Is Trypsin Inhibitor a Plant Defense Against the Larvae of the Forest Tent Caterpillar *(Malacosoma disstria)*?

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Abstract

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Forest tent caterpillars (*Malacosoma disstria*) are ubiquitous defoliating insects in North America. In Canada, trembling aspen is their favourite host tree, which can produce an inducible protein, trypsin inhibitor (TI), to inhibit trypsin, a major proteolytic enzyme in caterpillars' midguts.

To determine the digestion-inhibiting effect of TI on *M. disstria*, this study focused on the performance of second instar larvae with different levels of TI on both balanced and low protein diets. Indices of performance were growth, food consumption, nutrient utilization efficiencies and nitrogen concentration in the frass under the treatments of different diet quality and different levels of TI extracted from both soybean and aspen. Both protein deficiency and higher levels of TI impaired the growth of caterpillars, but the impairing effect of TI only appeared on the caterpillars fed on balanced diet.

Also, both soybean and aspen TI reduced food consumption and increased the nitrogen concentration in the frass, which was likely due to undigested protein in the frass. But only the aspen TI affected the nutrient utilization efficiencies in terms of decreased approximate digestibility (AD) and increased efficiency of conversion of ingested materials (ECI), which suggests a strategy used by caterpillars to utilize the nutrient more efficiently under aspen TI inhibition of the protein digestion.

This study confirmed the digestion-inhibiting function of TI as a defense of aspen against forest tent caterpillars. Also, it suggested that caterpillars were able to regulate their growth under severe protein deficiency, possibly as an adaptation to this defense.

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Chapter 1

General Introduction

Plants and herbivores have been co-evolving for thousands of years. Through this co-evolution, plants have developed numerous defence mechanisms to withstand the damage and stresses caused by herbivores, pathogens and also many abiotic factors (Birkett 1999). One of these mechanisms involves the production of secondary metabolites by host trees, like cyanogenic glycosides and phenols, especially tannins (Fitzgerald 1995).

A group of plant compounds known as secondary metabolites defend plants against a variety of herbivores and pathogenic microbes. They appear to have no direct function in growth and development, and also have restricted distribution in the plant kingdom (Taiz and Zeiger 2002). These compounds include three major groups: terpenes, phenolic compounds and nitrogen-containing compounds, where the last group exists in a large variety of plants (Taiz and Zeiger 2002). For instance, seeds and storage organs of plants, such as legumes, are particularly rich in nitrogen as a production of a variety of nitrogen-rich defensive compounds (Jongsma and Bolter 1997, Pilon *et al.* 2006).

Among the diverse plant defense components in the nitrogen-containing group, there are proteins that interfere with herbivore digestion (Taiz and Zeiger 2002) and nutritional absorption (Peumans and Van Damme 1995). The best known anti-digestive proteins produced by plants are the protease inhibitors (PIs) found in soybeans, tomatoes, and other plants, which can inhibit the digestion of dietary protein in herbivores' midguts by binding tightly and specifically to the active site of proteolytic enzymes (Taiz and

Zeiger 2002). PI ingestion leads to a reduction in the activity of specific digestive proteases, and affects the growth and survival of susceptible insects (Michaud 2000).

Nearly two thirds of the world's species are insects, and they are able to successfully exploit a wide range of food sources (Terra *et al.* 1996). The main herbivory pressure on plants comes from insects, and their digestive capacity depends on the enzymes present in the insect's gut (Terra *et al.* 1996). The knowledge of insect digestion is a prerequisite for developing methods of control that act via the gut, such as the use of transgenic plants to control phytophagous insects (Terra *et al.* 1996).

Digestive proteases in the midgut region catalyze the release of peptides and amino acids from dietary protein (Jongsma and Bolter 1997). They are divided into four subclasses: serine, cysteine, aspartate and metalloproteases, on the basis of their catalytic mechanism, as shown with specific pH value (Terra *et al.* 1996, Zhu-Salzman *et al.* 2003). Serine proteases are alkaline because they have serine and a histidine in the active site (Terra *et al.* 1996). They are the best-studied proteases present in various organisms (Pilon *et al.* 2006) and also the prevailing proteases in the majority of insect species (Terra and Ferreira 1994). Lepidoptera generally use serine proteases to digest proteins so they have alkaline midguts to provide optimal pH between 8 and 11.5 for the activity of this class of proteases (Jongsma and Bolter 1997). Insects normally contain serine proteinases in their midguts (McFarlane 1985), like trypsin and chymotrypsins, which act on a wide range of physiological process including digestion, protein activation or even antibacterial activities (Gormam and Paskewitz 2001, Ma and Kanost 2000).

Trypsin is a serine proteinase that preferentially cleaves protein chains on the carboxyl side of basic L-amino acids such as arginine or lysine. Trypsin isolated from

lepidopteran insects has higher pH optima, corresponding to the higher pH values found in their midguts, and is unstable in acid pH (Terra *et al.* 1996). However, other classes of proteases, like cysteine and aspartate proteases, which are slightly acid (pH 5-7), are found in some species of insects as well (Christeller *et al.* 1992, Terra and Ferreira 1994). The use of cysteine proteases may have been an evolutionary adaptation that allows insects to eat plant tissues that are normally high in serine protease inhibitors (Ryan 1990).

Serine protease inhibitors are common natural products in plants which affect the growth and development of lepidopteran larvae (Broadway 1997). Normally, they are found in seeds and reserve organs, but can also be induced by wounding in leaves (Schaller and Ryan 1995). Insects that feed on plants containing protease inhibitors may suffer reduced rates of growth and development (Taiz and Zeiger 2002). However, not all insects with serine proteases are susceptible to serine protease inhibitors (Broadway 1997). Larocque and Houseman (1990) demonstrated that serine protease inhibitors from various sources had different effects on growth of larvae of *Ostrinia nubilalis*. Thus, it is important to determine the interactions between plant inhibitors and insect proteases.

Trypsin inhibitors (TIs) are competitive inhibitors of serine protease in the midgut of insects produced by their host plants (Broadway 1995). Negative effects, such as reduced growth, delayed development and even mortality of TIs on insects, especially Lepidoptera, were found in different species of plants and also in the expression of TIs in transgenic plants (Johnston *et al.* 1993, McManus *et al.* 1999, Paulillo *et al.* 2000). Based on the studies that trypsin inhibitors in the insect diet reduced the average growths of larvae, it is suggested that the inhibitors interfere with proteolysis and effectively starve the larvae of protein (Johnston *et al.* 1993).

However, more and more studies suggest that not all trypsin inhibitors have inhibition effects on all insect species (Broadway 1997, Johnston *et al.* 1993, Lindroth *et al.* 1986). Moreover, some studies based on induced protease inhibitors demonstrate that several insect species become adapted and resistant to the defence of their hosts. It has been noted that there is a critical concentration for insects to be resistant to a trypsin-inhibitor benzamidine, where the insect adopts strategies to cope with the inhibition at the highest concentration, such that they performed worst on the 50% but not 75% (Pilon *et al.* 2006). It is possible that instead of eliminating proteolytic digestion in the midgut of insects, ingestion of protease inhibitors results in hyperproduction of proteolytic enzymes (Broadway 1995) or production of other types of proteases (Ryan 1990) under this adaptation. The ingestion of protease inhibitors has been proposed to sensitize feeding insects, which become resistant to the effects of the introduced inhibitor gene products in the hosts (Broadway 1995, Jongsma and Bolter 1997).

Trembling aspen (*Populus tremuloides*) is a widespread forest tree in North America and is susceptible to damage by a variety of lepidopteran pests. In many parts of its range, it succumbs to massive defoliation by periodic outbreaks of forest tent caterpillars (Haruta *et al.* 2001). Delayed inducible plant defense is proposed to be a driving force of insect outbreaks (Haukioja 1980). Protease inhibitors are often an inducible defense in leaves, thus understanding trembling aspen defenses is of ecological importance from both practical and theoretical perspectives (Haruta *et al.* 2001).

The use of naturally occurring plant protease inhibitors to target insect digestive enzymes has been considered as a means of insect pest management (Ahn *et al.* 2004). However, efforts to achieve host plant resistance by expressing protease inhibitors in

transgenic plants have been largely unsuccessful (Cloutier *et al.* 2000, De Leo *et al.* 1998, Jongsma and Bolter 1997). In the current study, the main aim is to test the effect of the naturally extracted trypsin inhibitor on the herbivore Lepidoptera *Malacosoma disstria* (forest tent caterpillar) larvae. Performance was based on body mass, nutrient utilization efficiency and nitrogen analysis of the frass.

The forest tent caterpillar (*Malacosoma disstria*) is one of the most ubiquitous defoliating insects found in North America (Parry and Goyer 2004). They are phytophagous insects whose geographic ranges exceed the distribution of any single primary host plant but may adapt to regional hosts and have better performance on these locally available species (Parry and Goyer 2004). The range of its habitat is from southern Texas (≈29° latitude) to northern Canada (≈62° latitude) and from the Atlantic to the Pacific Oceans (Brandt *et al.* 1996). Across this vast range, forest tent caterpillars use a diverse array of host species and occur in a variety of forest ecosystems with almost the same life history (Fitzgerald 1995, Parry and Goyer 2004). Oviposition and early-instar feeding occur on only one or a few hosts within a region. Across much of Canada, the primary host trees are trembling aspen (*Populus tremuloides*), oaks (*Quercus* spp.) and sugar maple (*Acer saccharum*) (Parry and Goyer 2004).

Forest tent caterpillars attack trees just as the season's new foliage is forming, and their seasonal development is closely tied to that of the host plant. During prolonged outbreaks, they may not just defoliate but also eventually kill their hosts (Fitzgerald 1995). However, when aspen forests experience heavy defoliation, individual trees exhibiting only light defoliation are commonly observed (Lindroth and Bloomer 1991). Variation in nutritional value and secondary compounds of foliage among trees could be the reason. It is

shown that the phenolic glycosides tend to reduce growth of *M. disstria* larvae fed low protein diet, which suggests an interaction effect between nutrition and secondary plant compounds (Lindroth and Bloomer 1991). Furthermore, it is suggested that the potency of a protease inhibitor can be influenced by the dietary quality, such as protein quality and quantity (Broadway and Duffey 1988, Oppert *et al.* 1993). Thus, protein deficiency may lead to the enhanced effect of protease inhibitors on caterpillars.

Insects can pre-ingestively regulate daily food consumption volumetrically and post-ingestively regulate the nutrient utilization efficiency to reach their growth target (Simpson and Raubenheimer 2001). They are able to regulate growth by respiring excess ingested carbohydrate and excreting excess ingested nitrogen (Simpson and Raubenheimer 1993). The nitrogen content of leaves has a marked influence on the growth rate and also the reproductive fitness of forest tent caterpillars since they do not feed at all as adults (Fitzgerald 1995, Despland and Noseworthy 2006). When nitrogen is in short supply, caterpillars have to process large quantities of food to meet their needs for protein (Fitzgerald 1995). Despland and Noseworthy (2006) also demonstrated that forest tent caterpillars perform best on the balanced food or slightly protein biased food. Since the trypsin inhibitor inhibits the digestion and even utilization of ingested protein, more deleterious effects from TIs are expected on forest tent caterpillars fed on the diet lacking protein.

The objective of this study is to determine the effect of naturally produced trypsin inhibitor on the performance of early instar forest tent caterpillars feeding on diet with three different levels of trypsin inhibitors (TIs). Since nutritional value plays important roles in the plant-insect interaction in nature (Lindroth and Bloomer 1991), at the same

time three types of nutritional diets will also be used to determine how the caterpillars will perform with the combined effect of the protein deficiency and TI inhibition. The three nutrition diets include two artificial diets with different protein to carbohydrate ratios and also hybrid poplar foliage, which is utilized as a control set of artificial diets to provide equivalent nutrition as in nature. Due to the inhibition of protein digestion by the trypsin inhibitor, we hypothesize that forest tent caterpillars will perform worse on diets with higher levels of TIs, especially on the protein deficient foods. Also, the nitrogen percentage in the frass is expected to be higher with higher levels of TI, because of the lack of efficient digestion of the protein in the presence of TI.

Chapter 2

Trypsin inhibitor effect on growth, food consumption and three parameters of nutrient utilization efficiency in forest tent caterpillars

2.1 Abstract

The inhibiting effect of plant trypsin inhibitors was tested by evaluating performance (growth, food consumption and three parameters of nutrient utilization efficiency (AD, ECD and ECI)) of 2nd instar forest tent caterpillars fed on both nutritional balanced diet (protein21%: carbohydrate21%) and protein deficient diet (protein14%: carbohydrate28%) with three levels of trypsin inhibitor (0%, 0.04% and 0.2%) extracted from both soybean and aspen.

Caterpillars performed worse on protein deficient diet in terms of reduced body mass at the moult to the next instar. At the same time, higher levels of TI (both from soybean and aspen) decreased food consumption. TI consumption decreased the growth of caterpillars fed on balanced diet but not of those on protein deficient diet, indicating growth regulation of caterpillars when both their ingestion and digestion of protein were limited.

Only aspen TI influenced the nutrient utilization efficiencies in terms of decreased approximate digestibility (AD) and increased efficiency of conversion of ingested material (ECI). AD decreased, as we predicted, due to the TI inhibition on digestion of protein, indicating the TI from aspen was more effective than the one from soybean. An unexpected

result of this study was the increased ECI with higher levels of TI, suggesting that caterpillars can increase their efficiency of converting ingested materials to body mass to regulate growth.

2.2 Introduction

In response to extrinsic and intrinsic factors, organisms can alter food intake, utilization and allocation actively and dynamically in an adaptive manner. For insect larvae, the growth rate, development time, final body weight and even survival will all be affected by food intake and also the quality of the food ingested (Slansky and Scriber 1985). Thus the knowledge of food intake responses by insects is important in understanding plant-insect interactions (Slansky & Rodriguez 1987). It is demonstrated by Slansky and Wheeler (1989) that increases in food consumption and utilization efficiencies allow an insect to reach its normal growth rate in response to reduced nutrient levels. But in other cases, growth rate may decline in spite of the increased consumption but to a lesser degree (Slansky and Wheeler 1989). However, on foods containing toxin extracts from plants, for example, azadirachtin, food intake is reduced or even suppressed depending on the insect species and the concentration of the extract (Martinez and van Emden 1999). The insect gut is not only the major digestive organ, but is a frontline of defence against a broad spectrum of dietary toxins and antinutritional factors (Ahn *et al.* 2004). Insects often possess multiple protease genes encoding various protease isoforms with different sensitivities to cope with dietary challenges. This capability of adaptation to plant defenses is a major obstacle in pest management (Moon *et al.* 2004).

The defensive function of inhibitors is suppressing insect digestive enzymes, consequently causing insect starvation (Zhu-Salzman *et al.* 2003), which directly influences the growth, development and even reproduction of insects. For instance, Sweet potato (*Ipomoea batatas*) trypsin inhibitor was shown to negatively affect second instar

larvae of *Spodoptera litura* in terms of reduced survival and decreased biomass (Yeh *et al.* 1997). However, some species of phytophagous insects have circumvented the effects of plant protease inhibitors (Harborne 1993). Negative impacts of dietary protease inhibitors vary with insect developmental stage (Orr *et al.* 1994), and normally, earlier instar larvae are more sensitive to dietary protease inhibitors. It is suggested that the soybean trypsin inhibitors affect growth and digestive physiology of *Spodoptera litura in vitro*, but ingestion of this inhibitor retards growth rate only in neonate larvae and has no effect on older larvae (McManus and Burgess 1995).

At the same time, protease inhibitors demonstrate a significant degree of specificity towards insect pests (Johnson *et al.* 1989, Broadway 1996). It is recorded that chronic ingestion of soybean trypsin inhibitor reduced the growth of *Agrotis ipilon* but not *Helicoverpa zea* (Broadway and Villani 1995). Thus, the appropriate inhibitors for the targeted digestive protease of the insect pest have to be selected (Oliveira *et al.* 2005). In concert with protease inhibitor specificity, the plant background, in which trypsin inhibitors accumulate, may also be an important factor in determining the efficacy of these inhibitors (McManus *et al.* 1999).

Furthermore, the concentrations of PIs vary depending on the source of material in plants (Jongsma and Bolter 1997). Soybean is an important crop, which has inducible serine protease inhibitors as defense against insect pests (Pilon *et al.* 2006). Dry soybeans have protein concentrations of 20-36% (w/w) with up to 6% of total protein consisting of PIs (Jongsma and Bolter 1997), which means up to 1.2-2.2% (w/w) of dry soybeans are PIs. However, protein concentrations are much lower in fresh soybeans (5%) so that the actual concentrations of PIs are only about 100-200 μM (Jongsma and Bolter 1997). In leaves, the

PI levels induced by feeding are even lower, in the range of 10-50 μM (Jongsma *et al.* 1994). Theoretically, the minimal concentration of PIs to achieve full inhibition of gut protease activity is in the range of 10-30 μM, corresponding to about 0.5-1.5% of total soluble protein in leaves. In most plant tissues, PI concentrations are only in slight excess over the insect proteases (Jongsma and Bolter 1997).

Trembling aspen (*Populus tremuloides*) is a widely distributed tree throughout most of Canada (Lindroth 1991). Trypsin inhibitors constitute an inducible protein-based defense system of trembling aspen against insect herbivores (Haruta *et al.* 2001). At least three to four Kunitz type TIs in trembling aspen were estimated by Haruta *et al.* (2001), which suggests the importance of a battery of TIs with differing specificities for gut proteases for defending against different herbivores with distinct digestive enzymes. Five distinct TI sequences in a genomic library were identified by Hollick and Gordon (1993) in hybrid poplar. The presence of several related TI genes in trembling aspen may, therefore, be an adaptation to the diversity of digestive enzymes in insects (Haruta *et al.* 2001).

Aspen trees are not only defoliated by forest tent caterpillars (Lindroth and Bloomer 1991) but also by other insects, like the tiger swallowtail butterfly (*Papilio glaucus*) (Lindroth *et al.* 1986). However, in many parts of their range, they are massively defoliated by periodic outbreaks of forest tent caterpillar (*Malacosoma disstria*) (Haruta *et al.* 2001). In the Great Lakes region, outbreaks of the forest tent caterpillar may cover thousands of square miles (Lindroth 1991). Since animals obtain energy and nutrients from food, diet can be considered a key factor that potentially affects all life-history components (Sterner and Schulz 1998, Taylor *et al.* 2005).

The caterpillars must process large quantities of food because they are able to capture only a small fraction of the total energy obtained from the food (Fitzgerald 1995). Of the energy contained in the food that the caterpillars ingest (*Ie*), an assimilated fraction (*Ae*) is used for growth (*Ge*), and the balance of the ingested energy is egested as faecal pellets (*Fe*) or used in respiration or the formation of silk (Fitzgerald 1995). There are three measures of nutrient utilization efficiency (Waldbauer 1968, Futuyma and Wasserman 1981):

1) ECI (the efficiency of conversion of ingested material), calculated as *Ge/Ie*, is the gross growth efficiency measuring the fraction of ingested material that is utilized for growth;

2) AD (approximate digestibility), calculated as *(Ie-Fe)/Ie,* is the fraction of ingested food that is assimilated;

3) ECD (the efficiency of conversion of digested material), calculated as *Ge/(Ie-Fe)*, is the net growth efficiency measuring the proportion of assimilated material used for growth.

There are studies of nutrient utilization efficiency under treatments with different qualities of food in other insect species (Futuyma and Wasserman 1981, Martinez and van Emden 1999, Slansky and Wheeler 1989). It is suggested by Martinez and van Emden (1999) that in *S. littoralis*, sublethal concentration of azadirachtin did not affect digestibility but reduced ECI and ECD because of increased energetic costs arising from a reduced ability to utilize dietary nitrogen. However, different results from azadirachtin were observed in another species, *Heliothis virescens* larvae, that digestibility was increased with a decreased rate of the passage of the food in the gut (Barnby and Klocke

1987). All the results suggest that AD, ECD and ECI are correlated in the digestion process but they are the results of various effects showing different feeding and utilization mechanisms. These indices will be used to explore the mechanisms by which TIs affect forest tent caterpillars.

This study focuses on the effect of trypsin inhibitor extracted from both soybean and the host aspen with three different levels: 0% (w/w percentage of wet weight), 0.04% (the normal level found in natural aspen foliage) and 0.2% (highest level used in transgenic plants). Meanwhile, two different nutrient ratios of protein and carbohydrate were used in the artificial diet to investigate the combinational effect of protein deficiency and TI inhibition: protein21%: carbohydrate21% (balanced diet) and p14: c28 (protein deficient diet) (Despland and Noseworthy 2006). The foliage of hybrid poplar painted with the same three levels of TI were fed to caterpillars in experiment 1 using soybean TI extracts, but not in experiment 2 with aspen TI extracts, because caterpillars did not perform very well on it during the first experiment. The development time, moulting weight, food consumption and the three nutrient utilization efficiency parameters (AD, ECD and ECI) were examined to determine the performance of second instar larvae of *M. disstria* under the different treatments. With higher levels of TI, we predicted decreased growth, food consumption and nutrient utilization efficiency, and also longer development time, especially on the diet when the protein was deficient.

2.3 Experiment 1

Effect of the trypsin inhibitor extracted from soybean

2.3.1 Methods and materials

2.3.1.1 Experimental insects and diets

Forest tent caterpillar colonies were reared from egg masses collected 40 km Northeast of Wabasca, Alberta (56°17.5'N, 113°93.9'W). The egg bands were soaked for 1 m 30 s in pure Javex (6% sodium hypochlorite), and then rinsed under cool running tap water for 5 minutes. After that, they were rinsed in 1% Javex (0.06% sodium hypochlorite) and then air-dried.

During the first instar, the caterpillars were separated into two groups and were reared on either modified artificial diet (Addy 1969) or hybrid (H11/11) poplar foliage. Once they moulted to the second instar, the larvae were split into 9 treatments, with 4 replicates of 10 larvae per treatment. The treatments consisted of three different diets with three different levels (0%, 0.04% and 0.2%) of Trypsin inhibitor (soybean extract from SIGMA-ALDRICH Co). The three different diets were two kinds of artificial diet with different protein to carbohydrate dry weight concentrations: 21% protein: 21% carbohydrate, 14% protein: 28% carbohydrate, as well as hybrid poplar foliage. The larvae initially reared on foliage were assigned to the foliage treatments, and the larvae reared on artificial diet were assigned to the artificial diet treatments. The three different levels of trypsin inhibitor were 0%, 0.04% (the normal level found in natural aspen foliage), and

0.2% (the highest level expressed in transgenic plants). Other components of the artificial diets were salt, cholesterol, anti-fungal agents, cellulose, vitamins and lipid. The diets were a composite of 6: 1 agar solution: dry ingredients ratio. The hybrid poplar plants, supplied by Université Laval (Quebec City), were reared in a greenhouse (20 °C) and leaves were freshly collected. They were then placed in flower picks containing water and painted on the back surface with trypsin inhibitor solution in different concentrations using a micropipette.

Before starting the treatment, the 2nd instar caterpillars were deprived of food for 2 hours right after they moulted (del Campo and Renwick 1999) and then the group weights for each replicate per treatment were recorded. Each piece of food, including the leaves, was weighed. The groups were kept in 10 cm (diameter) Petri dishes that were lined with a layer of paper towel and wax paper to maintain moisture. Then the Petri dishes were set in the growth chamber where the temperature was 22°C and the humidity was 70% on a 16:8 hour photoperiod. The caterpillars were checked daily and their moulting dates were recorded.

Three days after the initiation of the experiment, all the caterpillars were weighed individually on an analytical balance then put back into the growth chamber. Once each caterpillar moulted to the 3rd instar, it was weighed again. These caterpillars were then frozen and were placed in a drying oven at 40°C for two days to weigh in-group. When all caterpillars in a group moulted, the remaining food and frass were also dried in the oven at 40°C in order to record the dry weight for the calculation of the group food consumption and the three gravimetric parameters of the nutrient utilization efficiency.

Since the dry weight of the food and the caterpillars could not be obtained at the beginning, 12 pieces of artificial food, 10 hybrid poplar leaves and 12 2nd instar caterpillars, which were all under the same condition as the ones in the experiment, were picked out for a dry: wet regression. These were dried in oven at 40°C and the final dry weight was regressed against initial wet weight to obtain the slope of the relationship between the two.

2.3.1.2 Data Analysis

Nested two-way analyses of variance (ANOVAs) were used to determine the effects of the nutritional diets and the trypsin inhibitor on individual caterpillar growth (nesting replicates within treatments). This analysis also showed whether there were differences among replicates in each treatment.

Two-way factorial analysis of variance (ANOVA) was performed to test the effects of the nutritional diets and the trypsin inhibitor on the food consumption, which was calculated per group of each replicate. The food consumption was expressed as the dry weight of food ingested by the entire group in each replicate. Group values were also used to calculate the gravimetric indices.

The analyses of co-variance (ANCOVAs) were performed to determine the effects of the nutritional diets and the trypsin inhibitor on the caterpillar's process of digestion and nutrient utilization, which were measured in three gravimetric parameters: the approximate digestibility (AD), the efficiency of conversion of digested material (ECD) and the efficiency of the conversion of ingested material (ECI) (Waldbauer 1968). AD was

examined by analysis of covariance, comparing the amount of food digested between treatments with the amount of food ingested as the covariate. ECD was examined by analysis of covariance, comparing the amount of weight gain between treatments with the amount of food digested as the covariate, while ECI was examined by analysis of covariance, comparing the amount of weight gain with the amount of food ingested as the covariate.

Linear regressions were used to obtain the slope of the relationship between fresh weight and dry weight.

2.3.2 Results

2.3.2.1 Larval growth

Three days after the start of the experiment, the nested ANOVA showed that both the diet that the larvae were reared on and the trypsin inhibitor significantly influenced larval weight [effects of nutrition, $F_{(2,322)} = 401.440$, $P < 0.001$; effects of TI, $F_{(2,322)} = 3.486$, $P = 0.032$]. The interaction between nutritional diet and the TI was also significant [$F_{(4,322)} = 4.760$, $P = 0.001$]. The caterpillars reared on foliage have much lower larval weights than the ones reared on artificial diets. When looking at the artificial diets treatments (p21:c21 and p14:c28), only the TI and the interaction between diet and TI influenced the larval weight significantly [effects of TI, $F_{(2,215)} = 3.461$, $P = 0.033$; effects of interaction, $F_{(2,215)} = 5.029$, $P = 0.007$]. The different artificial diets did not have significant effects on the larval weight [$F_{(1,215)} = 1.925$, $P = 0.167$]. The caterpillars fed on the p21:c21 diet and the p14:c28 diet were of similar weights after three days of feeding. However, TI in the diet decreased caterpillar weight on the p21:c21 diet but not on the p14:c28 diet (seen in Fig. 1).

However, the difference among replicates for each treatment was significant as well (all treatments included, $F_{(27,322)} = 5.877$, $P < 0.001$; only the treatments with artificial food treatments included, $F_{(18,215)} = 5.382$, $P < 0.001$).

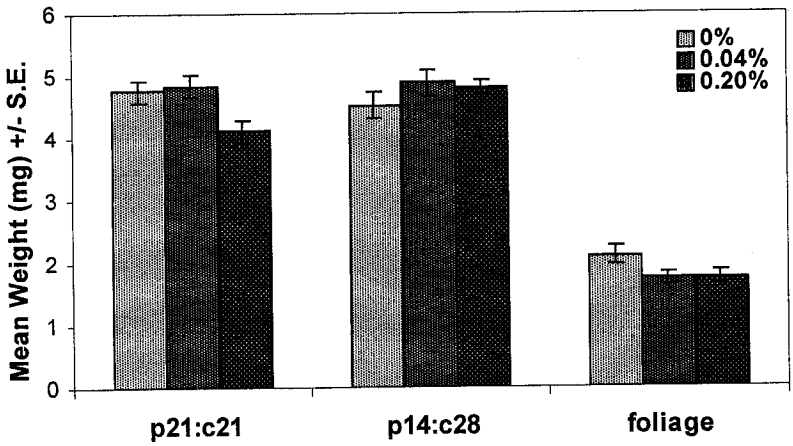

Figure 1: Individual 2nd instar larval fresh weights after 3 days of the beginning of the experiment for each treatment with different diets (p21:c21, p14:c28 and foliage) and different levels of TI (0%, 0.04% and 0.2%).

When the caterpillars moulted to the 3rd instar, 6 days after the initiation of the experiment, the nested ANOVA showed that both the diet and the level of trypsin inhibitor in the diet significantly influenced the larval weights [effects of nutrition, $F_{(2,286)} = 159.614$, $P < 0.001$; effects of TI, $F_{(2,286)} = 13.406$, $P < 0.001$, Figure 2.]. The interaction between nutritional diet and the level of TI also significantly affected the larval weights [$F_{(4,286)} = 6.570$, $P < 0.001$]. The caterpillars reared on foliage still had much lower larval weights than the ones reared on artificial diet. Among the artificial diet treatments, both the nutrition and the level of trypsin inhibitor had significant effects on the larval weight, as well [effects of nutrition, $F_{(1,215)} = 12.846$, $P < 0.001$; effects of TI, $F_{(2,215)} = 9.398$, $P < 0.001$]. Moreover, the interaction between the nutrition and the TI significantly influenced the larval weight [$F_{(2,215)} = 9.718$, $P<0.001$]. Caterpillars fed the p14:c28 diet had lower larval weight than caterpillars on the p21:c21 diet at moult to the third instar. The interaction between the nutritional diet and the TI showed the caterpillars fed on the

p21:c21 diet gained less weight when the TI level was higher but the ones fed on the p14:c28 diet performed similarly among the three levels of TI (Fig. 2). Although the difference among replicates for each treatment was significant (all replicates included, $F_{(27,322)} = 3.425$, $P < 0.001$; only the replicates with artificial food treatments included, $F_{(18,215)} = 3.519$, $P < 0.001$), it is not as significant as the difference among treatments because of lower F values.

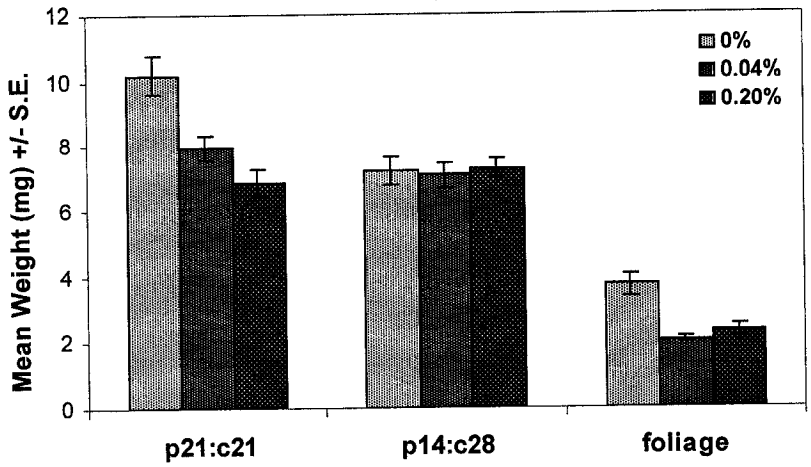

Figure 2: Individual 3rd instar larval fresh weight right after moulting for each treatment with different diets (p21:c21, p14:c28 and foliage) and different levels of TI (0%, 0.04% and 0.2%).

2.3.2.2 Food Consumption

The two-way factorial ANOVA showed that both nutritional diet and the level of trypsin inhibitor significantly affected the food consumption of caterpillars in each group [effects of nutrition, $F_{(2,27)} = 57.132$, $P < 0.001$; effects of TI, $F_{(2,27)} = 18.464$, $P < 0.001$; effects of interaction, $F_{(4,27)} = 3.443$, $P = 0.021$]. Since the caterpillars reared on foliage always performed unusually and did not eat much, the significant effect of nutrition was

only from the difference between artificial diet groups and foliage groups. When comparing the p21: c21 and p14: c28 diet groups, there was no significant difference [$F_{(1,18)} = 0.850$, P = 0.369], and no interaction effects [$F_{(2,18)} = 0.358$, P = 0.704]. However, the different levels of TI still had significant effect [$F_{(2,18)} = 20.344$, P < 0.001, Fig. 3]. The results suggested that the higher the level of TI, the less the caterpillars ate (Fig. 3).

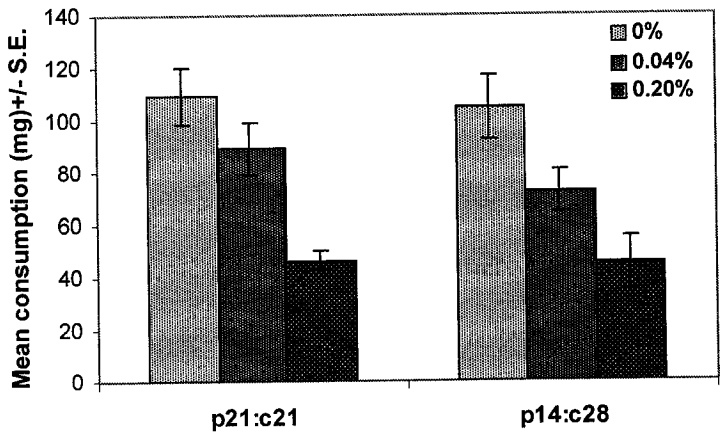

Figure 3: Dry mass of consumed food in each replicate during the whole 2nd instar for each treatment with different diets (p21:c21, p14:c28) and different levels of TI (0%, 0.04% and 0.2%).

2.3.2.3 Measures of nutrient utilization process

Looking at the three gravimetric parameters describing the digestion of food and the usage of energy, only the six treatments with artificial diet were analysed because caterpillars feeding with foliage performed poorly and were unhealthy. To determine the effect of nutrition and of soybean trypsin inhibitor on the three parameters, the analyses of co-variance (ANCOVA) were performed.

For the approximate digestibility (AD), no effect was seen either from the nutritional diets or different levels of TI (Table 1: nutrition and TI terms, and Fig. 4b). And no effect was seen from the interaction between the nutrition and the TI (Table 1: interaction term, Fig. 4b). The amount of digested material only depended on the total amount of ingested food (Table 1: ingested food term, and Fig. 4a).

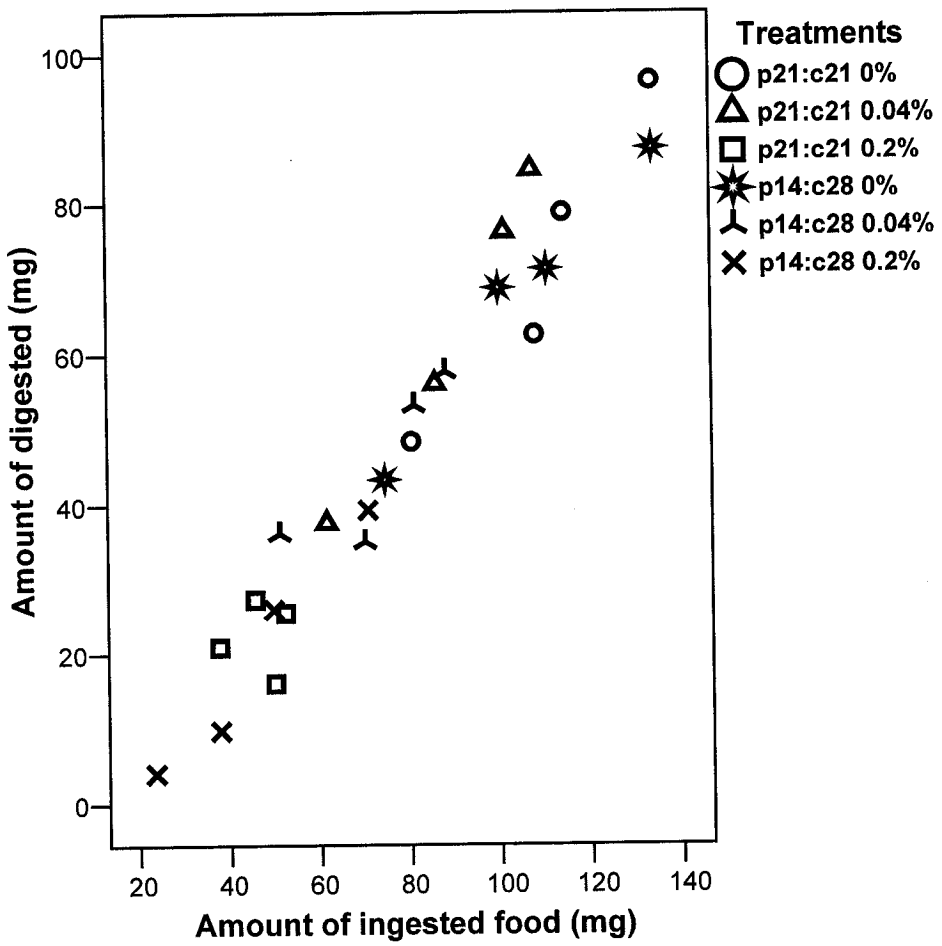

Figure 4: a) Scatter plot of the amount of dry digested material on the amount of dry ingested food for each group, which represents approximate digestibility (AD), for 2nd instar larvae reared on different diets (p21:c21 and p14:c28) with different levels of TI (0%, 0.04% and 0.2%) extracted from soybean.

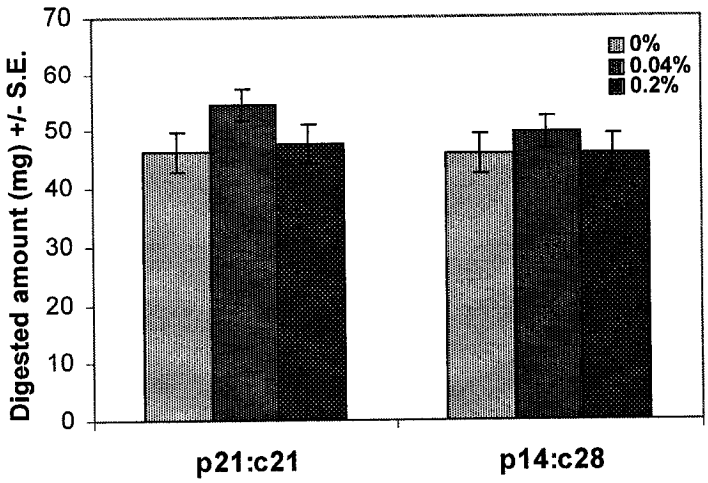

Figure 4: b) The amount of digested material (evaluated at the value of ingested food: 77.9546 mg). The estimated marginal means is the average dry amount of digested food corrected for the total amount of dry ingested food (co-variate).

Dependent Variable: Digested material

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Ingested food	4317.487	1	4317.487	137.167	< .001
Nutrition	31.872	1	31.872	1.013	.328
TI	174.199	2	87.100	2.767	.091
Interaction Nutrition * TI	19.973	2	9.987	.317	.732
Error	535.095	17	31.476		
Total	71736.030	24			
Corrected Total	15426.468	23			

Table 1: ANCOVA results for the effect of two factors on the amount of dry digested material, standardized for total amount of dry ingested food. The fixed factors were the nutrition and the TI, and the co-variate was the amount of dry ingested food. df = degrees of freedom, F is the test statistic, and Sig. is the p value ($\alpha = 0.05$).

The efficiency of conversion of digested material (ECD) was slightly, but not significantly, affected by the level of TI (Table 2: TI term, and Fig. 5b). Neither the nutritional diets nor the interaction between nutrition and TI had a significant effect (Table 2: nutrition and interaction terms, and Fig. 5). The amount of grown body mass was not affected by the total amount of the digested food (Table 2: digested food term, and Fig. 5a).

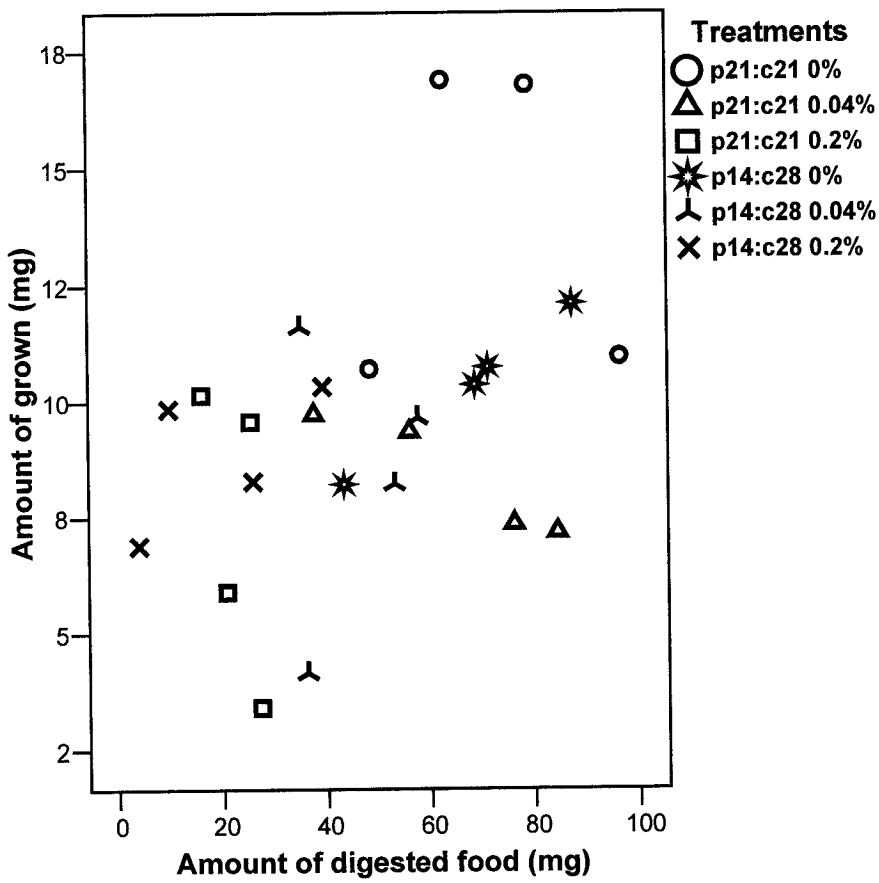

Figure 5: a) Scatter plot of the amount of dry grown body mass on the amount of dry digested food for each group, which represents the efficiency of conversion of digested material (ECD), for 2nd instar larvae reared on different diets (p21:c21 and p14:c28) with different levels of TI (0%, 0.04% and 0.2%) extracted from soybean.

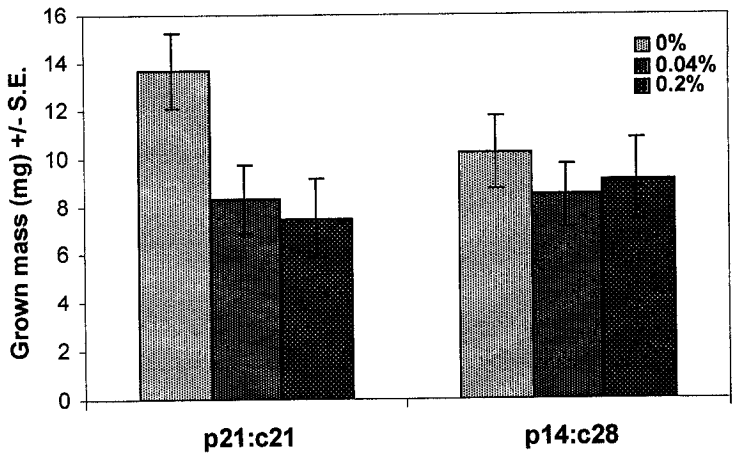

Figure 5: b) The amount of dry grown body mass in each replicate (evaluated at the value of digested food: 48.4379 mg). The estimated marginal means is the average dry weight corrected for the total amount of dry digested food (co-variate).

Dependent Variable: Grown body mass

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Digested food	.332	1	.332	.048	.829
Nutrition	1.762	1	1.762	.257	.619
TI	43.255	2	21.627	3.155	.068
Interaction Nutrition * TI	27.003	2	13.502	1.969	.170
Error	116.551	17	6.856		
Total	2399.969	24			
Corrected Total	226.743	23			

Table 2: ANCOVA results for the effect of two factors on the amount of dry grown body mass, standardized for total amount of dry digested food. The fixed factors were the nutrition and the TI, and the co-variate was the amount of dry digested food. df= degrees of freedom, F is the test statistic, and Sig. is the p value ($\alpha = 0.05$).

Neither the nutrition nor the level of TI has an effect on the efficiency of conversion of ingested material (ECI) (Table 3: nutrition and TI terms, and Fig. 6). There was no interaction between the nutrition and the TI (as seen in the term of interaction in Table 3). Moreover, the amount of grown body mass was not affected by the amount of ingested food (Table 3: ingested food term, and Fig. 6a).

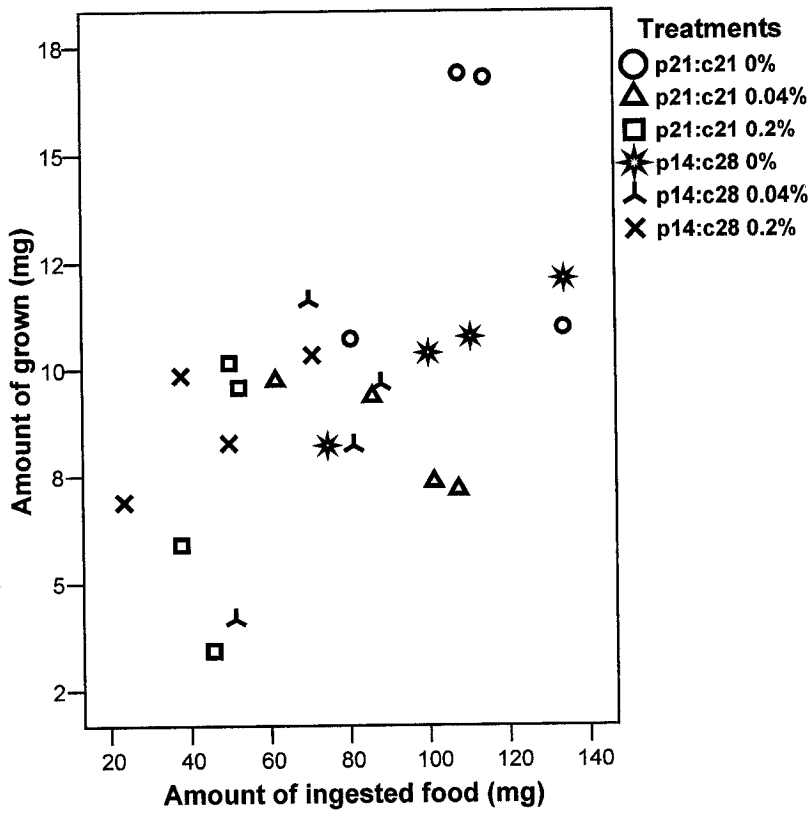

Figure 6: a) Scatter plot of the amount of dry grown body mass on the amount of dry ingested food for each group, which represents the efficiency of conversion of ingested material (ECI), for 2nd instar larvae reared on different diets (p21:c21 and p14:c28) with different levels of TI (0%, 0.04% and 0.2%) extracted from soybean.

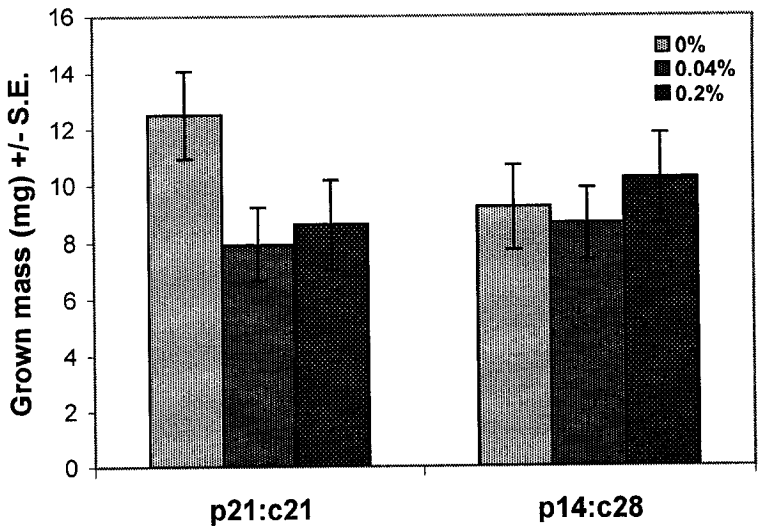

Figure 6: b) The amount of dry grown body mass in each replicate (evaluated at the value of ingested food: 77.9546 mg). The estimated marginal means is the average dry weight corrected for the total amount of dry ingested food (co-variate).

Dependent Variable: Grown body mass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Ingested food	12.175	1	12.175	1.977	.178
Nutrition	.594	1	.594	.096	.760
TI	22.302	2	11.151	1.810	.194
Interaction Nutrition * TI	27.320	2	13.660	2.218	.139
Error	104.708	17	6.159		
Total	2399.969	24			
Corrected Total	226.743	23			

Table 3: ANCOVA results for the effect of two factors on the amount of dry grown body mass standardized for total amount of dry ingested food. The fixed factors were the nutrition and the TI, and the co-variate was the amount of dry ingested food. df= degrees of freedom, F is the test statistic, and Sig. is the p value ($\alpha = 0.05$).

2.3.2.4 Larval development

Most caterpillars fed on the artificial diet moulted to 3rd instar on the same day, and most of the ones fed on the foliage of hybrid poplar did not moult at all. No difference in development time was observed among treatments during the 2nd instar.

2.4 Experiment 2

Effect of the trypsin inhibitor extracted from aspen

2.4.1 Methods and materials

2.4.1.1 Experimental insects and diets

Forest tent caterpillar colonies were reared from egg masses collected from the same place and prepared in the same manner as in Experiment 1.

During their first instar the caterpillars were reared on the same nutritional balanced modified artificial diet (Addy 1969). Once they moulted to the second instar, the larvae were split into 6 treatments, with 6 replicates of 10 larvae per treatment. The treatments consisted of two different diets with three different levels of trypsin inhibitor produced by aspen. The extract from aspen was sent by C. Peter Constabel (University of Victoria, see the reference in Constabel 1999). The two different diets are the same as in Experiment 1 with different protein to carbohydrate dry weight concentrations: 21% protein: 21% carbohydrate, 14% protein: 28% carbohydrate. The three different levels of trypsin inhibitor were still 0%, 0.04% and 0.2%. The other components of the artificial diets were the same as Experiment 1.

Before starting the treatment, the 2nd instar caterpillars were deprived of food for 2 hours (del Campo C. and Renwick 1999) and then the group weights for each replicate per treatment was recorded. Each piece of food was also weighed. The caterpillars were kept in 10 cm (diameter) Petri dishes that were lined with a layer of paper towel and wax paper to

maintain moisture. Then the Petri dishes were set in the chamber using the same conditions as Experiment 1.

Once the caterpillars moulted to the 3rd instar they were weighed individually, then dried in a drying oven at 40°C, and weighed again in-group. The remaining food and frass were also dried in the oven at 40°C in order to record the dry weight for calculation of the group food consumption and the three gravimetric parameters of nutrient utilization efficiency.

To determine the initial dry weight of food and the dry weight of live caterpillars, 20 pieces of artificial food, 18 2nd instar caterpillars and 18 3rd instar caterpillars, which were all under the same condition as the ones in the treatment, were used for a dry: wet regression.

2.4.1.2 Data Analysis

As in Experiment 1, nested analysis of variance (ANOVA) was used to determine the effects of the nutritional diets and the trypsin inhibitor on the caterpillar's growth, which was weighed individually. Two-way factorial analysis of variance (ANOVA) was performed to test the effects of the nutritional diets and the trypsin inhibitor on the food consumption, which were calculated in-group with ten caterpillars for each replicate per treatment. Analyses of co-variance (ANCOVAs) were performed to determine the effects of the nutritional diets and the trypsin inhibitor on the caterpillar's process of digestion and nutrient utilization, the AD, ECD and ECI.

2.4.2 Results

2.4.2.1 Larval growth

Six days after the initiation of the experiment, the caterpillars moulted to the 3rd instar. The nested ANOVA showed that both the nutritional diets and the level of trypsin inhibitor in the diet significantly influenced the larval weight [effects of nutrition, $F_{(1,276)} = 68.461$, $P < 0.001$; effects of TI, $F_{(2,276)} = 16.533$, $P < 0.001$, Figure 7]. The interaction between nutritional diet and the level of TI was also significant [$F_{(2,276)} = 4.506$, $P = 0.012$]. Caterpillars fed on the p14:c28 diet had lower larval weight than caterpillars on the p21:c21 diet after their 2nd instar (Figure 7). The interaction between the nutritional diet and the TI showed the caterpillars fed on the p21:c21 diet gained less weight when the TI level was higher but the ones fed on the p14:c28 diet performed similarly among the three levels of TI (Figure 7). Although the difference among replicates for each treatment was significant as well ($F_{(30,276)} = 6.341$, $P < 0.001$), it is not as significant as the difference among treatments because of lower F values.

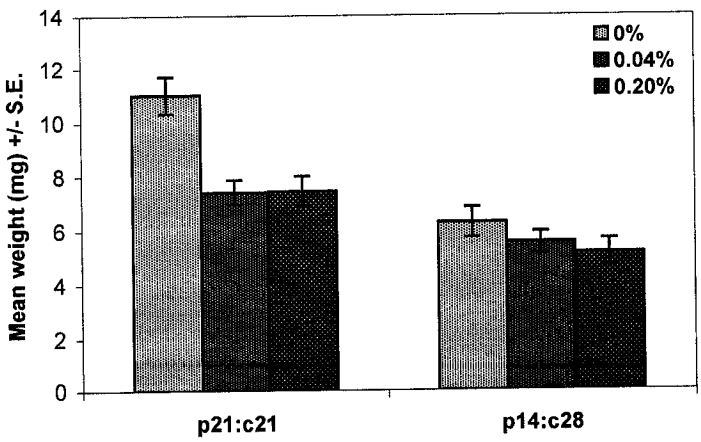

Figure 7: Individual 3rd instar larval fresh weight right after moulting for each treatment with different diets (p21:c21 and p14:c28) and different levels of TI (0%, 0.04% and 0.2%).

2.4.2.2 Food Consumption

Both the nutritional diets and the levels of trypsin inhibitor significantly affected larval food consumption in each group [effects of nutrition, $F_{(1,29)} = 6.129$, P = 0.019; effects of TI, $F_{(2,29)} = 6.933$, P = 0.003]. But there was no interaction between the two factors [$F_{(2, 29)} = 0.271$, P = 0.765]. The results suggested that the larvae had lower food consumption on the p14:c28 diet than on the p21: p21 diet (Figure 8). And also, the higher the level of TI, the less the caterpillars ate (Figure 8).

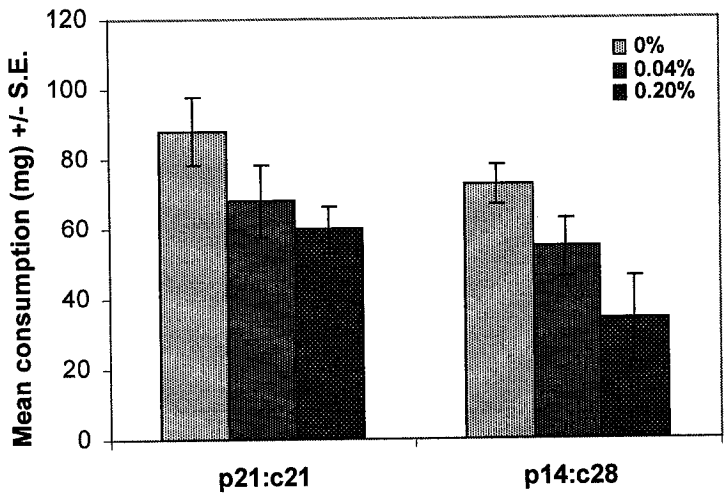

Figure 8: Dry mass of consumed food in each replicate during the whole 2nd instar for each treatment with different diets (p21:c21, p14:c28) and different levels of TI (0%, 0.04% and 0.2%).

2.4.2.3 Measures of nutrient utilization process

To determine the effect of nutrition and the level of trypsin inhibitor extracted from aspen on the three gravimetric parameters (AD, ECD and ECI), analyses of co-variance (ANCOVA) were performed.

Looking at the approximate digestibility (AD), the total amount of ingested food significantly affected the amount of digested material (Table 4: ingested food term, and Fig. 9a). The more the caterpillars ingested, the more they digested. No significant difference was observed between the two nutritional diets, but there was a significant difference among the different levels of TI (Table 4: nutrition and TI terms, and Fig. 9). With higher level of TI, the larvae digested less of the ingested material, suggesting a lower digestibility. At the same time, the interaction between the nutrition and the TI significantly affected the AD as well (as seen in interaction term in table 4, and Fig. 9b). Looking at the amount of digested material based on the same amount of ingested food, there was not much difference among caterpillars reared on the p21:c21 diet with three different levels of TI, but the difference was very obvious among caterpillars reared on the p14:c28 diet with three different levels of TI.

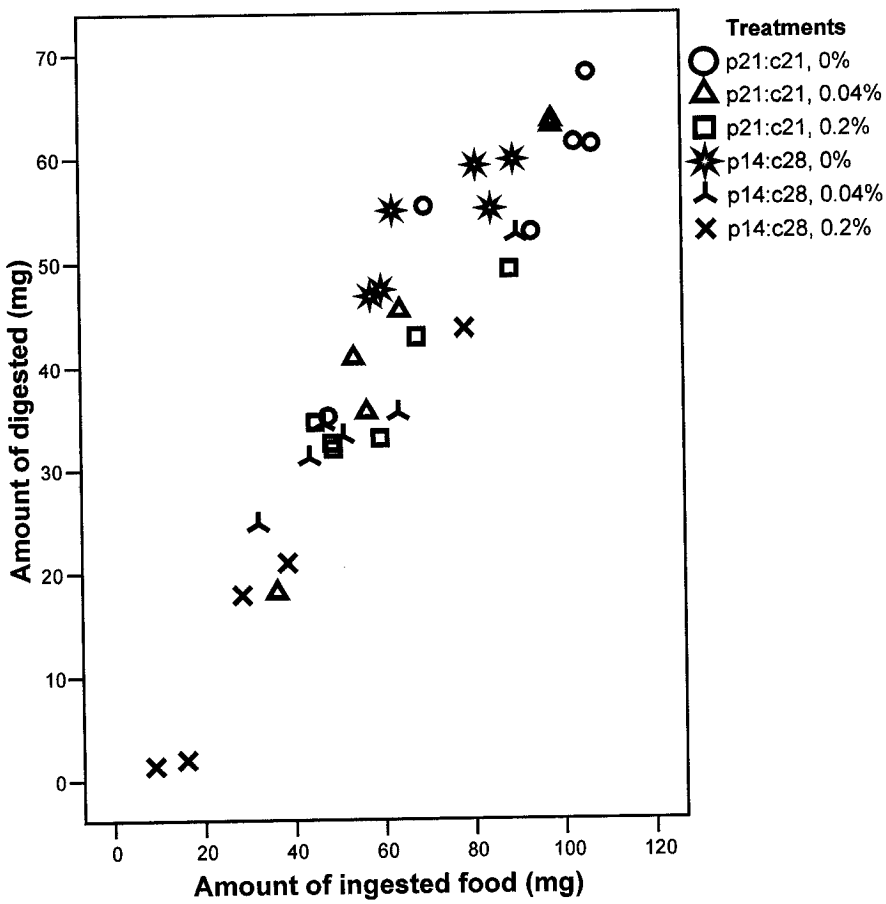

Figure 9: a) Scatter plot of the amount of dry digested material on the amount of dry ingested food for each group, which represents the approximate digestibility (AD), for 2nd instar larvae reared on different diets (p21:c21 and p14:c28) with different levels of TI extracted from aspen (0%, 0.04% and 0.2%).

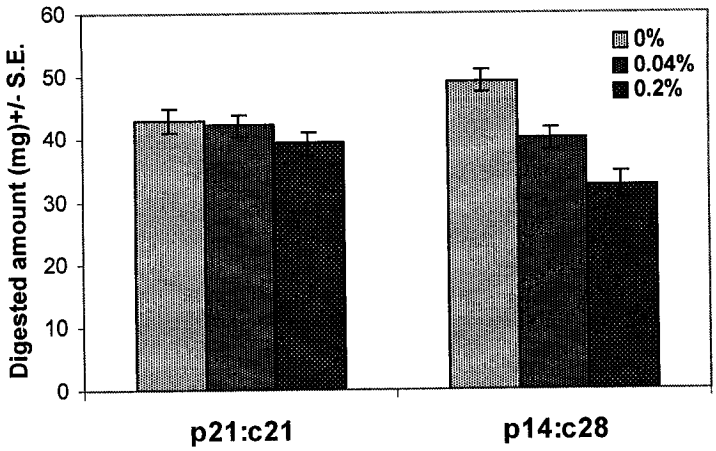

Figure 9: b) The amount of digested material (evaluated at the value of ingested food: 63.5806 mg). The estimated marginal means is the average amount of dry digested food corrected for the total amount of dry ingested food (co-variate).

Dependent Variable: Digested material

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Ingested food	3649.153	1	3649.153	194.041	<.001
Nutrition	6.800	1	6.800	.362	.552
TI	398.487	2	199.244	10.595	<.001
Interaction Nutrition * TI	247.794	2	123.897	6.588	.005
Error	526.570	28	18.806		
Total	69300.230	35			
Corrected Total	9640.721	34			

Table 4: ANCOVA results for the effect of two factors on the amount of dry digested material, standardized for total amount of dry ingested food. The fixed factors were the nutrition and the level of TI, and the co-variate was the amount of dry ingested food. df= degrees of freedom, F is the test statistic, and Sig. is the p value ($\alpha = 0.05$).

Looking at the efficiency of conversion of digested material (ECD), the amount of digested food affected the amount of grown body mass significantly, where more digested food led to more grown body mass (Table 5: digested food term, and Fig. 10a). Neither the nutrition diets nor the levels of TI had an effect on the ECD of larvae (Table 5: nutrition and TI terms, and Fig. 10). However, there was an interaction between the nutrition and the TI (Table 5: interaction term, and Fig. 10b) showing that the ECD of larvae was only increased with higher levels of TI on protein deficient diet but not on balanced diet.

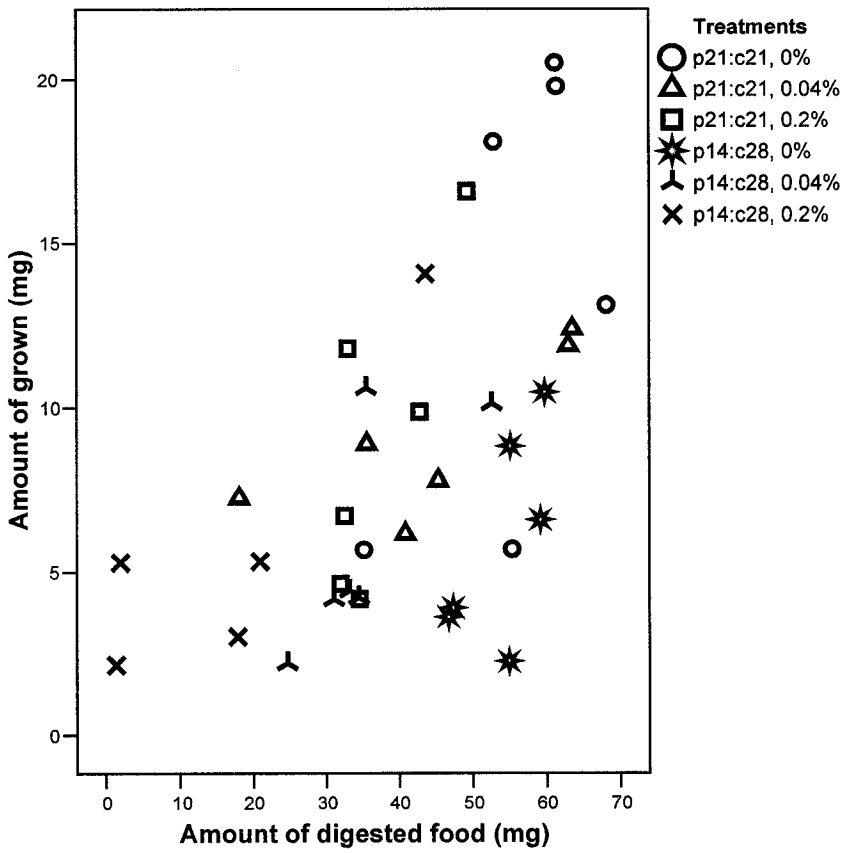

Figure 10: a) Scatter plot of the amount of dry grown body mass on the amount of dry digested food for each group, which represents the efficiency of conversion of digested material (ECD), for 2nd instar larvae reared on different diets (p21:c21 and p14:c28) with different levels of TI extracted from aspen (0%, 0.04% and 0.2%).

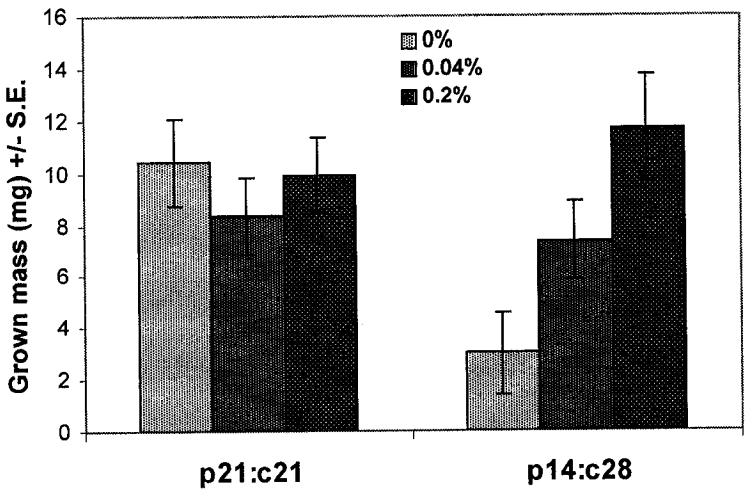

Figure 10: b) The amount of dry grown body mass in each replicate (evaluated at the value of digested food: 41.2863 mg). The estimated marginal means is the average dry weight corrected for the total amount of dry digested food (co-variate).

Dependent Variable: Grown body mass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Digested food	233.861	1	233.861	18.900	<.001
Nutrition	34.419	1	34.419	2.782	.106
TI	53.598	2	26.799	2.166	.133
Interaction Nutrition * TI	117.970	2	58.985	4.767	.017
Error	346.467	28	12.374		
Total	3303.915	35			
Corrected Total	862.124	34			

Table 5: ANCOVA results for the effect of two factors on the amount of dry grown body mass, standardized for total amount of dry digested food. The fixed factors were the nutrition and the level of TI, and the co-variate was the amount of dry digested food. df= degrees of freedom, F is the test statistic, and Sig. is the p value ($\alpha = 0.05$).

Looking at the efficiency of conversion of ingested material (ECI), the total amount of ingested food affected the amount of grown body mass significantly (Table 6: ingested food term, and Fig. 11a), where more ingested food led to more grown body mass. The ECI was not significantly affected by the nutritional diets, but it was affected significantly by the levels of TI (Table 6: nutrition and TI terms, and Fig. 11). The higher the TI level, the more weight the caterpillars gained based on the same amount of ingested food. Also, the interaction between the nutrition and the TI was seen (Table 6: interaction term, and Fig. 11b), which suggests that the caterpillars performed in different ways on the two nutritional diets with different levels of TI. On the p21:c21 diet, caterpillars had similar amount of grown body mass based on the same amount of ingested food; but on the p14:c28 diet, they gained more grown body mass based on the same amount of ingested food with higher level of TI.

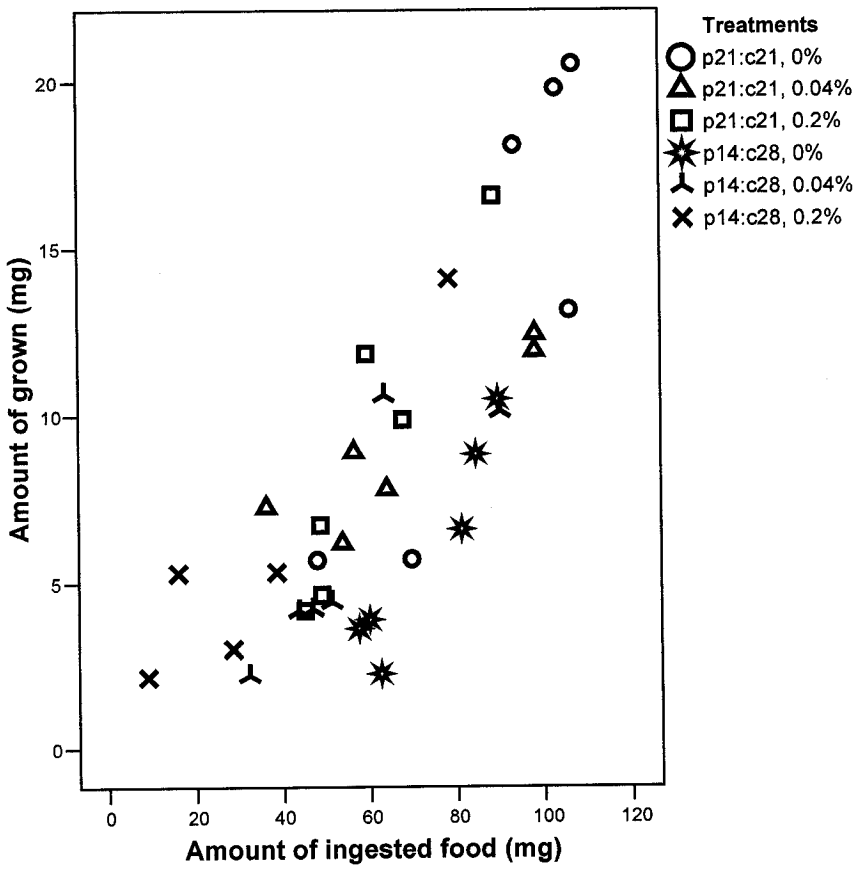

Figure 11: a) Scatter plot of the amount of dry grown body mass on the amount of dry ingested food for each group, which represents the efficiency of conversion of ingested material (ECI), for 2nd instar larvae reared on different diets (p21:c21 and p14:c28) with different levels of TI extracted from aspen (0%, 0.04% and 0.2%).

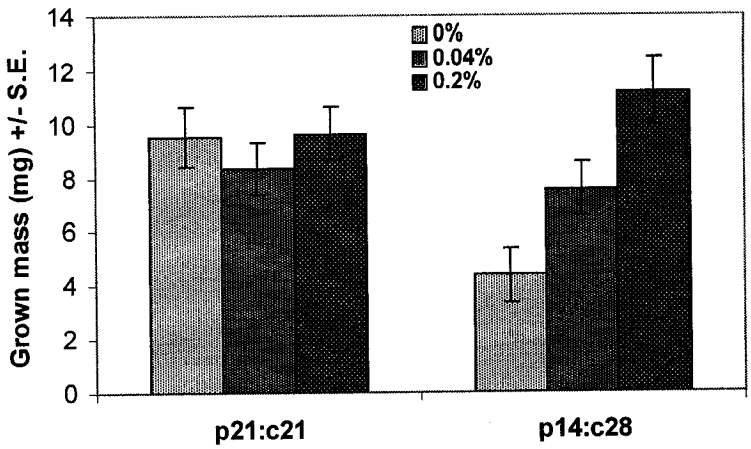

Figure 11: b) The amount of dry grown body mass in each replicate (evaluated at the value of ingested food: 63.5806 mg). The estimated marginal means is the average dry weight corrected for the total amount of dry ingested food (co-variate).

Dependent Variable: Grown body mass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Ingested food	417.798	1	417.798	71.976	<.001
Nutrition	15.603	1	15.603	2.688	.112
TI	50.986	2	25.493	4.392	.022
Interaction Nutrition * TI	66.343	2	33.171	5.715	.008
Error	162.530	28	5.805		
Total	3303.915	35			
Corrected Total	862.124	34			

Table 6: ANCOVA results for the effect of two factors on the amount of dry grown body mass, standardized for total amount of dry ingested food. The fixed factors were the nutrition and the level of TI, and the co-variate was the amount of dry ingested food. df= degrees of freedom, F is the test statistic, and Sig. is the p value ($\alpha = 0.05$).

2.5 Summary of the two experiments

In conclusion, from what we found in the two experiments, the trypsin inhibitor (TI) did have an effect on forest tent caterpillars in terms of decreased growth and food consumption. The TI extracted from the host tree, aspen, had stronger negative effects on forest tent caterpillars in terms of decreased digestibility and increased gross growth efficiency than the TI from soybean.

	***Experiment 1* with Soybean TI**		***Experiment 2* with Aspen TI**	
	Protein deficiency	Higher TI	Protein deficiency	Higher TI
Moult weight	*Decreased*	*Decreased*	*Decreased*	*Decreased*
Food consumption	*n.s.*	*Decreased*	*Decreased*	*Decreased*
AD	*n.s.*	*n.s.*	*n.s.*	*Decreased*
ECD	*n.s.*	*n.s.*	*n.s.*	*n.s.*
ECI	*n.s.*	*n.s.*	*n.s.*	*Increased*

n.s. = no significant effect was observed

Table 7: The summary result of experiment 1 and 2 comparing the effect of two kinds of TI on larvae growth, food consumption and three nutrient utilization efficiency parameters based on two diets (balanced and protein deficient).

2.6 Discussion

From our results, the caterpillars did not perform well when fed on the foliage of hybrid poplar, which is in contrast to our prediction. They did not grow much and most of them did not moult to third instar during the experiment. That foliage was collected from hybrid poplar and not trembling aspen could be one of the explanations. Lindroth *et al.* (1986) found that *Papilio glaucus canadensis* and *Papilio glaucus glaucus* were unable to survive and grow on each other's favoured plants. This differential utilization ability is possibly due to the plant secondary metabolites (Lindroth *et al.* 1986). It is mentioned by Robison and Raffa (1994) that different poplar hybrids are more or less suitable for forest tent caterpillars, which suggests that the hybrid poplar we used may be a non-suitable one to feed the larvae of forest tent caterpillars. Moreover, Hollick and Gordon (1993) suggest that protein-based defense such as protease inhibitors might also be induced in hybrid poplar. Since the leaves we fed the larvae were cut from the hybrid poplar right before using, they might have induced TI before we painted the TI solution on, so the TI concentrations would be higher than it supposed to be.

Meanwhile, leaf toughness could be another factor to impede larval feeding especially in early instars (Fitzgerald 1995). It is suggested that the caterpillar's herbivory is influenced not only by the varying quality of the foliage of different host species but also by the high sensitivity of these insects to within-tree differences in the age of foliage (Fitzgerald 1995). Normally, small larvae have difficulty penetrating quantities of non-nutritive fibre, which impedes the rate of food processing, so the increasing leaf toughness affects the growth of caterpillars (Fitzgerald 1995). Since forest tent caterpillars

hatch out synchronously with spring bud break and feed on the very young leaves, delay in hatching and feeding on older leaves would also slow their development (Parry *et al.* 1998). It is not possible to rear newly hatched larvae of *M. disstria* on leaves of their preferred host trees only several weeks older than foliage they normally feed on, because the caterpillars have great difficulty penetrating the leaf and grow little or not at all (Fitzgerald 1995). Even though we tried our best to collect leaves as young as possible, the foliage available for the experiment might have been too old for 2nd instar larvae to eat especially in a small group of 10, since the hybrid poplar we used were grown in the green house which does not give the plant natural seasonal conditions in bud development and leaf growth.

Looking at the larval growth, only three days after starting the experiment, adverse effects of TI extracted from soybean were observed on larval weight gain (Fig. 1) suggesting that TI can affect larval growth within three days. The protein deficiency did not take effect on larvae as fast as TI but the interaction showed the commencement of different TI effects based on different protein concentrations. Nevertheless, differences among replicates in each treatment were observed as well, which indicated great variance of performance between groups, possibly due to synergistic group effects. Once the larvae of *M. disstria* moulted to the third instar, their moult weight was obviously decreased by protein deficiency, but TI (from either source) differently affected larvae reared on the two diets (Fig. 2 and Fig. 7). On the balanced diet, as we predicted, larvae were much smaller when the TI levels went higher. However, in contrast to what we predicted, the larvae fed on protein deficient diet were of similar weight irrespective of TI treatment.

Protein digestibility affects not only the larva's weight gain but also the length of the larval period (Pilon *et al.* 2006). However, by looking at the moulting date of the

caterpillars in our study, no difference of development time was found. It might be because the larvae were only treated for one instar, which might not be long enough to retard the larvae from moulting.

It has been found that inhibition of digestive enzyme activities interferes with nutrient uptake and growth (Zhu-Salzman. *et al.* 2003). Thus we looked at food consumption of caterpillars during treatments as well. Lepidopteran larvae often compensate for dilute dietary protein by increasing consumption rates (Slansky and Wheeler 1989). Slansky and Feeny (1977) demonstrated that *Pieris rapae* caterpillars increased food consumption and nitrogen utilization efficiency as leaf nitrogen declined naturally or artificially. But this compensation behaviour was not observed on *M. disstria* in our study. In the first experiment that used soybean TI extract, dietary protein deficiency did not influence food consumption (Fig. 3), and therefore protein deficiency led to decreased growth of larvae fed on protein deficient diet compared to the balanced diet. However, in the second experiment using aspen TI extract, protein deficiency decreased the food consumption significantly (Fig. 8). Thus, less ingestion of food could have caused part of the reduced larval moult weight on the protein deficient diet in the second experiment using aspen TI.

Both soybean and aspen TI decreased food consumption on both diets (Fig. 3 and Fig. 8). This led to decreased growth with increased TI consumption on the balanced diet but not on the protein deficient diet, which indicated that the decreased food consumption only influenced the larval growth on the balanced but not the protein deficient diet. It has been suggested that in some cases, caterpillars can regulate their growth not just by increasing the food intake but also by increasing the growth efficiency (Slansky and

Wheeler 1989). Therefore, the larvae fed on protein deficient diet should have adopted some strategies for increasing the growth efficiency to reach the growth target when the low amount of ingested protein was also inhibited by TI to digest.

As for the reason that food consumption was decreased by TI, a down-regulation of the trypsin genes is observed in response to different kinds of protease inhibitors in the diet, which suggests that the insect is able to sense inhibitors in the diet and down-regulate trypsin as a result (Gatehouse *et al.* 1997). If the larvae of *M. disstria* are able to sense the inhibitors, then the reduced food consumption could be a reactive behaviour to protect them by avoiding dietary toxins. Furthermore, since smaller caterpillars eat less than the bigger ones, the decreased growth of caterpillars fed on balanced diet can reduce their food consumptions as well.

It has been demonstrated that food quality influences the feeding responses. Differences in the quality of leaves of the same tree species grown under different conditions may affect the efficiency of energy transfer in forest tent caterpillars (Fitzgerald 1995). Slansky and Wheeler (1989) found on *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) that not only the fresh weight food consumption, but also the approximate digestibility (AD) and the efficiency at which digested food is converted to biomass (ECD) all increased on the diluted diets especially the most dilute one. Thus, this insect exhibits post-ingestive compensatory responses to changes in the dietary quality.

Based on the result that reduced food consumption with higher levels of TI decreased larval growth on balanced diet but not protein deficient diet, we examined the three gravimetric parameters of nutrient utilization efficiency: AD, ECD and ECI to determine what compensatory response the caterpillars used to regulate their growth.

However, in the first experiment in our study, the protein deficiency did not affect any of these nutrient utilization efficiency parameters of larvae fed on diet with TI extracted from soybean. Only a slight but not significant trend of soybean TI was found on the AD and ECD, and ECI was not affected by the treatment at all. It confirmed that the TI effects on larval growth observed on balanced diet were due only to decreased food consumption. On protein deficient diet, the decreased food consumption did not lead to a decrease in growth; in this case, the slightly reduced decrease of ECD with TI increasing could compensate for the decreased food consumption to maintain similar growth.

To confirm these results, trypsin inhibitors extracted from aspen were used in the second experiment. Despite the fact that the protein deficiency still had no effect on the three nutrient utilization parameters, the aspen TI significantly affected AD and ECI this time, which suggested more effective inhibition of the TI extracted from the host tree of *M. disstria* than from soybean. As expected, the approximate digestibility (AD) was reduced by TI because of the inhibition of digestion from TI. This result was supported by Pilon *et al.* (2006) who looked at the effect of trypsin-inhibitor benzamidine, which has similar inhibition effect as TI, on larvae of velvetbean caterpillar (*Anticarsia gemmatalis*), and showed that protease inhibitors decreased both AD and food consumption. But the significant interaction between TI and protein deficiency shows that the AD did not decrease with higher levels of TI on the balanced diet as much as on the protein deficient diet. It is demonstrated in other studies that low levels of protein decrease activity of another digestive enzyme, esterase, and increase the toxicity of plant-produced secondary metabolites in *M. disstria* (Lindroth and Bloomer 1991).

In contrast to AD and also opposite to our expectation, the gross growth efficiency ECI was increased by higher levels of TI. And the same as AD, ECI increased much more with higher levels of TI on the protein deficient diet than on the balanced diet. This could explain the interaction between nutritional diet and TI on larval growth: the moult weight was reduced greatly by higher level of TI on the balanced diet, but kept in a similar value with different TI levels on the protein deficient diet. It suggested a compensation strategy used by the larvae of *M. disstria* in our study that they can increase the post-ingestive efficiency of converting ingested materials for growth (ECI) to keep surviving when both the ingestion and digestion were limited by TI and also the protein was not sufficient in the diet for their growth requirements at the same time. It seems the body weight they reached when they moulted on the protein deficient diet was the bottom line for their survival.

This study demonstrated very different effects of TI, especially aspen TI, on caterpillars fed on different nutritional diets suggesting the influence of protein content in the diet on TI effect. It has been suggested that both the quantity and quality of dietary protein influence the regulation of proteolytic enzymes (Liddle *et al.* 1986, Noriega *et al.* 1994, Sharara *et al.* 1993, Tsuzuki *et al.* 1991). Broadway and Duffey (1986) found that levels of protease activity in *Heliothis zea* and *Spodoptera exigua* change with protein content instead of meal size, suggesting that protease production should be dependent on protein content (Johnston *et al.* 1993). This dependence is because ingestion of dietary protein stimulates the synthesis and secretion of proteolytic enzymes in the digestive tract of animals (Lehane *et al.* 1996). For instance, trypsin secretion is determined by the quantity of amino acids produced during digestion of the meal (Lehane 1977). As a result, the deficiency of protein in the diet could lead to a decrease of trypsin production so that

just 0.04% TI was high enough to inhibit all the trypsin produced and also trigger the compensation responses to regulate the growth above the bottom line. This difference in the quantity of trypsin produced could explain why, on the balanced diet, inhibition of protein digestion did not trigger compensation. Therefore, on protein deficient diet, higher levels of TI did not make much difference on the larval growth in spite of their reduced food consumption and decreased digestibility.

Although our caterpillars didn't show the adaptive strategy by increasing diet consumption and protein digestibility (as AD) as demonstrated by Pilon *et al.* (2006) in the study on the velvetbean caterpillar (*Anticarsia gemmatalis*) exposed to the trypsin-inhibitor benzamidine, the mechanism they suggested for this adaptive strategy, hyperproduction of proteolytic enzymes, could probably explain the increased ECI in our study. In addition, it has been observed that ingestion of soybean trypsin inhibitor (STI) altered the number of enzymes and increased the level of activity of enzymes that were not susceptible to inhibition of STI (Broadway 1997). These findings suggest that TI were able to stimulate the activity or production of other proteolytic enzymes to overcome the TI inhibition of digestion, but at the same time, the increased cost for that led to a net loss of amino acids (Sacchi and Wolfersberger 1996) so that the larvae had to increase the efficiency to convert the limited amino acids to body mass.

De Leo *et al.* (1998) found that low levels of trypsin inhibitor increase growth of *Spodoptera littoralis* larvae (larvae are even bigger on the transgenic TI-expressing tobacco plants than on the control plants). But when the larvae were exposed to the transgenic plants with highest level of TI, deleterious effects were observed together with decrease of leaf damage. They suggest that the deleterious effects could be linked to a

shortage of available amino acids because of extensive protease synthesis; and the different performance between high and low level of TI suggests a sensitive threshold, above which the larvae are not able to overcome the inhibition just by over expressing digestive protease but have to produce new proteases, which is even a more considerable cost of available amino acids and energy, otherwise their growth will be impaired. This threshold was not observed in our study. However, it brought up a multi-layered resistant system of caterpillars to survive under the inducible inhibition of plant defense.

The effect of trypsin inhibitors induced in other plant tissues by hormone application or through transgenic expression has been studied (Broadway 1995, McManus 1999). The expression of the soybean trypsin inhibitor in transgenic tobacco leads to the result that larvae fed transgenic leaf tissue demonstrated greater mortality, and the survivors grew more slowly in terms of weight gain over time (McManus *et al.* 1999). However, the protease-inhibitors induced in potato leaves by methyl jasmonate are ineffective against the Colorado potato beetles (Bolter and Jongsma 1995). Broadway (1995) found that *Lymantria dispar* (gypsy moth) produce large quantities of trypsin-like enzymes that are resistant to natural and genetically produced protease inhibitors in plants. It is suggested that the modulation of the complement of midgut digestive enzymes contributes to rapid insect adaptation to plant defensive protease inhibitors (Ahn *et al.* 2004).

Insect adaptation and resistance to plant protease inhibitors has been reported in some species (Broadway 1995, Jongsma and Bolter 1997, Jongsma *et al.* 1994, Jongsma *et al.* 1996). Three mechanisms of adaptation to protease inhibitors are observed: overproduction of existing digestive proteases (De Leo *et al.* 1998), expression of

inhibitor-insensitive protease (Ahn 2004, Jongsma and Bolter 1997, Pilon *et al.* 2006), or proteolytic degradation of the protease inhibitor (Giri *et al.* 1998, Michaud *et al.* 1995). Two species of Lepidoptera specialists, *Pieris rapae* and *Pieris napi*, are observed to have evolved trypsin like enzymes that function in the presence of the trypsin inhibitors in their host plants (Broadway 1995). Another species, the velvetbean caterpillar (*Anticarsia. gemmatalis)* produces inhibitor-insensitive proteases to cope with hosts rich in trypsin inhibitors (Pilon *et al.* 2006). Ahn *et al.* (2004) demonstrated that proteolytic fragmentation of scN, a soybean cysteine protease inhibitor which impacts early developmental stages of cowpea bruchid larvae, can potentially free scN-inhibited proteases to resume their digestive capability, which represents a strategy insects use to cope with scN inhibition. Since the digestibility (AD) was still decreased by aspen TI in our study, which means the TI affected the digestion of protein in the larva midguts functionally, either hyperproduction of trypsin or synthesis of TI-insensitive protease are more likely to be used by our caterpillars fed on protein deficient diet to increase the ECI under high TI concentrations to keep the body mass above the bottom line for surviving.

Among the three parameters, due to the ability to examine how well the digested materials are absorbed and converted to body mass, ECD is presumably of greatest interest in determining whether the net effect of the digestion of food, the utilization of the energy converted, and the detoxification of secondary compounds results in greater efficiency or utilization of the plant (Futuyma and Wasserman 1981). But in our study, ECD was not affected significantly by the treatment from either experiment. Only an interaction effect between the nutrition and aspen TI was observed, which suggested a different influence from TI on ECD between two different nutritional diets. Actually, from the graph (Fig. 10b)

we could see an obvious increasing trend of ECD with higher levels of TI on the protein deficient diet, but little difference was seen on the balanced diet. This interaction effect might have lead to the non-significant statistical result of the effect of TI on ECD. And it was expected that the TI inhibition did increase ECD when the protein was deficient, since the ECI was increased by TI on the protein deficient diet even though the AD was decreased.

Different plant species have different types of defense so there are no universal defense proteins (Constabel 1999). The selection pressures on insects are to evolve proteases that are largely insensitive to host plant PIs, and the evolution of insects will often be considerably faster than plants (Jongsma and Bolter 1997). TI genes of aspen, which are systemically wound- and herbivore-induced, evolve more rapidly than other genes to be resistant to the adaptability of insect pests and their multiple digestive enzymes (Haruta *et al.* 2001). The inducible protein-based defense system established by trembling aspen represents a complementary function instead of a trade-off (Haruta *et al.* 2001) to balance the cost for growth and defense. The significant effect from aspen TI in our study confirmed the defense function of TI in trembling aspen against forest tent caterpillars.

Chapter 3

Trypsin inhibitor effect on the nitrogen concentration in the frass of forest tent caterpillars

3.1 Abstract

The nitrogen concentration in the caterpillar frass was analyzed to evaluate the proportion of undigested protein to confirm the TI inhibition effect on digestion. Frass was collected from 2nd instar larvae of forest tent caterpillars in the two experiments in Chapter 2, and also in a new experiment with the same treatments (two diets: nutritional balanced diet and protein deficient diet; three levels of TI from both soybean and aspen: 0%, 0.04% and 0.2%).

As expected, significantly lower concentrations of nitrogen were found in the frass from caterpillars fed on the protein deficient diet compared to the ones on the balanced diet. Meanwhile, frass nitrogen concentrations increased with higher levels of TI, indicating that more nitrogen was egested when the protein digestion was inhibited by TI. This confirms our prediction of increased nitrogen concentration in frass because of higher proportion of undigested protein under inhibition of TI.

However, it was noticed that this increase of N concentration with higher levels of TI was not as great as expected. Based on the results found for nutrient utilization efficiency in Chapter 2, it was suggested that this reduced increase in frass nitrogen was

due to the increased ECI. This indicates a higher efficiency of converting nutrients to body mass so that more nitrogen than expected is utilized when TI was present. This presumably reflects changes in gut enzymes in response to TI.

3.2 Introduction

A large part of the leaf consists of non-digestible components, which pass through the digestive tract without being absorbed and are eventually egested as faecal pellets by caterpillars (Fitzgerald 1995). Part of the energy content of the leaf that is assimilated is used for maintenance (respiration) and is lost in the process, and only the remaining energy is stored as caterpillar body or made available for reproduction (Fitzgerald 1995).

Plant leaves are considered to be rich in carbohydrate but low in protein (Slansky and Scriber 1985). Proteins are essential for the growth and maintenance of tissues, as well as for structural support, storage, transport, movement, and in the maintenance of cellular metabolism (Campbell and Reece 2002). Nitrogen is often bound up in plant proteins, and protein content is often measured by nitrogen content (Campbell and Reece 2002). Nitrogen levels in aspen foliage are not much greater than 2% N, which is 12.5% protein since 16% of protein is nitrogen (Lindroth and Bloomer 1991). Nitrogen is regularly considered as a limiting nutrient in the natural herbivore diet because in general there are low levels of nitrogen in plant tissue relative to nutritional requirements of the insect (Campbell and Reece 2002). Insect herbivores need to find ways to utilize nitrogen in an efficient and productive manner (Campbell and Reece 2002).

Few lepidopterans consume protein as adults so they rely heavily on the protein store in larvae stages (Telang *et al.* 2003). It was found in *Heliothis virescens* by Telang *et al.* (2003) that caterpillars can regulate both nutrient consumption and post-ingestive physiology to achieve greater growth; only when the nitrogen intake is above a given value was a greater amount of nitrogen found in frass with increasing nitrogen intake.

Furthermore, they presented the post-ingestive partitioning of nutrients into pre- and post-absorptive components, and found that nitrogen egested by both *Heliothis virescens* and *Estigmene acreain* in excess of growth requirements is mainly of post-absorptive origin (Telang *et al.* 2003), indicating that even the extra nutrient is absorbed and metabolized before being egested.

To confirm the results from experiment 1 and 2 that the digestion of dietary protein was inhibited by trypsin inhibitor in terms of less moult weight partly caused by lower digestibility, we analyzed the oven-dried frass collected from experiment 1 and 2. We also set up a new experiment using the 2nd instar caterpillars reared under the same treatments as in experiment 1 and 2 to collect freezer-dried frass for analysis.

This study focuses on the nitrogen analysis of the frass to investigate the proportion of undigested protein in the frass. Since the ingestion of trypsin inhibitor could inhibit the digestion of the protein, the undigested protein was predicted to be egested in the frass. Under the inhibition of TI, the percentage of nitrogen is predicted to be higher in the frass from larvae reared on the diet with higher levels of TI. Also, due to the lack of protein, lower concentrations of nitrogen are predicted to be found in the frass from larvae reared on the protein deficient diet.

3.3 Methods and materials

3.3.1 Sample collection

Nitrogen analysis was performed to determine the concentration of nitrogen in the larval frass for each treatment. All the oven-dried frass produced by 2nd instar larvae in experiment 1 and experiment 2 were collected. They were used to calculate the digested amount of food so they were oven-dried together with the leftover food and 3rd instar larvae right after they moulted. These samples were considered as replicate 1. Since freeze-drying can minimize the loss of nitrogen during the sample preparation rather than oven drying, it was utilized for preparing samples of another replicate to confirm the result from replicate 1. Thus we reared 300 freshly moulted 2nd instar caterpillars on 12 treatments the same as experiment 1 and 2 (with two different diets (p21:c21 and p14:c28) and three different levels (0%, 0.04%, 0.2%) of TI extracted from both soybean and aspen) to collect the frass and freeze-dry them once the larvae moulted to 3rd instar. These samples constituted replicate 2.

In replicate 2, there were 25 caterpillars for each treatment in order to collect enough frass to do the nitrogen analysis. The minimum amount required for CHN analysis is 1 mg, but the loss of sample amounts in the preparation, especially during the grinding, required much more than the minimum. Since the frass collected from experiment 1 and 2 had the minimum amount of 6.7 mg of one replicate in each treatment, which had 10 caterpillars, we mixed the frass from all replicates under the same treatment to one sample for the CHN analysis.

3.3.2 CHN analysis

All the dried frass samples were crushed with liquid nitrogen, then weighed on an electrobalance (1-2 mg) and sealed in pressed 6 x 4 mm tin capsules. The tin capsules were dropped into a combustion chamber filled with catalytic material at approximately 1000°C (Perkin-Elmer Series II CHNS/O Analyzer 2400). As the sample entered the combustion chamber, a fixed amount of oxygen was injected into the constant stream of helium, which was used as the carrier gas. All of the reaction products including nitrogen, carbon dioxide, water and sulphur dioxide were then separated and detected. The chromatographic responses were calibrated against pre-analyzed standards (cysteine: Perkin Elmer organic analytical standard), and the CHN elemental contents were reported in weight percent (Gnaiger and Bitterlich 1984). These analyses were done with the help of Dr Yves Gélinas, Department of Chemistry and Biochemistry (Concordia University).

3.3.3 Data analysis

A three-way factorial analysis of variance (ANOVA) was performed to determine the effects of nutritional diets, trypsin inhibitor and plant source on the concentration of nitrogen in the frass.

3.4 Results

The results from three-way ANOVA suggested that both the nutrition and the level of TI significantly affected the concentration of nitrogen in caterpillars' frass (Table 7: nutrition and TI level terms, and Fig. 12). The caterpillars treated with protein deficient diets had a lower percent of nitrogen in their frass. At the same time, the caterpillars fed the higher level of TI had a higher percent of nitrogen in their frass (as seen in Fig. 12). However, no significant difference was seen between the two sources of TI. The trypsin inhibitor extracted from both soybean and aspen had the same effect on the concentration of nitrogen in the larval frass (Table 7: TI source term, and Fig.12). No interaction was seen among the three factors (as seen in all interaction terms in Table 7).

Dependent Variable: N% in frass

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nutrition	1.978	1	1.978	34.991	< .001
TI level	.489	2	.244	4.323	.039
TI source	.179	1	.179	3.158	.101
Interaction Nutrition * TI level	.006	2	.003	.056	.946
Interaction Nutrition * TI source	.155	1	.155	2.746	.123
Interaction TI level * TI source	.174	2	.087	1.539	.254
Interaction Nutrition * TI level * TI source	.021	2	.010	.185	.833
Error	.678	12	.057		
Total	87.270	24			
Corrected Total	3.680	23			

Table 8: Three-way ANOVA results for the effects of three factors on the concentration of nitrogen in caterpillars' frass. The fixed factors were the nutrition, the level of TI and the source of TI. df= degrees of freedom, F is the test statistic, and Sig. is the p value (α =0.05).

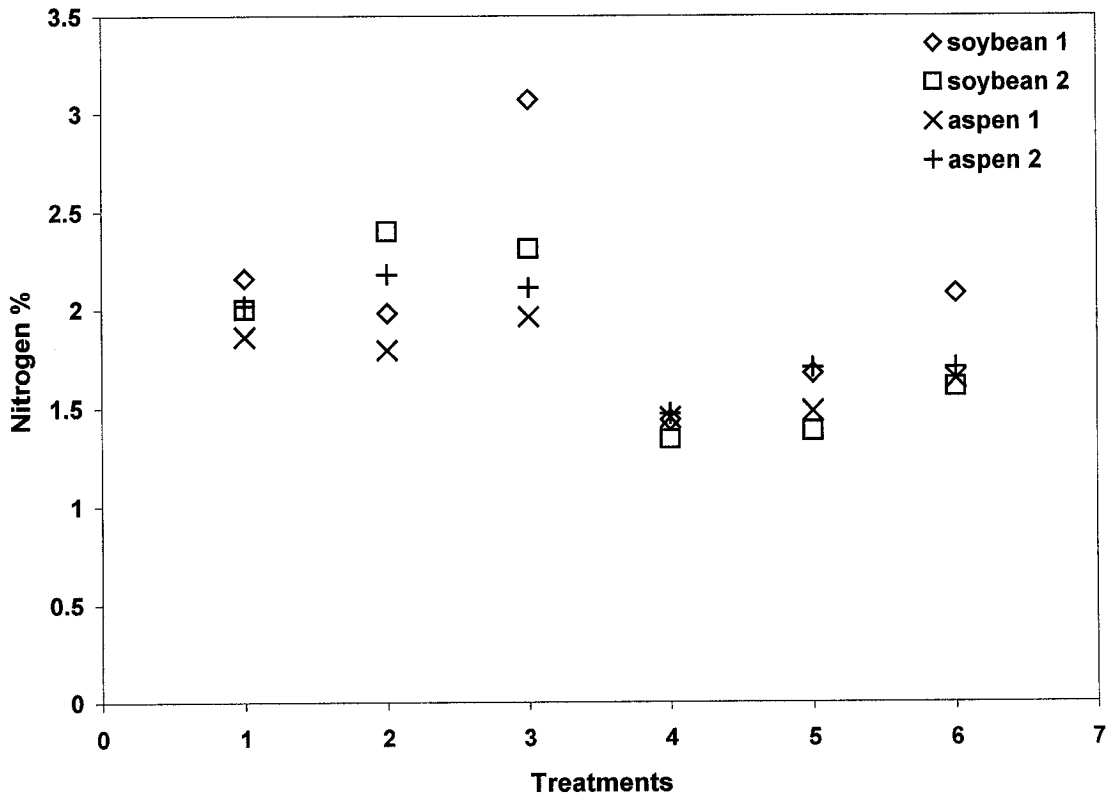

Figure 12: The concentration of nitrogen in the frass of caterpillars treated on different diets with three levels of TI from different sources. Treatment 1: p21: c21 0% TI; Treatment 2: p21: c21 0.04% TI; Treatment 3: p21: c21 0.2% TI; Treatment 4: p14: c28 0% TI; Treatment 5: p14: c28 0.04% TI; Treatment 6: p14: c28 0.2% TI. Each symbol represents one sample.

3.5 Discussion

Nitrogen is a vital nutrient for growth and reproduction in insects (Mattson 1980). As we predicted, the percentage of nitrogen in the frass of 2nd instar caterpillars reared on the protein deficient diet was lower than the ones reared on balanced diet because of less ingestion of nitrogen (Fig. 12). It is supported by Telang *et al.* (2003) where *Estigmene acreain* egests proportionately less nitrogen only at low nitrogen-intake levels.

Furthermore, as we predicted, with higher levels of TI in the food ingested, larvae egested frass with higher concentration of nitrogen, presumably because of the TI inhibition on protein digestion (Fig. 12). It is shown in *Mytilus edulis* (Bivalvia: Mollusca) by Hawkins *et al.* (1983) that the high nitrogen content in the faecal samples represents relatively undigested material. Also, they suggest that the percentage of nitrogen within frass is inversely correlated with the rate of faecal egestion, because higher faecal egesting rate was observed when the nitrogen was absorbed very well and little nitrogen was egested. As shown by the approximate digestibility [AD: (*Ie-Fe*)/*Ie*] in Chapter 2 that digested materials (*Ie-Fe*) increased with the amount of ingested materials (*Ie*), the amount of faecal output (*Fe*) also increased with the food consumption. Therefore, according to Hawkins *et al.* (1983), the concentration of nitrogen in the frass should inversely correlate with food consumption as well, which corresponds to what we found. It means that the nitrogen concentration went higher in the frass when the food consumption was reduced, possibly because the egestion rate was reduced as well, due to the insufficient digestion or absorption of nitrogen.

However, it was noticed that the effect from TI was not as significant as the effect from protein deficiency (Table 7). Looking at Fig. 12, the difference in nitrogen concentrations among different levels of TI was not as much as we expected. One of the explanations is that much of the faecal nitrogen was of a post-absorptive nature (Telang *et al.* 2003), indicating that digested nitrogen can be egested out as well without sufficient absorbing. It suggests that the absorption is also a critical part in determining the concentration of nitrogen in the frass. It was found that in *Heliothis virescens,* most of the ingested protein was digested and absorbed across the midgut because only a small proportion of faecal nitrogen was accounted by protein and amino acid and the most was in a post-absorptive form as uric acid nitrogen (Telang *et al.* 2003). Although the form of nitrogen in the frass in our study was not investigated, the inverse correlation between food consumption and nitrogen concentration in the frass mentioned above suggests that the nitrogen in the frass is counted in two sources: the pre-absorptive and also the post-absorptive.

Furthermore, it has been found that food quality can influence nitrogen utilization in insects. Slansky and Wheeler (1989) found in *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) that the relative nitrogen consumption rate declined with dietary dilution, but the nitrogen utilization efficiency increased with dietary dilution so that the relative nitrogen accumulation rate was stabilized at all the diets. Whether this nitrogen utilization efficiency increased with higher levels of TI to result in less difference of nitrogen concentration in the frass among TI treatments is not clearly known yet. But based on what we found with ECI and ECD in Chapter 2, the increased utilization efficiency of nutrients was mostly likely the reason. It means that caterpillars increased nutrient utilization

efficiencies to utilize the nitrogen more efficiently instead of egesting it when the digestion of nitrogen was inhibited.

Other mechanisms used by animals to regulate the utilization of nitrogen are observed. In herbivores, when nitrogen is not contained in sufficient amounts in the diet, bacteria lysed by stomach lysozymes are used as sources of nitrogen (Fujita 2004). Not much work examining the nitrogen concentration in herbivore frass under the TI inhibitions has been done to confirm the inhibition of protein digestion. In terms of the lessened increase of nitrogen concentration in the frass with higher levels of TI, our study confirmed TI effects on protein digestion of forest tent caterpillars and also demonstrated the adaptive regulation of nutrient utilization in these caterpillars under TI inhibition.

Chapter 4

General Discussion

To protect themselves against insects and other herbivores, plants have evolved a variety of defense mechanisms (Haruta *et al.* 2001). Multiple mechanisms show the evolution of resistance within herbivore populations and represent defenses specialized against particular plants (Haruta *et al.* 2001). However, present methods for pest control are based on the use of agrochemicals, which cause environmental damage, contamination of operators during the handling period, and selection of resistant species, thus it is necessary to develop environmentally friendly methods of insect control (Paulillo *et al.* 2000). Therefore, more and more research has focused on the utilization of the plant defense system as a pest control mechanism. Our study confirmed the defensive function of trypsin inhibitor (TI), an inducible plant protein that is able to inhibit the digestion of dietary protein of forest tent caterpillars (*M. disstria*).

From our study, 2nd instar larvae of *M. disstria* were considerably impaired by TI in terms of their weight gain due to reduced food consumption and also to reduced digestibility, which was decreased only by aspen TI but not soybean TI, when they were fed on the nutritionally balanced food. But when protein was deficient in the diet, instead of performing even worse under the inhibition of TI, they increased the efficiency of converting ingested food to body mass to regulate their growth by maintaining the same weight no matter the levels of TI in the diet.

Additionally, the second part of our study analyzed the nitrogen in the frass, which indicated just a slight increase of nitrogen concentration with higher levels of TI. This

suggests that even though the undigested protein was predicted to be egested in the frass leading to an increase of the nitrogen concentration with higher levels of TI, more efficient absorption and utilization of digested nitrogen lessened this increase of nitrogen concentration. As a result of the efficient absorption and utilization of digested nutrient, the ECI was increased to regulate the growth for larval survival. However, this regulation of growth was only observed in the experiment using the TI extracted from the host tree, aspen, but not from soybean, which indicates the defense and anti-defense interactions between host trees and herbivores (Broadway 1995, 1997, Thaler *et al.* 2001).

Furthermore, the increased ECI can reflect the regulation of digestive protease production and activity inside of larvae midguts as an adaptation strategy (Constabel *et al.*, unpublished data). The midguts of the experimental caterpillars were dissected after the treatments and enzyme-assayed by C. Peter Constabel *et al.*, University of Victoria. Their enzyme assays on these caterpillars showed that soybean TI reduced the activity of digestive proteases but increased the proportion of soybean TI resistant protease activity without changes in trypsin levels. The increased resistant protease activity could explain the non-changed digestibility on both diets and also non-decreased growth on protein deficient diet from soybean TI in our study, because there were other kinds of digestive enzymes to digest the protein.

Conversely, still without changes in trypsin levels, the aspen TI we used increased the proteases activity, which was even higher for larvae fed on protein deficient diet than balanced diet, explaining the increased ECI and the maintained growth with higher levels of aspen TI on the protein deficient diet. However, there was no change in the proportion of aspen TI resistant proteases, which indicates the different changes in the midgut protease

activity between soybean TI and aspen TI. It has been found that not all protease inhibitors enhance the level of protease inhibitor resistant enzymes, even if those protease inhibitors inhibited a significant portion of enzyme activity (Broadway 1997). Moreover, synthesis and secretion of PI resistant enzymes are regulated by the ingestion of PIs in a dose and time dependant manner, and have been demonstrated in several different species of larvae treated on the soybean TI (Broadway 1997). All these findings suggest a complex system responsible for the regulation of proteolytic enzymes in the midgut of larval Lepidoptera.

Our study confirmed the inhibition effect of the trypsin inhibitor (TI) as a defense of the host tree, aspen, against forest tent caterpillars. Although the caterpillars demonstrated adaptive regulation of growth by increasing nutrient utilization efficiencies to deal with this inhibition effect under severe protein deficiency, the TI is still considered as an environmentally friendly and manipulatively practical method of pest control in the forest.

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