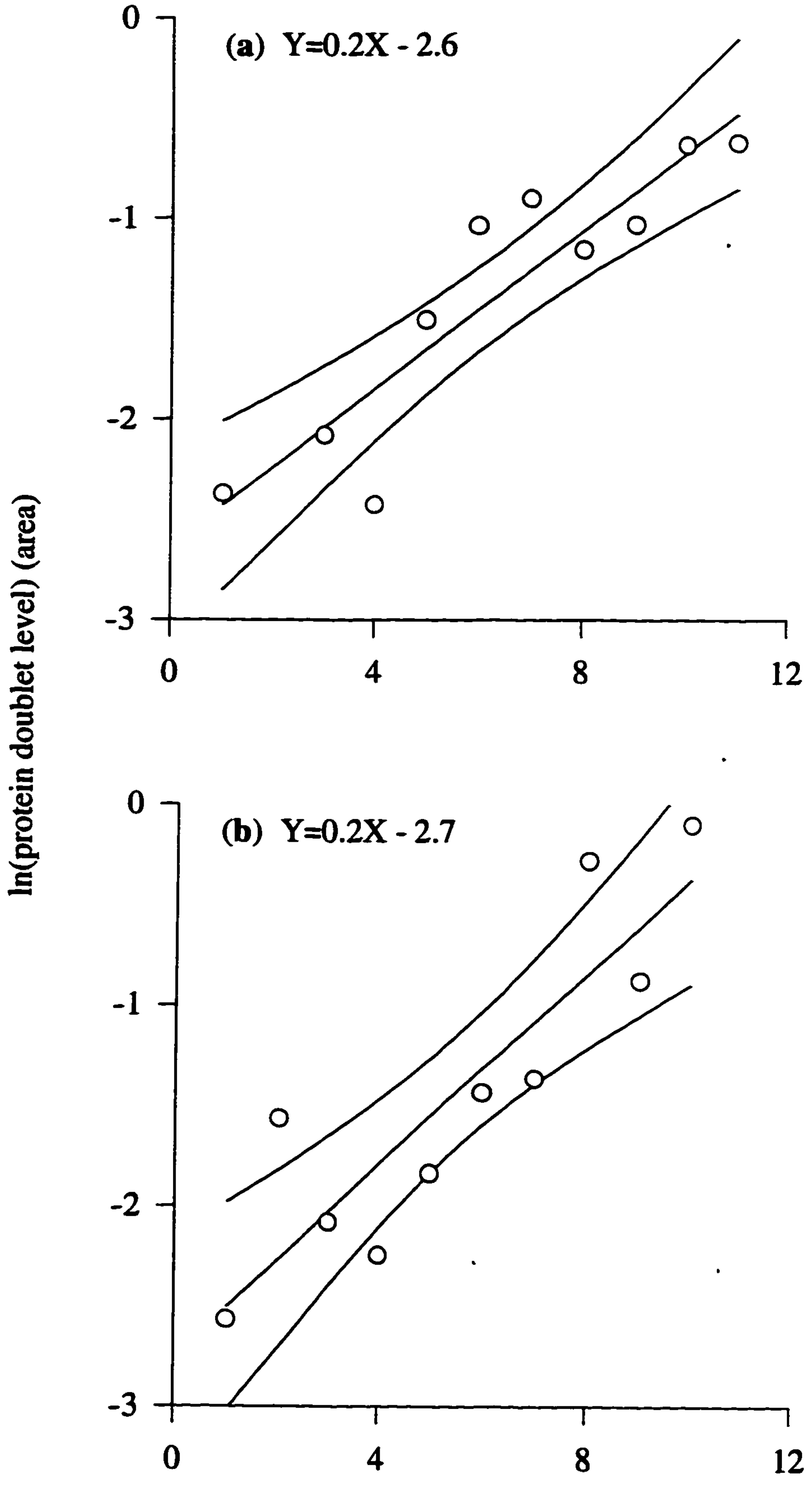
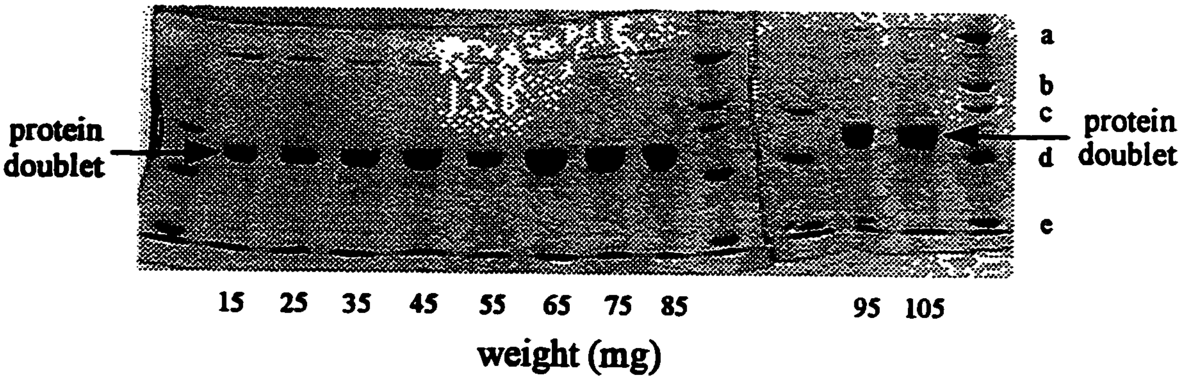

0
-1
-2
-3
-4
(a) Y=1.0X - 5.9
2.5
3.5
4.5
5.5
0
-1
-2
-3
-4
(b) Y=1.4X - 7.0
2.5
3.5
4.5
5.5
ln(protein doublet level) (area)
ln(weight) (mg)

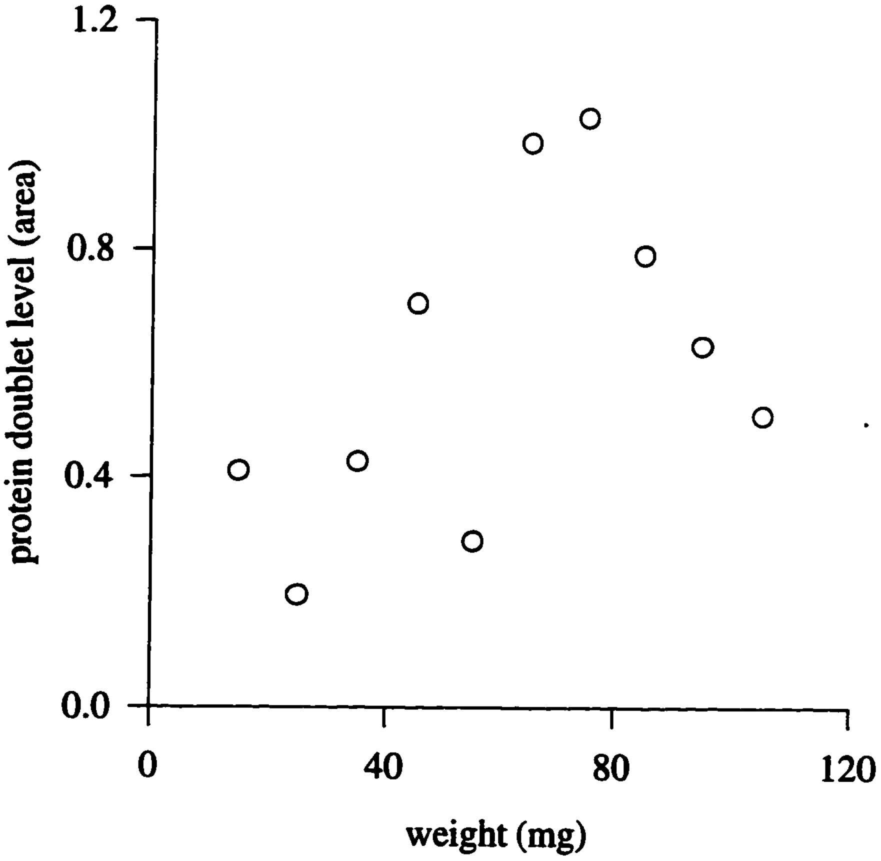
Figure 10. The effect of larval age on protein doublet levels in (a) intact larvae and (b) fat bodies in the European corn borer. 95% confidence intervals are plotted. (r^2=0.8, F=47, p<0.001, residual mean square error=0.1, n=11 for intact larvae (a); r^2=0.8, F=27, p=0.001, residual mean square error=0.2, n=10 for fat bodies (b))



(a) Y=0.2X - 2.6
(b) Y=0.2X - 2.7
0
-1
-2
-3
0
4
8
12
ln(protein doublet level) (area)
age (days)

Figure 11. (a) SDS-PAGE and (b) plotted levels showing the effect of larval weight on protein doublet levels in the haemolymph of the European corn borer. Each lane in (a) has 2 µg of protein and each data point in (b) represents pools of larvae. Protein doublet levels for (b) were estimated using densitometric scanning and determined as the area under the curve of the corresponding gels; in this case, the gel read in (a). Molecular weight markers for (a) are: a, myosin (M_r 220,000); b, β-galactosidase (M_r 116,250); c, phosphorylase b (M_r 92,500); d, bovine serum albumin (M_r 66,200); and e, ovalbumin (M_r 45,000). The larval weights shown in (a) are intervals of 10 mg; for example, 15 indicates an interval of 15-25 mg. *115 includes any insects >115 mg.


protein doublet
a
b
c
d
e
protein doublet
15
25
35
45
55
65
75
85
95
105
weight (mg)


1.2
0.8
0.4
0.0
protein doublet level (area)
0
40
80
120
weight (mg)

The ANCOVA results show that there is an interaction between ln(larval weight) and protein quality (casein, gluten, zein or haemoglobin) on the amount of total protein in final instar corn borers (MS (mean square)=0.4, df=3, residual mean square error=0.06, $p<0.001$, n=335). Since this means the homogeneity of slopes assumption was not met, separate regressions were performed for each diet type (Figure 12). The intercepts for the regressions of the four diets range from -4.7 mg/insect to -5.7 mg/insect and the slopes range from 1.3 to 1.6 /insect (Figure 12). By contrast, protein doublet level results show that there was homogeneity of slopes (no significant interaction between ln(larval weight) and protein quality; MS=0.2, df=3, residual mean square error=0.5, p=0.7, n=44). There was a significant effect of ln(larval weight) on protein doublet levels (MS=18, df=1, residual mean square error=0.5, $p<0.001$, n=44) with no significant effect of protein quality (MS=0.3, df=3, residual mean square error=0.5, p=0.6, n=44; Figure 13). The intercepts of the four diets range from -5.9 to -8.1 area/mg and the slopes range from 0.8 to 1.4 /mg (Figure 14).

3.3 Discussion

The results of this study show that the protein doublet found in European corn borer has a native molecular weight of 460 kDa. The levels in intact insects increase throughout larval development and diminish with adult development. These two results satisfy the criteria for a storage protein (Kanost *et al.*, 1990). Other storage proteins are often markedly diminished in the adults at or within three days of emergence (de Bianchi *et al.*,

Figure 12. The effect of larval weight on the amount of total protein in intact final instar European corn borers fed diets differing in protein quality. The diets contained different protein sources as follows: (a) casein ($r^2=0.9$, $F=2.3 \times 10^3$; $p<0.001$; residual mean square error=0.06; n=187); (b) gluten ($r^2=0.9$, $F=4.2 \times 10^2$; $p<0.001$; residual mean square error=0.06; n=58); (c) zein ($r^2=0.9$, $F=4.3 \times 10^3$; $p<0.001$; residual mean square error=0.04; n=43); and (d) haemoglobin ($r^2=0.8$, $F=2.4 \times 10^3$; $p<0.001$; residual mean square error=0.09; n=47). 95% confidence intervals are plotted.

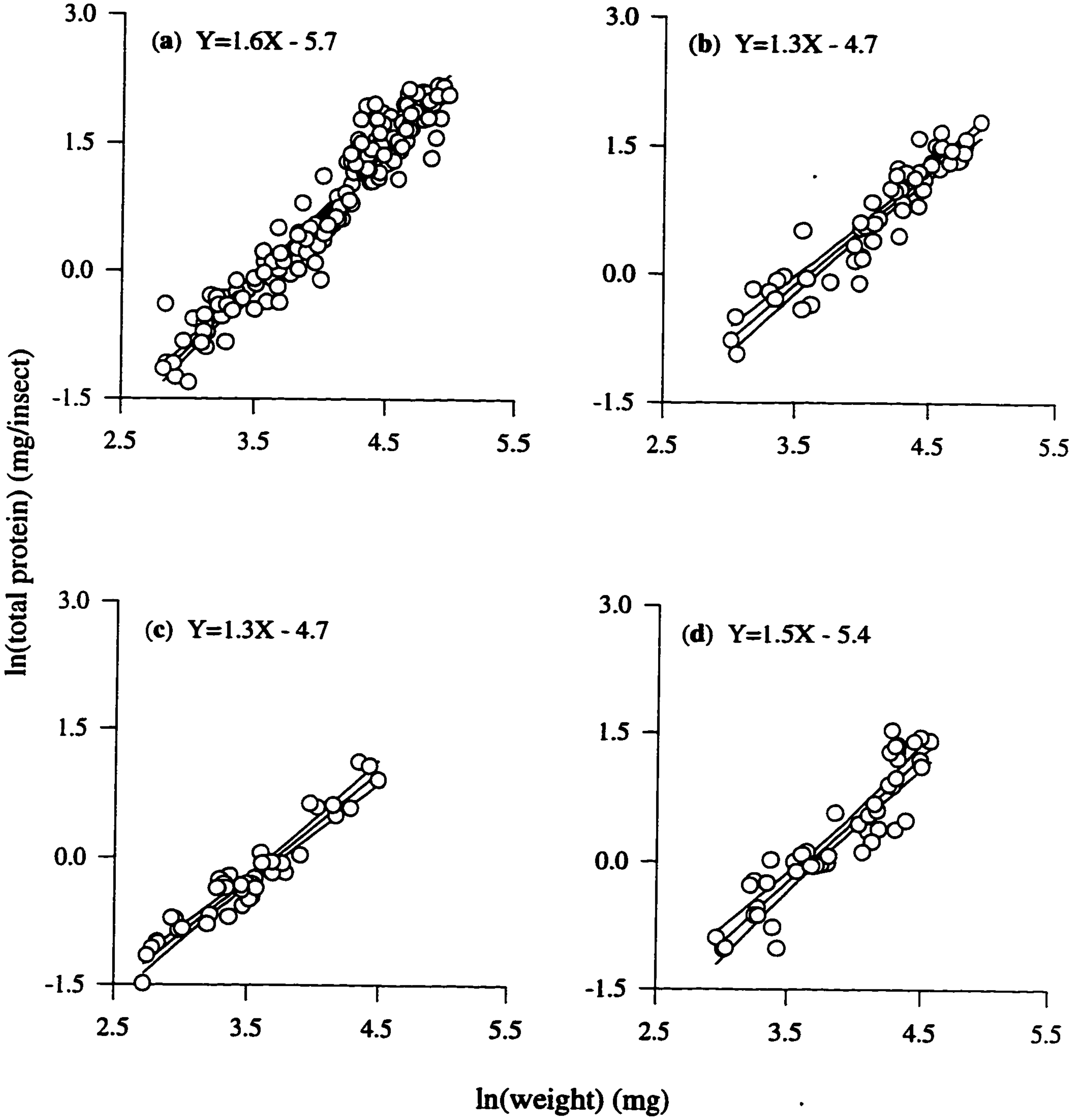

(a) Y=1.6X - 5.7
(b) Y=1.3X - 4.7
(c) Y=1.3X - 4.7
(d) Y=1.5X - 5.4
3.0
1.5
0.0
-1.5
2.5
3.5
4.5
5.5
ln(total protein) (mg/insect)
ln(weight) (mg)

Figure 13. The effect of larval weight on protein doublet levels in intact final instar European corn borers fed diets differing in protein quality (c=casein, g=gluten, z=zein, h=haemoglobin) (r^2=0.4, F=35; p<0.001; residual mean square error=0.5; n=44).

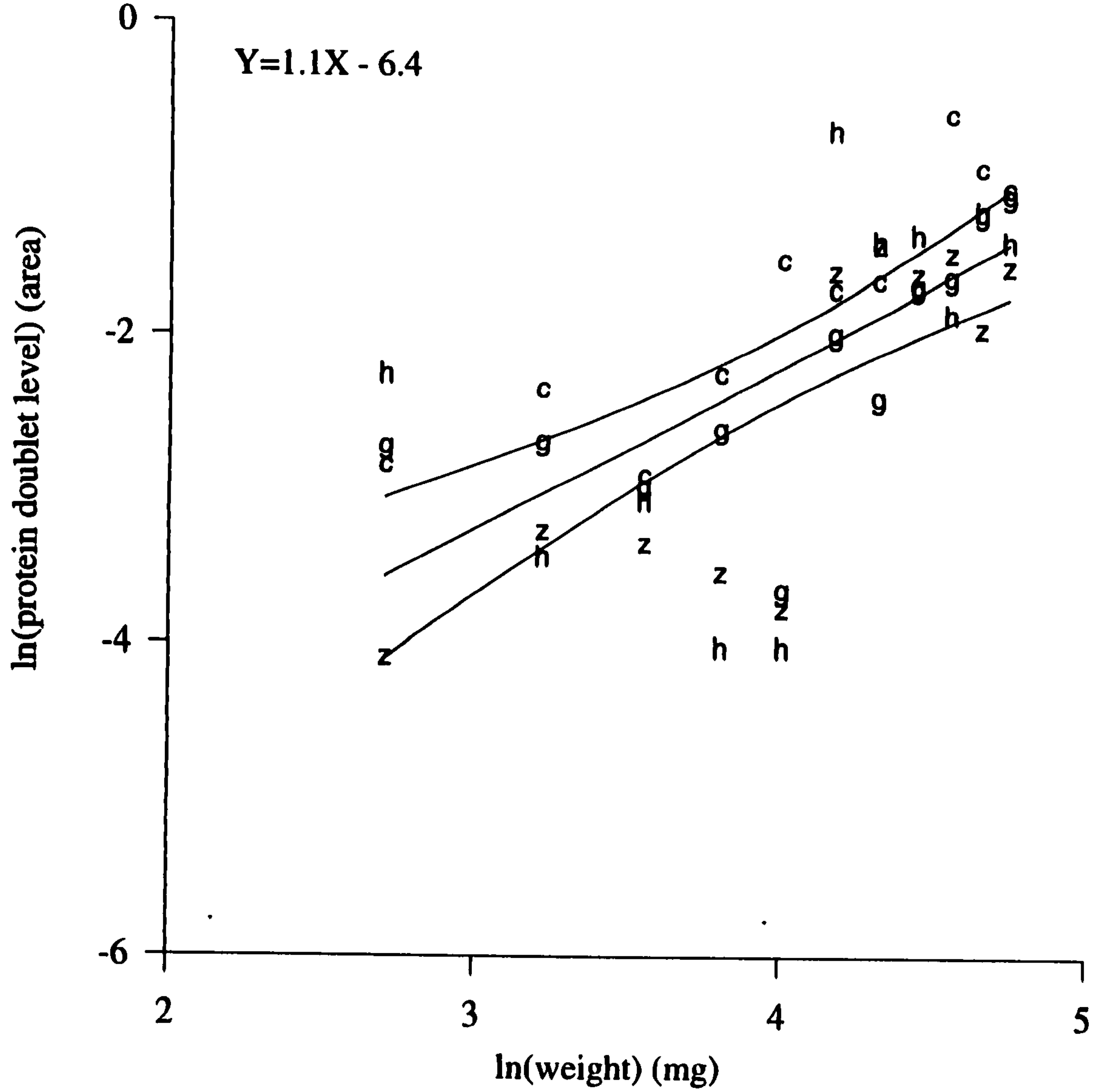
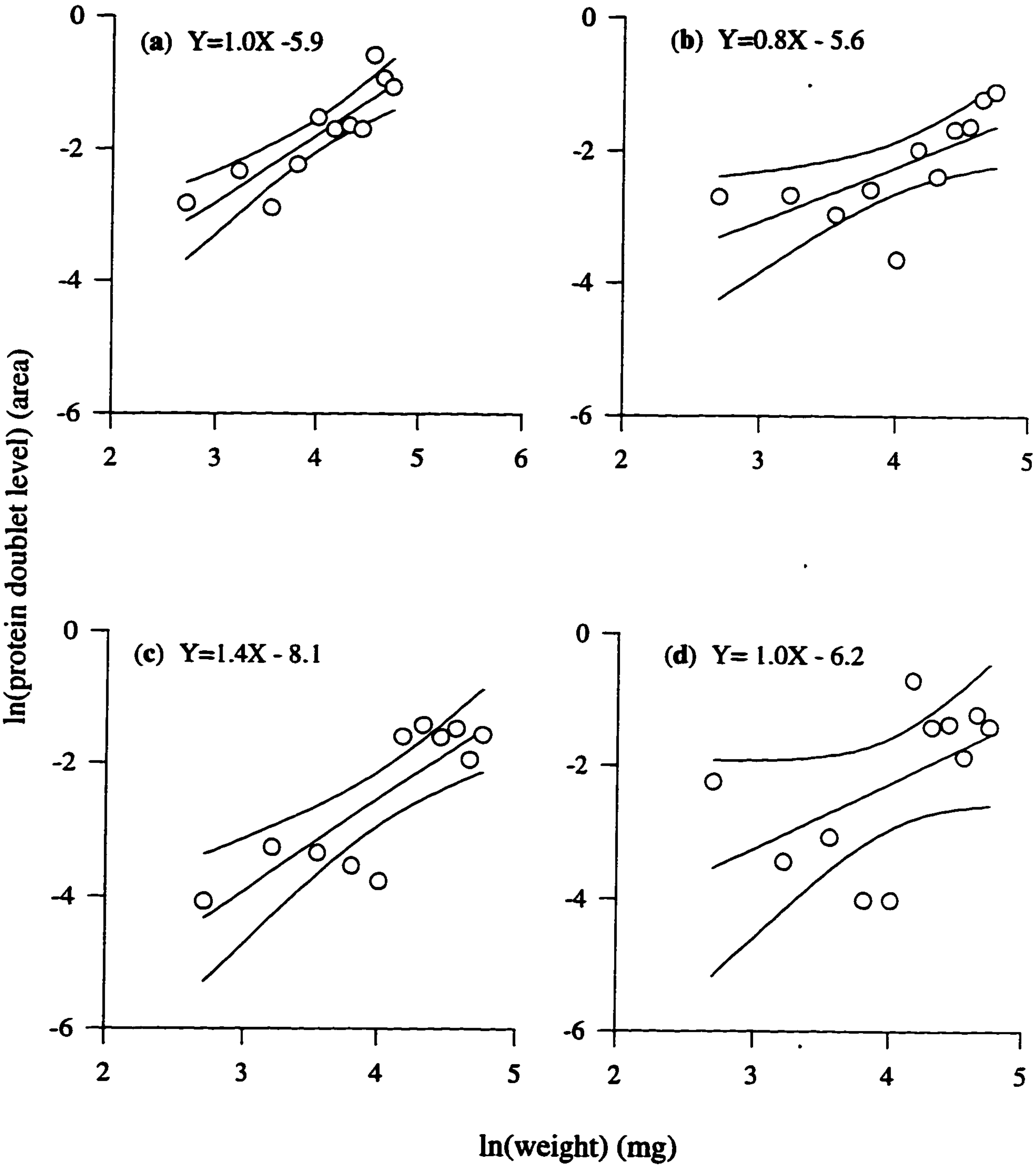

Y=1.1X - 6.4
ln(protein doublet level) (area)
0
-2
-4
-6
2
3
4
5
ln(weight) (mg)
c
g
h
z

Figure 14. The effect of larval weight on protein doublet levels in intact final instar European corn borers fed diets differing in protein quality. The diets contained different protein sources as follows: (a) casein (r^2=0.8, F=29; p<0.01; residual mean square error=0.1; n=11); (b) gluten (r^2=0.5, F=7.7; p<0.01; residual mean square error=0.4; n=11); (c) zein (r^2=0.7, F=20; p<0.01; residual mean square error=0.4; n=11); and (d) haemoglobin (r^2=0.3, F=3.6; p>0.05; residual mean square error=1.1; n=11).


(a) Y=1.0X -5.9
(b) Y=0.8X - 5.6
(c) Y=1.4X - 8.1
(d) Y= 1.0X - 6.2
0
-2
-4
-6
2
3
4
5
6
ln(protein doublet level) (area)
ln(weight) (mg)

1983; Telfer *et al.*, 1983; Levenbook and Bauer, 1980). However, Munn and Greville (1969) found levels of arylphorins in *Calliphora erythrocephala* adults up to 6 days after emergence. In this study, the storage protein in male corn borers cannot be detected by 3 days after emergence, however, in female adult corn borers the storage protein appears to persist longer, but does begin to diminish two days after emergence and is very difficult to detect by five days after emergence (Figure 2). An egg protein in the corn borer has a similar molecular weight as the storage protein and appears to overlap the storage protein, on the gel, in female adults (Figure 2). It is therefore possible that the storage protein level indicated for adult females in Figure 1b is actually a measurement of an egg protein and not the storage protein. This egg protein does not appear to be a vitellogenin since lepidopteran vitellogenin subunits are composed of a large (180 kDa) and a small (50 kDa) molecular weight subunit (Kunkel and Nordin, 1985). However, the lipophorin found in *M. sexta* also has a small and a large subunit but in this case the small subunit is approximately 81 kDa (Pattnaik *et al.*, 1979) suggesting that the egg protein on the gel done in this study may be a lipophorin.

The native molecular weight of 460 kDa and the two subunit weights of 81 and 87 kDa suggest the storage protein examined in the corn borer is a heterohexamer. Although the two bands representing the arylphorin storage protein of other lepidopterans are generally found in equal amounts (Tojo *et al.*, 1980; Kramer *et al.*, 1980), the staining intensities of the two bands found in this study differ. The 81 kDa subunit stains darker than the 87 kDa band suggesting that the 81 kDa band is a major band and the 87 kDa subunit a minor band. Similar results were found in the gypsy moth (*L. dispar*) with the lighter molecular weight subunit being the major band (Karpells *et al.*, 1990). That fact that the storage protein found in this study is an heterohexamer and accounts for up to 80% of the total protein in the haemolymph (Figure 3) suggests it is an arylphorin, but further immunological studies and amino acid analysis are required to determine this.

The storage protein levels show a pattern of increasing to the intermoult period, days 5-7 or weights of 75-85 mg, in the haemolymph during the final instar and decreasing prior to pupation (Figure 3a,b; 11a, b). The fat-body storage protein taken from the same insects as those used for haemolymph collection in the corn borer shows an increase in storage protein levels to pupation (Figure 9b). This suggests that the fat body sequesters the storage protein at the time the storage protein declines in the haemolymph, and is consistent with the fat body synthesizing the storage protein, releasing it into the haemolymph and taking it back up just before pupation. The pattern of increasing and decreasing with larval weight (Figure 11b) has no statistical support (i.e., there is no significant second order regression term). However, the insects at different weights were at various levels of head capsule slippage. A cyclic pattern of increasing to the intermoult period with a decrease in storage protein levels during the molting has been found in other insects (Karpells *et al.*, 1990; Riddiford and Hice, 1985; Nagata and Kobayashi, 1990b). If for the two points that seem to deviate from the pattern at 25-35 mg and 55-65 mg, one or more of the pooled insects was still in some stage of head capsule slippage (still moulting), the pooled levels of storage protein for these two weight intervals may be substantially less.

Larval weight does not increase linearly with larval age in intact larvae in the final instar of the European corn borer. There is an increase in weight with age until shortly before pupation at which time the larval weight decreases (Figure 4); the same pattern is shown by the amount of total protein in intact larvae (Figure 5a). The findings that both larval age and larval weight account for a large amount of the variation in total protein suggest that both may be good indicators of protein levels. Larval weight accounts for 90% of the variation in the amount of total protein in intact insects while larval age accounts for 80%. This suggests that larval weight is possibly a better indicator of the amount of total protein than larval age but the difference is slight.

In contrast to intact larvae, the amount of total protein in the fat body increased with both larval age and weight (Figure 5b, 6b). Levenbook and Bauer (1980), when studying *Calliphora vicina*, also found fat-body total protein continued to increase with age prior to pupation. They suggested this result may be due to preformed polypeptide chains in the fat body that are not released to the haemolymph. As well, in *Calliphora stygia*, it was found that although the storage protein is taken up by the fat body prior to pupation, other proteins are still being synthesized (Kinnear and Thomson, 1975). The increasing relationship found here between fat-body total protein and larval age is consistent with the fat body both synthesizing proteins as well as sequestering them from the haemolymph.

The haemolymph total protein concentrations differ from that of either fat body or intact larvae. The results of only a small amount of variation in total protein concentration accounted for by either larval weight (Figure 8) or larval age (Figure 7) is expected since the haemolymph is constantly receiving proteins synthesized by the fat body and then having them removed when required for use by the insect.

Both larval weight and larval age account for a large amount of variation in storage protein levels and the residual mean square errors are similar for both (Figure 9a,b; Figure 10a,b). These findings suggest that for burrowing insects where larval stages are difficult to identify at least based on head-capsule slippage, using weight as an indicator of total protein or storage protein levels is certainly as good as using the insect's age.

The results of the ANCOVA suggest that there is no additional effect of diet type on storage protein levels when corrected for larval weight. Nagata and Kobayashi (1990b) found that in *B. mori*, storage protein levels in the haemolymph decreased on an artificial diet containing low amounts of protein. In studies using parasitized *M. sexta*, storage protein levels were decreased in the host haemolymph (Beckage and Kanost, 1993). Because parasites consume the host's nutrients, these authors suggest that the decrease in storage protein levels may be due to a lack of nutrients available to the host. Both these studies also found that larval weights were lower for either the insects fed the poor diet or parasitized insects, however they did not consider the weight of the larvae when statistically examining the other effects. Considering this, my findings agree with the literature. However, the sample size used in this experiment was very small and the power of the test may be low therefore further investigations are required to determine this.

In summary, both larval age and larval weight are excellent predictors of the amount of total protein in the European corn borer. Larval weight is also a good indicator and predictor of storage protein levels in this insect. Measurements of larval weight to predict these levels are simple and less time consuming than the biochemical techniques required to determine levels of storage protein. Monitoring levels of storage protein in insects may give further insights into the developmental progress of the insect and may aid in biological control of these pests. Assuming the relationship between larval weight and storage protein levels in the field also holds, using a simple technique such as weighing the insect to estimate levels of storage proteins may save a great deal of time.

Chapter 4 The European corn borer, *Ostrinia nubilalis*, can partially compensate for nutritional changes in its artificial diet

4.1 Introduction

Insects can compensate for changes in both protein quality and quantity in their diets by pre-ingestive or post-ingestive compensation or both (Slansky and Scriber, 1985; Simpson and Simpson, 1990; Slansky, 1993 for reviews). Pre-ingestive compensation includes behavioural changes such as increasing food consumption (Slansky and Scriber, 1985; Simpson and Simpson, 1990) or prolonging development by increasing the time spent feeding (Slansky and Scriber, 1985). In post-ingestive compensation, physiological processes of digestion and absorption of nutrients are altered (Simpson and Simpson, 1990; Zanotto *et al.*, 1993). Since lepidopteran females must accumulate protein for egg production as well as for maintenance (Slansky and Scriber, 1985), their ability to compensate for changes in dietary nutrition may be more pronounced than for males.

Frequently, lepidopterans compensate for nutritionally unbalanced foods, but may attain lower pupal weights than insects fed control diets (Slansky and Wheeler, 1992; Naeem, *et al.*, 1992; Hayes, *et al.*, 1992). Reproductive success is affected by both the size and weight of a pupa (Slansky and Scriber, 1985; Slansky, 1993) and insect weight has been found to significantly affect metamorphosis in many lepidopteran species (Nijhout, 1975; Morita and Tojo, 1985; Ochieng'-Odero, 1990). Nijhout (1975) defines a critical weight as a minimum species-specific size or weight that must be attained before pupation can occur and produce a viable, reproductive adult. Ochieng'-Odero (1990) defines a larval critical weight as the weight that allows metamorphosis to occur. The author also predicts a pupal and adult threshold weight below which larvae cannot pupate or pupae cannot emerge as viable adults. In many lepidopterans, larval and pupal weights are greater for females than males (Slansky and Scriber, 1985; Slansky 1993).

In this study I alter growth and development of the European corn borer, *O. nubilalis*, by modifying the protein quality and quantity in its artificial diet. Changes in protein quality involve substituting different proteins in a standard diet, and changes in protein quantity involve increasing or decreasing the amount of one specific protein. In the field, protein quantity ranges from approximately 2% to 10% during the final instar. The questions I wish to address are: 1) can the corn borer compensate for changes in protein quality and quantity in its artificial diet? 2) if I find that compensation does occur, does the corn borer use pre-ingestive or post-ingestive compensation or both? 3) of the five different proteins I use, what is the 'best' or 'worst' protein in terms of quality and quantity for the corn borers to consume? Although zein is one of the main proteins corn borers feed on in the field during the last instar, Broadway and Duffey (1988) suggest that zein should be the worst because its amino acid profile is very poor and that casein should be the best due to the high amounts of lysine and arginine in casein (see Appendix I for a list of proteins used); 4) do male and female corn borers compensate for these changes in the same way? and, 5) is there a suggestion of a critical pupal weight -- is there a threshold pupal weight?

4.2 Results

Only two insects survived on the 8.0% haemoglobin diet (1 male and 1 female) and only 2 males survived on the 21.5% haemoglobin diet. Because of this, the analyses after determining sex differences, on the 21.5% diets only, were restricted to females, and all insects fed the haemoglobin diets were omitted from further analyses.

4.2.1 Sex Differences

Significant differences were found between males and females in the mean amount of food consumed, mean larval duration and mean dry pupal weights (Table 1). Females consume on average 0.4 mg more food, remain in the larval stage approximately 1 day longer and obtain pupal weights of approximately 0.2 mg greater than males (Table 1).

4.2.2 Effects of Protein Quality and Quantity

Both quality and quantity of protein in the artificial diet of European corn borers affect the amount of food consumed, larval duration and pupal weights (Table 2). The only interaction between diet quality and diet quantity seen was with the amount of food consumed (Table 2). Multiple comparisons suggest the only significant differences in the amount of food consumed were by larvae fed the casein diets and those fed either the albumin or zein diets (Table 3). Significant differences found in the length of the larval

Table 1. The effects of sex on various parameters for final instar European corn borers (values in the table are means±standard errors)

Sex	ln(food consumed) (mg) t=-6.8	ln(larval duration) (days) t=-2.9	ln(dry pupal weight) (mg) t=-4.1
Female (n=107)	5.19±0.04	2.47±0.03	3.07±0.02
Male (n=79)	4.81±0.04	2.35±0.03	2.91±0.03

$p<0.016$ and df=184 for all; t-tests were done to compare the two means in each column

Table 2. The effects of protein quality (casein, albumin, gluten or zein) and quantity (21.5% or 8.0%) on various parameters for final instar European corn borers (values in the table are the mean squares)

Source of variation	df	food consumed	ln(larval duration)	ln(dry pupal weight)
protein quality	3	2.5×10^4**	0.6**	0.8**
protein quantity	1	1.2×10^5**	1.6**	1.8**
interaction between protein quality and protein quantity	3	9.2×10^3*	0.000	0.04
error	140	2.5×10^3	0.03	0.04

*$p<0.016$, **$p\leq0.001$, n=148; where there are no asterisks, there are no significant effects ($\alpha=0.05$)

Table 3. The effects of protein quality (casein, albumin, gluten or zein) and quantity (21.5% or 8.0%) on various parameters for final instar European corn borers (values in table are means±standard errors)

Diet type	food consumed (mg)	ln(larval duration) (days)	ln(dry pupal weight) (mg)
casein (n=53)	$187\pm8^{a,b}$	$2.43\pm0.03^{a,d}$	$4.34\pm0.03^{a,d}$
albumin (n=45)	239 ± 8^{a}	2.53 ± 0.03^{b}	4.29 ± 0.03^{b}
gluten (n=34)	220 ± 10	$2.57\pm0.04^{c,d}$	$4.13\pm0.0^{c,d}$
zein (n=16)	250 ± 13^{b}	$2.83\pm0.04^{a,b,c}$	$3.92\pm0.05^{a,b,c}$
21.5% (n=99)	190 ± 6^{d}	2.47 ± 0.02^{e}	4.30 ± 0.02^{e}
8.0% (n=49)	258 ± 8^{d}	2.71 ± 0.03^{e}	4.04 ± 0.03^{e}

within each column, the lowercase superscript letters identify significant pairwise differences in the means; rows that have matching superscripts differ significantly from each other ($p<0.05$); for example, in the ln(larval duration) column, the superscript a's in the first and fourth row indicate a significant difference in the mean larval duration of casein- and zein-fed insects duration were with zein-fed insects and casein-, albumin- or gluten-fed insects and between gluten-fed insects and casein-fed insects (Table 3). Significant differences in pupal weights were seen with insects fed the zein diets and those fed the casein, albumin or gluten diets; and, gluten-fed insects and insects fed the casein diets (Table 3). Comparisons between the 21.5% and 8.0% diets show that food consumed, larval duration and pupal weights of insects fed the 8.0% were all less than for insects fed the 21.5% diets.

There were no significant interactions between protein quality and quantity in the ANOVAs used to compare approximate digestibility (AD) or efficiency of converting digested food to biomass (ECD) on different diets (Table 4). Both AD and ECD differed with protein quality and protein quantity (Table 4). Multiple comparison tests show that AD for insects fed the zein diets differed significantly from those fed the casein, albumin or gluten diets (Table 5). ECD for insects fed the casein diets differed significantly from those fed the albumin, gluten or zein diets (Table 5). AD and ECD also differed between the 8.0% and 21.5% diets (Table 5).

4.2.3 Weight Results

There was no significant interaction term in the ANOVA performed to determine if pupal weights of insects that survived were higher than for those that died (Table 6). Multiple comparisons show that those pupae that survived had a higher mean dry weight at

Table 4. The effects of protein quality (casein, albumin, gluten or zein) and quantity (21.5% or 8.0%) on various physiological factors for final instar European corn borers (values in the table are the mean squares)

Source of variation	df	AD	ECD
protein quality	3	0.1**	0.06**
protein quantity	1	0.2**	0.2**
interaction between protein quality and protein quantity	3	0.004	0.01
error	140	0.007	0.009

**$p<0.001$; $n=148$; $r^2=0.3$ for both AD and ECD; AD=approximate digestibility; ECD=efficiency of converting digested food to biomass; where there are no asterisks, there are no significant effects ($\alpha=0.05$)

Table 5. The effects of protein quality (casein, albumin, gluten or zein) and quantity (21.5% or 8.0%) on various physiological factors for final instar European corn borers (values in table are means±standard errors)

Diet type	AD	ECD
casein (n=53)	0.40 ± 0.01^{a}	$0.32\pm0.01^{a,b,c}$
albumin (n=45)	0.41 ± 0.01^{b}	0.26 ± 0.01^{a}
gluten (n=34)	0.39 ± 0.02^{c}	0.24 ± 0.02^{b}
zein (n=16)	$0.32\pm0.02^{a,b,c}$	0.24 ± 0.02^{c}
21.5% (n=99)	0.42 ± 0.01^{d}	0.31 ± 0.01^{d}
8.0% (n=49)	0.32 ± 0.02^{d}	0.22 ± 0.02^{d}

AD=approximate digestibility; ECD=efficiency of converting digested food to biomass within each column, the lowercase superscript letters identify significant pairwise differences in the means; rows that have matching superscripts differ significantly from each other ($p<0.05$); for example, in the AD column, the superscript a's in the first and fourth row indicate a significant difference in the mean AD of casein- and zein-fed insects

Table 6. Comparison of mean dry weights of dead and live European corn borer pupae when grouped by protein quality (casein, albumin, gluten or zein) and quantity (21.5% or 8.0%)

Source of variation	df	MS
dead and live pupae	1	1.6*
grouping variable (protein quality and quantity)	7	0.9*
interaction between (dead and live pupae) and the grouping variable	7	0.04
error	332	0.04

*$p<0.001$; MS=mean squares; where there are no asterisks, there are no significant effects ($\alpha=0.05$)

2.9(±0.01) mg (n=268) compared to those that did not survive 2.7(±0.02) mg (n=80; data were ln transformed; $p<0.001$). The logistic regression shows a significant effect of dry weight on whether pupae survived or died (Figure 15).

The log linear model to determine if mortality was independent of either protein quality or quantity demonstrated a significant three-way interaction term ($G^2=11.7$, $p=0.019$, df=4, n=457). This suggests that the model with the three-way interaction term was the best fitting model and because of this interaction, two separate models were fit. The results demonstrate that mortality was not independent of either protein quality ($G^2=35.8$, $p<0.001$, df=10) or protein quantity ($G^2=88.8$, $p<0.001$, df=16). Table 7 shows percentage mortality on each diet.

4.3 Discussion

4.3.1 Sex Differences

Female European corn borers eat more, have a longer larval duration and greater pupal weights than males. These results are consistent with findings on Southwestern corn borers, *Diatraea grandiosella* (Ng *et al.*, 1993), and are expected since the European corn borer has non-feeding adults and sex differences are usually greater in insects with non-feeding adults (Slansky and Scriber, 1985).

Figure 15. The effect of dry pupal weight on whether pupae survived (1) or died (0). The logistic regression performed on this data shows a significant effect ($p<0.001$) of dry pupal weight on survival [logit (# pupae survived/total # pupae) = -1.93(±0.55) + 0.18(±0.03)dry pupal weight]. The distributions of pupal dry weights that survived or died are shown as notched box plots (Wilkinson *et al.*, 1992b). The points above the whiskers identify extreme points that lie beyond 1.5 times the interquartile distance.

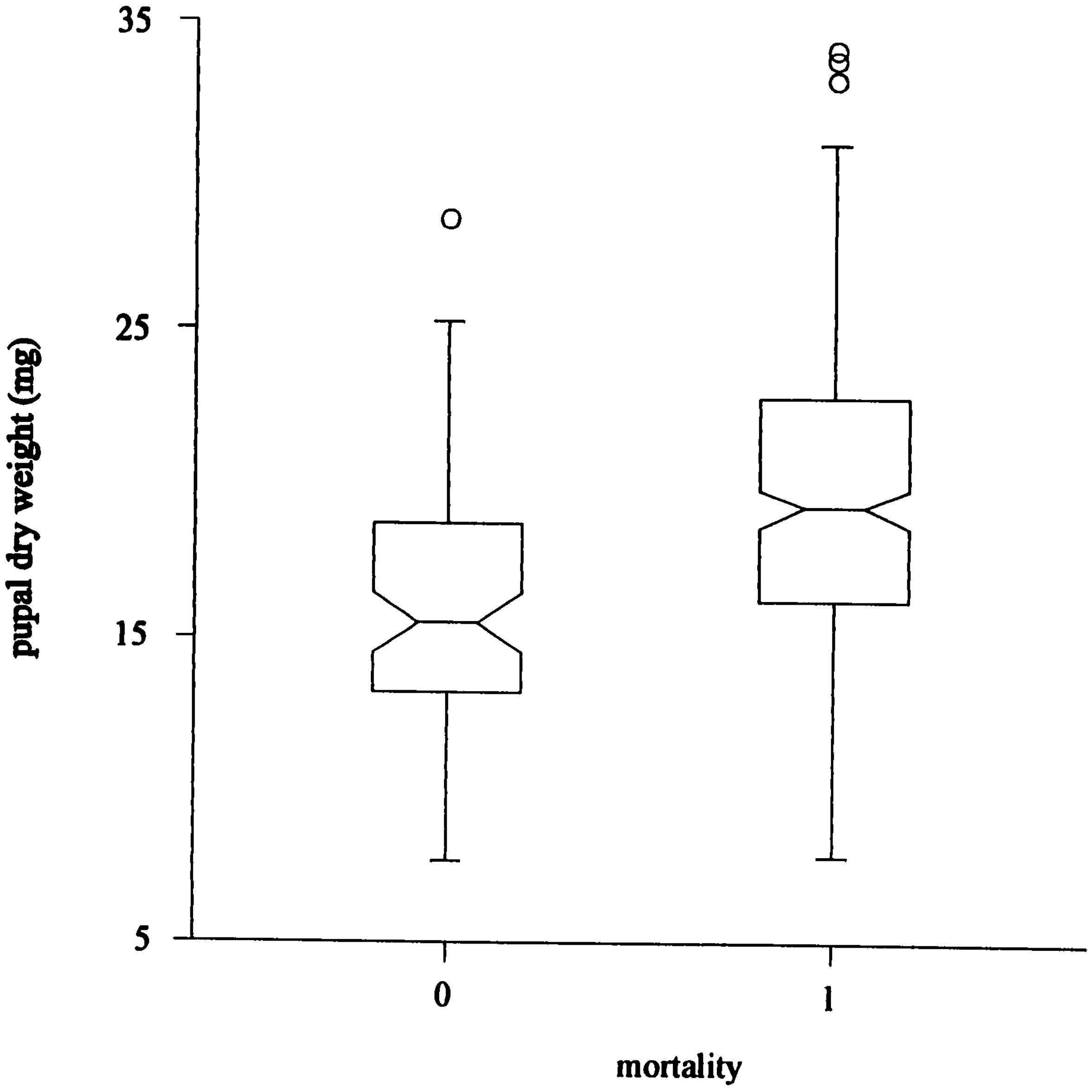

35
25
15
5
pupal dry weight (mg)
0
1
mortality

Table 7. The effects of protein quality (casein, albumin, gluten, zein or haemoglobin) and quantity (21.5% or 8.0%) on percent mortality for final instar European corn borers

Diet type	casein	albumin	gluten	zein	haemoglobin
21.5%	33 (n=59)	46 (n=63)	54 (n=57)	78 (n=49)	90 (n=71)
8.0%	66 (n=45)	57 (n=47)	82 (n=44)	70 (n=42)	94 (n=33)

numbers not in brackets are the % dead of the total number of insects, n

4.3.2 Effects of Dietary Protein Quality and Quantity

Protein quality depends on both relative and absolute amounts of amino acids and the secondary and tertiary structure of the protein (Broadway and Duffey, 1988). Female European corn borers fed the zein diet and 8.0% diets compensate pre-ingestively by eating more and increasing development time, but, they still attain lower pupal weights than those insects fed the standard casein diet. Females fed the albumin diet had similar pupal weights to those fed the casein diet though they consumed more suggesting that albumin offers similar nutrition as the standard casein diet. Gluten-fed females have lower pupal weights and a longer larval duration than casein-fed females and all but two insects died on the 8.0% haemoglobin diet. These results suggest that the order of 'best to worst' dietary protein is casein>albumin>gluten>zein>haemoglobin. The percentage mortality data correspond to this pattern for the 21.5% diets and vary slightly for the 8.0% diets (Table 7). Similar results were found using casein-, gluten- and zein-based diets fed to *Spodoptera exigua* (Broadway and Duffey, 1988).

Broadway and Duffey (1988) found a reduction in insect growth associated with gluten and zein diets and attributed this to lower amounts of lysine and arginine found in these two proteins. Gluten, which has higher amounts of both lysine and arginine than zein, supported insect growth better than that of zein (Broadway and Duffey, 1988). That haemoglobin is a 'worse' protein than zein is somewhat surprising because haemoglobin is richer in lysine, arginine and histidine than zein. Lysine, arginine (Broadway and Duffey, 1988), cysteine (Broadway and Duffey, 1988; Felton *et al.*, 1992), histidine, and methionine (Felton *et al.*, 1992) which have all been strongly correlated with larval growth are believed to be the most important amino acids required in an insect's diet. In one study, phenylalanine was found to reduce consumption, increase growth rate and efficiency of converting ingested food to biomass in the larvae of *Schistocerca gregaria* (Bernays and Woodhead, 1984), however, Broadway and Duffey (1988) found no correlation between the aromatic amino acids and larval growth. When comparing the amino acid composition of zein and human haemoglobin, haemoglobin appears to have higher percentages of lysine, arginine and histidine than zein and both have similar amounts of cysteine (Appendix I). Only methionine and phenylalanine are slightly higher in zein than haemoglobin. Since the haemoglobin used for these experiments was denatured, the tertiary structure of the haemoglobin cannot be what is interfering with consumption. I suggest that perhaps the odour or the texture (which was slightly rougher when rubbed between fingers than that of the other diets) of the haemoglobin diet act as a phagodeterrent.

Both zein and gluten are non-nutritious for other lepidopteran species. For example, growth of *Heliothis zea* and *S. exigua* when fed a gluten diet was found to be no better than for insects fed a diet containing no added protein (Bloem and Duffey, 1990). Gluten only weakly supported growth of these two species and this was attributed to the low digestibility of gluten (Bloem and Duffey, 1990). Horie and Watanabe (1983) found that *B. mori* larvae had severely retarded growth and low levels of proteins in the haemolymph when fed diets containing either zein or gluten. They found an increase in uric acid excretion on these two diets and concluded that there was an imbalance of amino acid composition in gluten and zein. Broadway and Duffey (1988) found 90% mortality in zein-fed *S. exigua*. Although mortality of European corn borers fed the zein (21.5%) diet in this study was high at 78%, the insects that survived managed to compensate, albeit at lower pupal weights. I find that gluten-fed corn borers compensated though they attain lower pupal weights than the insects fed the standard diet. Lower pupal weights may result in reduced reproductive success, but any insects that survived would likely still contribute to the next generation (Slansky and Scriber, 1985). My results suggest that the European corn borer may be less sensitive to changes in protein quality than other lepidopterans. These differences tend to support the idea that responses to nutritional changes are likely species-specific. It also suggests that altering the protein quality of the European corn borer's diet may not be a good means of controlling this insect.

Insects can compensate for changes in food quality by altering consumption , selecting another food or altering their digestive and metabolic efficiencies (Simpson and Simpson, 1990). This study shows the European corn borer used pre-ingestive compensation by both altering the amount of food it consumes and remaining in the larval stage longer possibly attempting to attain more nutrients. However, whether larvae compensated post-ingestively remains in doubt. My results suggest that insects fed the ‘poorer’ diets did not increase ECD in response to the changes in protein quality or quantity and in fact the highest ECD values were found on the casein and 21.5% diets. Similar results were found with *Spodoptera eridania* where ECD was lower for insects fed a zein diet when compared to a standard diet (Karowe and Martin, 1989). These authors also found that

AD was lower on a zein diet which is similar to my results where AD was lower only on the zein and 8.0% diets and did not differ on the other three. That approximate digestibility was lower on the zein diets may be due to the larger amount of food passing through the gut of the zein-fed insects as they increased consumption. The rate at which the food passed through the insect's guts fed poorer diets may have been such that there was less time allocated to digestion and assimilation of the food (Slansky and Scriber, 1985).

It is clear from this study that the European corn borer could partially compensate for nutrient poor diets using pre-ingestive compensation processes. Compensation was only partial because had the insects fully compensated I would have found similar mean pupal weights on all diets. Pupal weights were lower for insects fed the gluten and zein diets when compared to the standard casein diet and also lower for those insects fed the 8.0% diets when compared to the 21.5% diets. That pupal weights were lower on poor quality and quantity diets and the finding that pupal weight of insects that survived was higher than those that died suggests weight at pupation does effect survival. The logistic regression results confirm this and suggest that higher weight pupae have a greater chance of surviving to the adult stage (Figure 15). The data plotted suggest there is no critical pupal weight in the corn borer.

Chapter 5 General Discussion

5.1 Overall Conclusions of Thesis

I hypothesized that storage proteins in the European corn borer are an important factor determining the onset of pupation and becoming a viable adult and that insect weight is an indicator of storage protein levels and thereby the onset of pupation and success of metamorphosis. My first objective was to identify and characterize the storage protein in the corn borer and follow the normal pattern of storage protein levels throughout the final instar to adult emergence. The protein doublet found in the corn borer is indeed a storage protein. It has a native molecular weight of 460 kDa and shows a general pattern of increasing levels to pupation and diminishing with adult development. The storage protein is likely an arylphorin because it is a heterohexamer and is the most abundant protein in the larval haemolymph. In the haemolymph, the normal pattern throughout the final instar is one of increasing to the intermoult period with a decrease prior to pupation consistent with the fat body sequestering the storage protein from the haemolymph just prior to pupation. In the final instar, storage protein levels increase to pupation in both the fat body and intact larvae. There is a large amount of variation in storage protein levels accounted for by larval weight. These results support the hypothesis that larval weight is an excellent predictor and indicator of storage protein levels in the European corn borer.

My next objective was to alter the protein quality and quantity in the diet of the corn borers in an attempt to affect development events by reducing growth and storage protein levels. That weight is correlated to the onset of pupation and success of metamorphosis is strongly suggested by these studies. Pupal weights of insects that survived were greater than for those that died, consistent with a correlation between pupal weight and successful adult emergence. Insects remained in the larval stage longer on poor diets and mortality was higher on poor diets. These findings suggest that corn borers may have a 'critical weight' that they need to attain before they can pupate and are correlated with the onset of pupation being affected by the insect's weight.

My final objective was to determine if storage protein levels were affected by the protein quality in the insect's diet. There was no significant effect of protein quality on storage protein levels after correcting for the weight of the insect. Larval weight in the corn borer accounts for a large amount of the variation in storage protein levels. Larval weight ultimately affects pupal weight (Slansky and Scriber, 1985) so that lower weight pupae that died likely had lower levels of storage protein than those that survived because of the strong correlation between weight and storage protein levels.

5.2 Research Implications

The European corn borer appears to compensate quite well to changes in dietary protein at least in the final instar. This suggests that decreasing the quality or quantity of protein in the insect's diet may not be a good way to control this pest and other methods need to be investigated. In the field, levels of protein in the plants are often lower than that provided by an artificial diet. As well, the quality of protein in a plant varies within a season (Slansky and Scriber, 1985). If insects can compensate even in the field for low levels of protein, increased crop damage could occur as insects remain in the larval stages longer attempting to attain sufficient nutrients for survival and reproduction.

It is difficult to suggest a breeding strategy for corn that would best control the corn borer. Though some corn borers are bivoltine, if the protein in the plant is of very poor quality or quantity and causes the insects to remain in the larval stage longer, it may mean that they will be unable to reproduce again before the season is complete. Similarly, if their weight is sufficiently reduced and affects fecundity, damage in future years may also be less.

In some lepidopterans, storage proteins are not synthesized until after the first instar or later (or at least have not been detected until that time). Keeping storage protein levels to a minimum or undetected would prevent the insects from developing successfully. I think we need to target the early instars to have successful control of the corn borers.

Because larval weight is strongly correlated with storage protein levels, larval weight may be used as a predictor of storage protein levels and therefore save a lot of time and expense in experiments involving estimating storage protein levels.

5.3 Further Research

The densitometer used to measure storage protein levels only gives an estimate of these levels especially since the readings were performed on an impure protein. To better quantify the relationship between weight and storage protein levels, a method such as enzyme-linked immunosorbant assay (ELISA) or rocket immunoelectrophoresis should be used. These methods use an antiserum made from the pure storage protein and allow for more accurate quantification of the storage protein in insects. Purifying the storage protein would aid in determining if the third band that appears late in development is actually an immunoreactive storage protein band or some other storage protein. To clearly identify the storage protein in the corn borer as an arylphorin, further investigations are required. Amino acid analysis on the pure storage protein of the corn borer would indicate whether there is a high content of aromatic amino acids, a feature of arylphorins.

The effects of altering protein quality and quantity in the insect's diet generally showed that the insects could compensate quite well for these changes. However, when the insects were started on the diets containing different proteins as egg masses, high mortality was observed. This was not quantified because the actual number of eggs was not recorded, only the number of egg masses used per experiment. The number of egg masses used for each diet type was similar. Fewer than 60 insects were able to be collected for diets containing gluten, zein or haemoglobin where greater than 187 were collected for casein diets. Food consumption is lower during the early instars, so studying the effects of nutrition on early instar larvae rather than the final instar may show greater effects of altering the dietary nutrition. I think it is necessary to try to eliminate this pest early on before it has the chance to do a great deal of damage to the corn as it does in its final instar when consumption very high.

Studies should be conducted in actual field experiments to determine if the corn borer can compensate for changes in protein quality or quantity on their natural foods and to determine if the relationship between storage proteins and larval weight holds. As well, determining if there is a critical weight or a ‘critical level of storage protein’ in this insect may aid in understanding the biology of this pest.

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Appendix I. Approximate amino acid (%) composition of proteins used in experiments involving protein quality

amino acid	casein (Lawrie, 1970)	albumin (Brown, 1975)	gluten (Bonner, 1950)	zein (Bonner, 1950)	haemoglobin (mammalian) (Darnell *et al.*, 1986)
arginine	4.2	3.4	7.9	1.6	2.1
lysine	8.2	8.6	2.5	0.0	7.6
histidine	3.0	2.8	3.9	0.8	6.6
phenylalanine	5.8	3.9	4.5	6.6	5.2
cysteine	0.4	5.9	4.0	1.0	1.0
methionine	3.3	0.5	2.3	2.5	1.0

Appendix II. Percent of total soluble protein that was extracted from samples when determining the amount of total protein

Trial	Extraction 1	Extraction 2	Extraction 3	Extraction 4	Extraction 5
1	0.71	0.16	0.06	0.04	0.03
2	0.74	0.14	0.05	0.04	0.02
3	0.62	0.22	0.05	0.06	0.04
4	0.67	0.20	0.06	0.04	0.02
5	0.73	0.15	0.05	0.04	0.02
6	0.76	0.13	0.05	0.04	0.03
average	0.70	0.19	0.05	0.04	0.03
approximate % extracted in 3 extractions			94%		

(see Materials and Methods section 2.2.3 for details)