THE EFFECTS OF GLUCOSINOLATES IN CANOLA MEAL FEEDING REGIMENS ON LIVER AND THYROID PHYSIOLOGICAL RESPONSES AND PRODUCTIVE PERFORMANCE PARAMETERS IN THE LAYING HEN

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Yuqiong Wang

In Partial Fulfilment of the

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ABSTRACT

Yuqiong Wang. Ph. D., The University of Manitoba, May, 2003. The effects of glucosinolates in canola meal feeding regimens on liver and thyroid physiological responses and productive performance parameters in the laying hen. Major Professor; Lloyd Campbell.

Long-term (4-8 months) experiments were conducted to evaluate the feeding value of a new low-glucosinolate canola meal for laying hens and to investigate the physiological responses in the liver and the thyroid to diet glucosinolate levels in various canola meal feeding regimens. An experiment was conducted to investigate the effects of glucosinolates on fertility and hatchability in breeder laying hens and to assess the possible influence of iodine supplementation.

The importance of glucosinolate hydrolysis products in the physiological response of laying hens to dietary glucosinolates was exemplified by the observations that hepatic glutathione and thyroid weight showed more pronounced response in birds fed diets designed to produce a maximum amount of glucosinolate hydrolysis products. A significant linear relationship between diet glucosinolate levels and hepatic glutathione content was observed. These data along with the data that a high rate of egg production and no mortality due to liver hemorrhage were noted in laying hens fed meal produced from the new low-glucosinolate

cultivar indicate that the glucosinolate content should be reduced in future canola varieties. The corresponding meals could then be used in laying hen diets based on their nutritive value with no need for an upper-limit constraint. An increase in T_3 and a decrease in T_4 in response to the increasing glucosinolate levels were noted from the regression analyses indicating that the biological active hormone form, T_3, rather than T_4 tended to be produced as a compensatory response to ingested glucosinolates. No adverse effects were noted on hatchability and fertility of laying hens in canola meal feeding regimens indicating that canola meal can be used as a protein supplement in breeder laying hen diets. However, a reduction in body weight and plasma T_4 of day-old chicks were noted although the latter response could be alleviated by adding supplemental iodine to the canola meal diet.

DEDICATION

I am dedicating this thesis to my parents, Wentin Wang and Xiuqing Chen, and my brothers, Yulin Wang and Yuwei Wang, whose unconditional love and understanding are beyond description. My dad's insight and persistency inspired me to achieve. My mom's courage in handling difficulties and enthusiasm toward life encouraged me to look forward. The consistent support of Lin and Wei pulled me through many difficult times.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CHB	1-cyano-2-hydroxy-3-butene
SCN	Thiocyanate ion
OZT	5-vinyloxazolidine-2-thione
GST	Glutathione-S-transferase
GSH	The reduced form of glutathione
GS_X	Total glutathione
GSSG	Oxidized form of glutathione
min	Minutes
I	Iodine
T_3	Triiodothyronine
T_4	Tetraiodothyronine
EDTA	Ethylenediamine tetraacetic acid
DCNB	3, 4 dichloronitrobenzene
NADPH	Nicotinamide adenine dinucleotide phosphate
NAD	Nicotinamide adenine dinucleotide
SCWL	Single comb white leghorn

Chapter I

1.0 GENERAL INTRODUCTION

In Canada extensive plant selection programs to reduce the glucosinolate content of rapeseed in an attempt to improve the nutritional quality of the meal began in the mid 1950s. Low-glucosinolate rapeseed varieties now called canola (containing 30 μmol glucosinolates per gram oil free meal) were first made available for commercial use in the 1970s. Nutritive value of rapeseed meal and canola meal were extensively evaluated in various species and the meals were shown to be an excellent protein supplement with a well balanced amino acid pattern and rich in sulfur amino acids. However, antinutritive effects were demonstrated in these studies. The effects were more pronounced for rapeseed meal in comparison to canola meal and hence glucosinolate content was recognized as a major antinutritive factor in rapeseed meal and canola meal feeding regimens. Over the years increased understanding of glucosinolate metabolism in animals was helpful in explaining the toxicity of glucosinolates, but more information is needed.

Mortality due to liver hemorrhage was a major antinutritive effect of glucosinolates in rapeseed meal feeding regimens in laying hens and this effect became less severe with the development of canola. Consequently occurrence of liver hemorrhage became a non-sensitive indicator of the antinutritive effects of canola meal feeding regimens. Other more sensitive

indicators for evaluating the effects of glucosinolates are needed. Thyroid enlargement and alterations of thyroid hormone levels have been reported in rapeseed/canola meal feeding regimens in various species. Liver is the most important detoxifying organ in the body and glucosinolates, as a foreign compound could influence the liver detoxification enzyme system (ie alterations in concentrations of glutathione, glutathione-S-transferase and cytochrome P_{450}). In this regard changes in physiological parameters relating to thyroids and livers of laying hens could be used as biomarkers for the effects of glucosinolates. Measurements of alterations of these physiological parameters in the liver and thyroid will help to understand the development of liver hemorrhage. In collaboration with the performance characteristics, the data could be used as a guideline for the use of canola meal in animal diets.

In pigs, the effects of feeding rapeseed meal on reproductive efficiency have been throughly studied. In general, sows show only slight antinutritive effects due to high-glucosinolate rapeseed meal consumption. In contrast, fetuses show a marked sensitivity to rapeseed meal consumption by sows with enlarged thyroid and depressed blood T_4 levels. These negative effects can be counteracted by iodine supplementation. In poultry, studies have reported enlarged thyroids in hatched chicks from eggs produced by hens consuming rapeseed meal/canola meal although no effects on fertility and hatchability of eggs were indicated. In general, the information on canola meal feeding in breeder laying hens is not as well documented as that for pigs and the effects of iodine supplementation have not been studied to any extent, consequently more research is needed.

In this current study a series of experiments will be conducted in which laying hens will be fed canola meal based diets varying in glucosinolate content. An attempt will be made

to establish the relationship between dietary glucosinolate level and physiological parameters relating to the liver xenobiotic detoxification system and thyroid status. In addition, a hatchability study will be conducted to investigate the effects of canola meal feeding regimens and dietary iodine supplementation on fertility and hatchability in breeder laying hens.

Chapter II

2.0 LITERATURE REVIEW

2.1 INTRODUCTION

Glucosinolates are a class of sulphur-containing glycosides found mainly among the Cruciferae family. Glucosinolate concentrations vary throughout the plant with seeds containing a higher concentration than the vegetative parts (Duncan, 1992). As a major antinutritive factor in rapeseed meal and canola meal, the presence of glucosinolates limits the usage of rapeseed meal and canola meal in animal diets, especially in diets for pigs and poultry. Many studies have been conducted to evaluate the feeding value of rapeseed meal and canola meal and guidelines are provided for practical use of these products. However, the physiological effects of glucosinolates and their breakdown products are still not clear despite the extensive research on the structure, biosynthesis and taxonomic distribution of glucosinolates. The following review will give a brief introduction of glucosinolate structure and breakdown, and then concentrate on glucosinolate metabolism, glucosinolate toxicity in general, goitrogenicity, liver hemorrhage in the laying hen, and the effects of glucosinolates on liver detoxification enzyme systems.

2.2 STRUCTURE

The general structure of glucosinolates elucidated by Ettlinger and Lundeen (1956) (Figure 1) shows that the significant features of the structure are a sulfonated oxime grouping. Almost all glucosinolates contain β-D thioglucose groupings, but one exception is that a sinapic acid may be esterified at 6 position (Linschied et al., 1980). The side chain, R (Figure 1) may comprise aliphatic (saturated and unsaturated) and aromatic (indole) groupings. Indole groups are common among the aromatic representatives and a terminal unsaturated bond is typical among aliphatic glucosinolates (Duncan, 1992). Of approximately 100 different side chains found in glucosinolates, only six are present in significant quantities in rapeseed (Bell, 1984).

2.3 MYROSINASE BREAKDOWN

Glucosinolates can be hydrolyzed by myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1), an enzyme present in all glucosinolate-containing plants. Myrosinase consists of a group of isoenzymes and exists in multiple molecular forms in many plants. The physical and chemical properties of each molecular form are distinctly different from one another. The stability of glucosinolates in the intact tissues led to the conclusion that the location of glucosinolates and myrosinase are in distinct compartments. Myrosinase was found and verified to be located in myrosin cells (Thangstad et al., 1990 and Höglund et al., 1992). The cellular location of glucosinolates still remains unsolved. At least three possibilities for the location of the substrate and enzyme are proposed: (1) in different cells; (2) in different compartments of the same cell; and (3) in the same compartment of the same cell, but in an

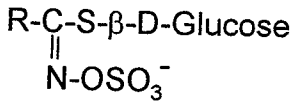

FIGURE 1. The basic structure of glucosinolates. R, the side chain, may comprise aliphatic (saturated and unsaturated) and aromatic (indole) groupings (Adapted from Ettlinger and Lundeen, 1956).

inactive form (Bones et al., 1996).

Glucosinolates and myrosinase come into contact with each other when plant cells are damaged by cutting or chewing (Fenwick et al., 1983). Myrosinase catalyzes the hydrolysis of glucosinolates (I) by splitting off glucose (Figure 2). The main product of hydrolysis is an unstable aglucone (II), which further breaks down to a wide range of products, the exact nature of which depends on the hydrolysis media involving factors such as pH, temperature, metallic ion concentration, the presence of co-factors (Duncan, 1992) and the chemical structure of the glucosinolate side chain (Fenwick et al., 1983). The side chain determines the chemical nature of the products of the enzyme hydrolysis and thereby plays a key role in the biological effects and potencies of glucosinolates as toxic compounds. Most glucosinolates can be divided into three classes (Figure 2). The first class comprises those with either an alkyl or alkenyl side chain producing primarily isothiocyanates at pH 5 to 7 (III). The second being the same as the first class but with a β-hydroxyl substituent in the side chain, forms unstable hydroxyisothiocyanates which spontaneously cyclize to oxazolidinethiones (such as IV). The third class possessing an indole nucleus gives rise to unstable isothiocyanates which degrade to inorganic thiocyanate ion (V) and an alcohol (VI). Nitrile production is favored among all three groups in more acid conditions (VII , VIII, IX) (Fenwick et al., 1989).

As discussed above, canola seed contains myrosinase and during the process of oil extraction the interaction of this enzyme with its glucosinolate substrate can result in the production of hydrolysis products. In order to minimize glucosinolate hydrolysis, rapeseed cooking is employed to inactivate myrosinase. The cooking cycle usually lasts 15-20 minutes

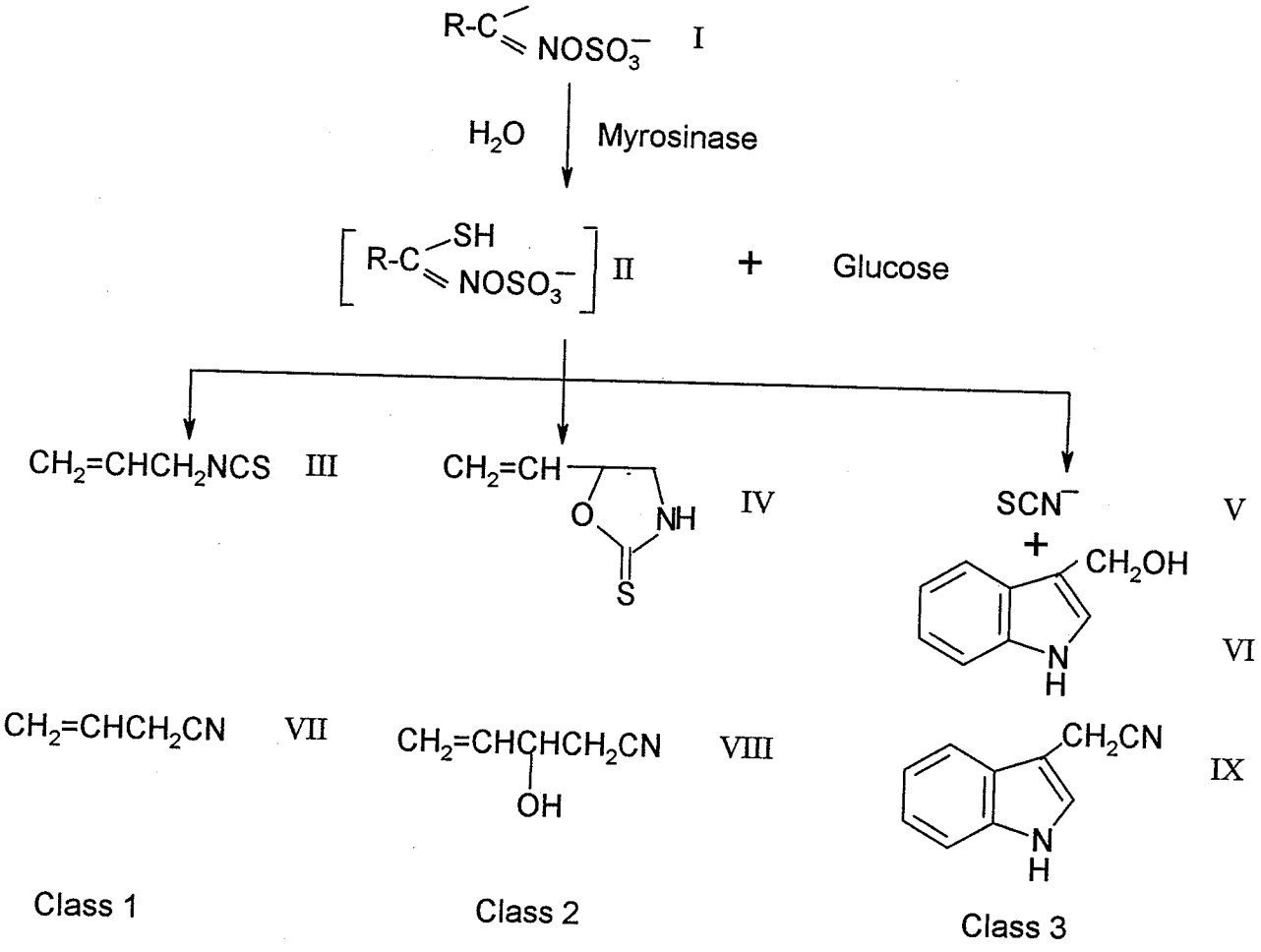

FIGURE 2. Hydrolysis of glucosinolates (I) by myrosinase enzyme to glucose and unstable aglucones (II) which further degrade to isothiocyanates or nitriles, the nature of which depends upon the chemical structure of the side chain (R). There are three general side chain classes: class 1, alkyl or alkenyl side chain; class 2, as class 1 but with β-hydroxyl group in the side chain; class 3, indole side chain. Examples of the hydrolysis products within each class are: prop-2-enyl isothiocyanate (III); 5-vinyloxazolidine-2-thione (IV)(cyclized isothiocyanate); thiocyanate ion (V) and indole-3-carbinol (VI) (products of an unstable isothiocyanate); 1-cyanoprop-2-ene (VII); 1-cyano-2-hydroxybut-3-ene (VIII); 3-indolylacetonitrile (IX). (Adapted from Fenwick et al., 1989).

at a temperature of 80-105°C, with an optimum of about 88°C (Canola Meal Feed Industry Guide, ed. By D. Hickling, Canola Council of Canada, Winnipeg, MB, 2001). In commercial canola processing, an initial moisture content of 6-10% is common for canola seed (Carr, 1995). In the processing procedure, the moisture of the canola flakes is adjusted to 5.0-6.5% at the cooker discharge point, immediately prior to entering the screw press (Unger, 1990). At this moisture level, myrosinase activity is at a low level since the enzyme is most active at a moisture level of 13% and above (Maheshwari et al., 1981). To minimize the hydrolysis of glucosinolates in cooking stage, the temperature in the cooker should be reached as quickly as possible at the start of cooking. Myrosinase activity may be still present after the cooking, screw pressing and extraction operations according to the tests conducted at various stages of processing (Unger, 1990). However, myrosinase is completely inactivated in the desolventizing-toasting stage (105°C) (Slominski et al., 1985). Consequently, canola meal should be myrosinase-free and contain intact glucosinolates as the major form of glucosinolates.

2.4 THERMAL DEGRADATION

As discussed above, heat is applied in the flaking, cooking and desolventizing-toasting stages during commercial canola oil processing. It was reported that 40-60% percent of aliphatic glucosinolates were degraded (Daun, 1986), and up to 100 percent of indole glucosinolates could be decomposed during commercial processing (Campbell and Cansfield, 1983b). Free thiocyanate ion (SCN) is a major thermal decomposition product of indole glucosinolates (Slominski et al., 1987b and Sosulski et. al., 1984). Indoleacetonitriles (3-

indoleacetonitrile and 4-hydroxy-3-indole acetonitrile) were identified as additional thermal degradation products as well as some unidentified breakdown products. Campbell and Slominski (1990) further investigated the extent of thermal decomposition of indole glucosinolates during processing of canola seed, and reported that indoleacetonitriles (3-indoleacetonitrile and 4-hydroxy-3-indoleacetonitrile) and SCN together accounted for 45-60% of the degraded indoles in the samples studied. It was also reported in the same study that thermal degradation of indole glucosinolates occurred mainly in the desolventization stage of seed processing with minor decomposition of indole glucosinolates happening prior to this stage of processing and little or no effects shown during meal drying.

2.5 METABOLISM OF GLUCOSINOLATES

There is a paucity of published information on the fate of glucosinolates as antinutritive compounds in animals or on the mode of action of glucosinolates and glucosinolate metabolites. This is partly due to the practical analytical problems associated with the determination of the reactive breakdown products in the complex medium of digestive fluid (Duncan, 1992) and the difficulties of working with surgically modified animals (Maskell, 1994). Improvement in isolation techniques, the use of isotopically labeled compounds (Duncan, 1992) and the significant interest in glucosinolates and their degradation products as potential anticancer agents in human nutrition will increase research in this area.

The metabolism of glucosinolates has been investigated from several aspects. Experiments were conducted to determine the extent of disappearance of intact glucosinolates from rapeseed or other bound forms (e.g. *Brassica* forages) in the digestive tract of animals.

Slominski et al. (1988) conducted balance trials with intact and caecectomised hens to determine the recovery of intact glucosinolates in the excreta of laying hens consuming 40% commercial canola meal or rapeseed meal. Based on the fact that 86% of intact glucosinolates was recovered, the authors suggested that not more than 14% of the ingested intact glucosinolates may be absorbed during passage through the upper parts of the gastrointestinal tract of laying hens. The view that absorption was responsible for the disappearance of intact glucosinolates in the upper gastrointestinal tract was also shared by early researchers (Lo and Hill, 1971, Bell et al., 1971, Ochetim et al, 1980). Slominski et al. (1988) further indicated that a very low absorption of intact glucosinolates occurred in the laying hen, based on the very low intact glucosinolate concentration in the blood of hens fed canola meal. In this study the only intact glucosinolate detected was progoitrin (2-OH-3-butenyl-glucosinolate). Freig et al. (1987) investigated the excretion patterns in feces and urine of intact glucosinolates by using colostemized roosters and deposition of intact glucosinolates in bile, liver and kidney tissues by using intact roosters. The results showed that the concentration of intact glucosinolates and aglucones were low in urine, bile, liver and kidney samples, supporting the conclusion that the absorption of intact glucosinolates was limited. In addition to the low absorption rate, the low concentrations of intact glucosinolates could also be due to the binding to proteins and metabolism during the passage of the intestinal epithelium or during the first pass in the liver (Michaelsen et al., 1994). In pigs, Maskell (1990) suggested that about 60% of most glucosinolates reach the large intestine undegraded, according to the recovered concentrations of intact individual glucosinolates in ileal digesta. In follow-up studies, Maskell et al. (1994) did a series of *in vitro* experiments

to simulate digestion in the stomach and small intestine in pigs. It was found that the decline in total glucosinolate content of rapeseed was 14% after simulated gastric digestion and 32% when followed by a 4 h simulated digestion in the small intestine. These *in vitro* experiments indicated that degradation may play a role in the loss of glucosinolates in the upper part of gastrointestinal tracts in addition to absorption. In the same study, the major degradation product resulting from myrosinase hydrolysis of progoitrin, namely vinyloxazolidine-2-thione (OZT), was not detected in any of the incubations. Therefore, it was suggested by the authors that progoitrin was degraded by a pathway other than that catalyzed by myrosinase. It was reported that part of the degradation in the upper gastrointestinal tract could be caused by low pH alone or in combination with metal ions present in the feed, which may catalyze hydrolysis of glucosinolates (Olsen et al., 1981, Searle et al., 1984 and Daxenbichler et al., 1966).

From the above studies, it would appear that a small extent of absorption and degradation of glucosinolates occurs in the upper digestive tracts of animals. Consequently, there must be other sites for the majority of glucosinolates to be metabolized resulting in a negative impact on animal growth and development. Slominski et al. (1988) studied the hydrolysis of intact glucosinolates in *in vitro* incubations with contents from various segments of the gastrointestinal tracts of laying hens. It was demonstrated that significant hydrolysis of intact glucosinolate was present only in caecal or caecal plus colon samples and this confirmed that caeca are the main site for decomposition of intact glucosinolates within the gastro-intestinal tract of laying hens.

Maskell et al. (1994) conducted a series of *in vitro* experiments to simulate digestion

in the stomach, small intestine and caecum of the pig. Glucosinolates were incubated with pepsin-HCL and contents of the small intestine and caecum of pigs fed on a soybean meal based diet. It was found that the losses of total glucosinolates in rapeseed meal during the peptic, small intestine incubations and during 4h of caecal incubation under control conditions amounted to 14.7, 15.8 and 78.8% of the glucosinolates present at the start of the incubations. These results also pointed to the caecum as a major site of glucosinolate degradation. It can be concluded that the majority of the degradation of glucosinolates happens in the lower parts of the gastrointestinal tract, mainly the caecum.

The glucosinolate, (2R)-2-hydroxybut-3-enyl glucosinolate (progoitrin), is the most abundant glucosinolate regardless of the total intact glucosinolates in rapeseed meal, usually accounting proportionately for at least 60% of all intact glucosinolates present. Of the nitriles produced from the hydrolysis of progoitrin, (2R)1-cyano-2-hydroxy-3-butene (CHB) is by far the most important quantitatively (Daxenbichler et al., 1967; Lo and Hill, 1972). Nitriles and OZT are generally considered the most physiologically active derivatives. It is important to study OZT and nitriles so that the deleterious effects of glucosinolates can be better elucidated. In poultry, Smith and Campbell (1976) determined the hydrolysis products of progoitrin and found that nitrile compounds existed in all areas of digestive tracts in much larger relative amounts than OZT. Maskell et al. (1994) conducted an *in vitro* study to investigate the production of OZT when rapeseed meal was incubated with myrosinase or with caecal contents of the pig fed rapeseed meal. It showed that OZT was produced more rapidly in the presence of myrosinase and OZT concentration reached a plateau within 2 hours which was maintained for at least 8 hours. There was no degradation of OZT in the presence

of myrosinase alone. However, when incubated with the ceacal contents from rapeseed meal-fed pigs, the lag-phase of the production of OZT was shorter and the rate of OZT degradation after the peak was fast. Therefore, the authors suggested that myrosinase alone could not degrade OZT, while the microbial population could degrade OZT as well as produce it from progoitrin.

Lawrence et al. (1995) investigated the potential production and absorption of CHB in cannulated pigs fed different levels of total intact glucosinolates induced by including rapeseed meal with different levels of total intact glucosinolates but with similar individual glucosinolate proportions. 1-cyano-2-hydroxy-3-butene was not found in the feces of pigs given a rapeseed meal-based diet, however, it was found in ileal digesta in highly variable concentrations among individual animals when intakes of total glucosinolates were 23.7μmol or above and the peak concentrations of CHB tended to occur at between 2.5 and 5.5h after feeding. It was concluded from the study that the foregut, up to the point of the terminal ileum, was the most likely production site of CHB from the hydrolysis of glucosinolates. The absence of CHB in feces does not preclude the possibility of CHB passing into the lower gut and being rapidly absorbed or being broken down further to yield other compounds. On the other hand, some glucosinolates could escape the hydrolysis in the foregut and be broken down in the hindgut with the CHB produced having either or both of the fates described above. Another possible route of nitrile disappearance was via glutathione conjugation (Silver et al., 1982). These studies shed some light on the production sites of OZT and nitriles, but how these findings are connected to further metabolism of these compounds is not clear. Future work is needed to better understand the negative effects of glucosinolates in animals.

The hydrolysis of glucosinolates is known to be affected by many factors (Duncan, 1992). Numerous studies have been done to investigate the influence of dietary supplementation of metal ions on the nutritive value and potential effects of rapeseed meal. Bell et al. (1967) found in *in vitro* experiments that the concentrations of OZT and isothiocyanates in rapeseed meal were reduced when rapeseed meal was soaked with copper (as copper sulphate solution) followed by drying. Lüdke et al. (1988) and Schöne et al. (1988) found that pig performance and thyroid status (thyroid weight and thyroid hormone levels) were improved or normalized in comparison with soybean meal fed pigs when the pigs were fed rapeseed meal treated with copper in addition to iodine supplementation.

Roman (1991) studied the effects of copper and probiotics on glucosinolate concentrations in ileal digesta and the feces of growing pigs. A small reduction of total intact glucosinolate concentrations in ileal digesta was found in pigs fed rapeseed meal with added probiotics (*Lactobacillus acidophilus* bacteria) in comparison with rapeseed meal-fed pigs. A significant decrease of total intact glucosinolate concentrations in ileal digesta was observed in pigs fed rapeseed meal with copper addition. The concentrations of total intact glucosinolates in feces, the individual intact glucosinolates expressed as proportions of total intact glucosinolates in the diets and ileal digesta and glucosinolate hydrolysis products (OZT, isothiocyanates or nitriles) in ileal digesta were similar for the three rapeseed meal treatments. This study indicates that glucosinolate hydrolysis was extensive and was not significantly affected by addition of the probiotics to rapeseed meal diets but was increased by the addition of copper sulphate. Furthermore, the study also indicates that the glucosinolates and/or their hydrolysis products were either absorbed from the intestinal rumen and/ or converted to other

products which were not determined.

In the same study (Roman, 1991), the researchers conducted an *in vitro* experiment in which the effects of the indigenous flora were minimized. Rapeseed meal was aerobically incubated for 4h with ideal digesta from the pigs fed soybean meal diet. Aliquots were taken for the incubation with or without copper. It was found that the same amount of glucosinolates were hydrolyzed irrespective of copper sulfate addition. The relative proportions of individual glucosinolates were similar to the original rapeseed meal. Concentrations of OZT and isothiocyanates were low and similar with and without copper and nitriles were not detected. The authors suggested that the action of copper may be mediated either by interaction with indigenous flora of the foregut or by an effect on absorption of glucosinolates from the gut.

From the above studies, it can be seen that metal ions affect glucosinolate breakdown and may assist the absorption of intact glucosinolates and glucosinolate hydrolysis products in the animal. The metal ions may have an influence in concert with microflora in the gastrointestinal tract. Copper supplementation in combination with iodine can normalize the thyroid status, and the clarification of these effects of metal ions can help to understand glucosinolate metabolism better.

Michaelsen et al. (1994) investigated the absorption of intact glucosinolates (sinigrin, glucoberin, glucotropaeolin and sinalbin) in the form of active and passive transport across the intestinal wall *in vitro* using the intestine of rat and hamster as everted sacs. Transport of glucose and individual intact glucosinolates across the wall of everted intestinal sacs from the mucosal to the serosal side was assessed by measurements of glucose and intact glucosinolate

concentrations in the serosal and mucosal incubation media before and after 40-90 min incubation under physiological conditions. In Experiments to detect active transport of glucosinolates and glucose, glucose was found to be absorbed actively throughout the entire length of hamster and rat intestine based on the concentration difference on the serosal and mucosal sides. No increase in the intact glucosinolate concentration on the serosal side nor decrease on the mucosal side was observed in the everted sacs from both hamsters and rats indicating that intact glucosinolates could not be actively transported by means of the glucose transport system. However, sinigrin and glucotropaeolin were found to be passively transported across the small intestine of the hamster. Passive transport of glucosinolates across the wall of sacs made from large intestine was slower than that of sacs made from small intestine. The rate of absorption seemed to depend on the lipophilicity of the glucosinolate side chain. These researchers suggested that glucosinolates forming ion pairs with more lipophilic cations such as sinapine or alkaloids might be absorbed more easily, perhaps even actively. Therefore, individual glucosinolates may have different effects on overall toxicity of glucosinolates due to the different transport mechanism across the gut wall which results from their different chemical structures and substances present in the digestive medium.

Microorganisms are of importance with regard to glucosinolate degradation in the lower parts of the intestinal tract. Greer and Deeney (1959) was one of the first researchers to suggest that intestinal microflora were involved in glucosinolate hydrolysis. In later studies, Greer (1962) found microflora in the caecum and colon of the rat and the fowl (Marangos and Hill, 1974) were shown to possess a 'myrosinase-like' potential. Oginski et al. (1965)

reinforced this hypothesis when they showed that human fecal strains belonging to *Enterobacteriaceae* were able to convert progoitrin into goitrin *in vitro*. All these experiments were done *in vitro*. Slominski et al. (1987a) studied the disappearance of intact glucosinolates from the gastrointestinal tracts of cecectomized hens and antibiotic-fed hens. It was found that cecectomized hens as well as the inclusion of antibiotics in the experimental diets dramatically increased glucosinolate recovery, with approximately 80% intact glucosinolates recovered in the former case, indicating the importance of hind gut microflora in glucosinolate hydrolysis. Nugon-Baudon et al. (1988) used germ-free and conventional rats and chickens and chimera rats/chickens (germ-free animals inoculated with the microflora of an animal belonging to another species) to study the role of intestine flora in rapeseed meal toxicity and the characteristics of intestinal microflora in the two species. It was found that the rat intestinal microflora were responsible for the dramatic growth depression and the slight hypertrophy of glucosinolate target organs (liver, kidneys) in conventional rats. The growth of young birds (two-week-old) was not sensitive to the bacteriological status since there was no difference between the conventional vs germ-free birds. However, older conventional birds (ten-week-old) had shown an advantage to a soybean meal diet vs a rapeseed meal diet with regard to weight gain. The chicken flora was responsible for a much more important goitrogenic effect in conventional chicken and chimera rats. The rat flora induced a moderate increase of thyroid weight both in conventional rats and chimera chickens.

Rabot et al. (1993a) studied the influence of different strains of human fecal flora on rapeseed meal toxicity in gnotobiotic rats and the specificity of the symptoms (e.g. the reduced feed intake, the changes of internal organs, and the changes of thyroid hormone

levels) observed according to the rat bacteria status supported the hypothesis that bacteria yielded specific derivatives in relation to specific enzyme properties. Campbell et al. (1995) conducted an experiment to study the fate of ingested glucosinolates in germ-free and conventional rats fed rapeseed meal diet. Both total intact glucosinolates and indole glucosinolates showed a reduction from stomach contents in both types of animals. However, the majority of intact glucosinolates disappeared in the lower part of gastrointestinal tracts in conventional animals and remained virtually unchanged in germ-free animals indicating a marked effect of intestinal flora on intact glucosinolates. The common negative effects associated with glucosinolate intake such as elevated organ weights, increased xenobiotic metabolizing enzymes in liver tissue along with a depression in plasma T_4 level were observed in conventional animals but not in the germ-free animals. These data indicated that the significant differences between germ-free and conventional animals could be attributed to the influence of intestinal microflora on dietary glucosinolates and it was suggested that glucosinolate derived products absorbed from gastrointestinal tract were responsible for the effects observed. In addition to myrosinase-like activity of intestinal microflora, microorganisms of *Lactobacillus* strain from a chicken crop were shown to induce goitre in gnotobiotic rats fed rapeseed meal diet (Nugon-Baudon et al., 1990b). It can be concluded that microflora in the digestive tract have a profound influence on the rapeseed meal toxicity. The microorganism of different animal species or of different strains in the same species have a distinct impact on the toxicity of glucosinolates.

2.6 TOXICITIES

Rapeseed meal/ canola meal is an excellent protein supplement, rich in sulfur-amino acids. However, reductions of animal performance and changes to vital organs (thyroid, liver, and kidney) have been reported to be associated with high dietary levels of rapeseed meal/canola meal inclusion. Glucosinolates are believed to be mainly responsible for these symptoms. Better understanding of glucosinolate toxicity will help to provide information for the safe utilization of rapeseed meal/canola meal.

In past studies, the most common way of assessing the toxicity of glucosinolates was to correlate toxicity with dietary glucosinolate levels. Gradient levels of glucosinolates were attained by combining different ratios of rapeseed meal with soybean meal or using "high glucosinolate" and "low glucosinolate" rapeseed meal. In these studies, toxicities were evaluated by gross productive characteristics and often corroborated by the measurements of organ weight (i.e. thyroid, liver, kidney). Most of the studies provided little information regarding the actual mechanism of toxicity of glucosinolates but were useful from a practical standpoint in helping to determine the safe inclusion levels of rapeseed meal in diets for different animal species. These studies indicated that glucosinolates and their breakdown products could be responsible for the problems associated with rapeseed meal feeding. In addition to the influence of glucosinolates, the effects of non-antinutritive attributes of rapeseed meal/ canola meal (less energy and lysine in comparison with soybean meal) and other minor antinutrients such as sinapine, tannins, phytate and fibre could not be ruled out.

More specific studies on glucosinolate toxicities have involved the treatment of rapeseed meal in various ways to produce particular groups of toxic metabolites followed by

an evaluation of the toxicities of these products in animal studies. For example, rapeseed meal rich in either nitrile hydrolysis products or OZT were produced through controlling the hydrolysis conditions of glucosinolates. When fed to rats or chicks, both caused depression in live-weight gain but the mode of action was different. The OZT-rich rapeseed meal caused an increase in thyroid weight, while the nitrile-rich rapeseed meal led to kidney enlargement (Srivastava et al., 1975). Other similar studies in assessing glucosinolate toxicities have been conducted by extracting rapeseed meal with specific solvents and adding the extracts to glucosinolate-free diets. Lee et al. (1984) demonstrated a depression in the voluntary feed intake of growing pigs consuming soybean meal with added intact glucosinolate extracts. Lo and Bell (1972) prepared a rapeseed isolate which contained largely butenyl isothiocyanate and butenyl cyanide. It was shown that live weight gain and intake were depressed in rats fed this mixture. Thyroid weight was unaffected but tracer studies with ^{125}I suggested that biosynthesis of thyroid hormones were affected. The toxicity of specific hydrolysis products of glucosinolates was stressed in these studies, although the biochemical basis for this toxicity remains unsolved.

Improved methods for the isolation of intact glucosinolates and glucosinolate derived products supplied sufficient amounts of these compounds in relatively high purity which in turn facilitated a series of animal studies. In this regard, Vermorel et al. (1986) studied the antinutritive effects of individual glucosinolates in rats, including gluconapin and progoitrin, two major components of high-glucosinolate rapeseed cultivars, and glucobrassicin, a typical indole glucosinolate in rapeseed. Thyroid weights were only significantly affected by the diet containing progoitrin which also produced small increases in the weights of the livers and

kidneys. At the level fed in this study none of the glucosinolates resulted in a reduction in feed intake, growth rate or plasma thyroid hormone levels. In other studies with isolated glucosinolates antinutritive and toxic effects of six glucosinolates (gluconapin, epiprogoitrin, glucoiberin, glucoraphanin, glucocheirolin, and glucotropaeolin) were investigated in a balance trial with rats (Bjerg et al., 1989). Epiprogoitrin was the only glucosinolate that affected the relative size of livers. Glucoraphanin and glucotropaeolin significantly affected the relative weight of kidneys. A tendency was observed with all glucosinolates with regard to kidney weight. Kidney weights were reduced at lower concentrations of glucosinolates and the opposite phenomenon happened at higher concentrations. The effects on the kidney was in agreement with the study done by Vermorel et al.(1986) who demonstrated that low levels of glucobrassicin, glucotropaelin and gluconapin caused a reduced kidney weight whereas a high concentration of progoitrin caused increased relative kidney weights though none of the changes were highly significant. Relative thyroid weights were reduced by gluconapin, epiprogoitrin, glucoiberin, and were not affected by glucoraphanin, glucocheirolin, and glucotropaeolin. Other workers have not noted an influence from epiprogoitrin (Van Etten et al., 1969 and Nishie et al., 1982) whereas an increased relative thyroid weight has been seen with progoitrin (Vermorel et al., 1986). It can be seen that individual glucosinolates have different effects on internal organs (thyroid, liver and kidney). Progoitrin is a powerful goitrogenic agent and also has influence on livers and kidneys. Epiprogoitrin is a potent inducer of liver enlargement although kidneys tended to decrease in weight with low concentrations of glucosinolates studied and increase with high concentrations. The different effects of these glucosinolates are likely in relation to their structure and degradation

products.

The addition of active myrosinase to various glucosinolate containing diets represents a further method of elucidating the toxic mechanism of glucosinolates. Vermorel et al. (1988) studied the antinutritional effects in rats fed low-glucosinolate and high-glucosinolate rapeseed meal with and without myrosinase addition to the diets. The experimental diets also included a diet containing progoitrin with added myrosinase and a pair-fed control diet. Live weight gain was significantly reduced by both types of rapeseed meal and the addition of myrosinase accentuated this effect during the second half of the 30-day experimental period. Liver weight was increased by 58% in the diet containing progoitrin with added myrosinase. Thyroid weights were found to be greatly increased by the feeding of rapeseed meal or progoitrin together with myrosinase although an increase in the size of thyroid was also observed in the absence of the enzyme. The major antinutritional effects became evident after only 10-15 d perhaps explaining the lack of such effects in short term experiments (Bille et al., 1983). The myrosinase effect in this study is in accordance with the study conducted by Bjerg et al. (1989) who reported that the myrosinase supplementation aggravated the negative effects on internal organs caused by individual glucosinolates alone. The results support the view that progoitrin is a major factor limiting the more extensive use of rapeseed meal as an animal feed and that myrosinase plays an important role with respect to the glucosinolate toxicity.

In summary, individual glucosinolates and/or their degradation products can have specific antinutritional or toxic effects. Among the glucosinolates, progoitrin is of major significance in relation to the antinutritive effects in rapeseed meal. The effects are most likely

due to its hydrolysis products, i.e. hydroxynitriles and OZT. The hydrolysis products of glucosinolates act on different target organs. The thyroid is the major target organ for isothiocyanates and specifically OZT, while liver and kidney are influenced by nitriles to a great extent. Since the hydrolysis products of glucosinolates play an important role in the toxicity of glucosinolates, it is necessary to consider hydrolysis media in digestive tracts, such as pH, content of specific metal ions and the microflora of the gut.

2.7 GOITROGENICITY

The first evidence that rapeseed contained goitrogenic properties was reported by Kennedy and Purves (1941) in rats. Bell and Belzile (1965) reported that rapeseed could cause goitrogenicity in swine and poultry and demonstrated that the response was dependent upon the levels and nature of glucosinolates present in the diet. In later studies, thyroid enlargement was extensively reported as a goitrogenic effect of glucosinolates in poultry fed crambe or rapeseed meal diets (Srivastava et al., 1975, Papas et al., 1979a and Fenwick et al., 1980a). The changes of thyroid histopathology induced by glucosinolates have been described for various species (Bell et al., 1972 and Wight et al., 1985a). It was found that low levels of intake initiated an increase in follicular epithelial cell height and foamy cytoplasm and continued exposure led to hypertrophy with thyroid weight increasing 2-5 times. Loss of follicular colloid and development of hyperplasia were typical results of high glucosinolate intake for prolonged periods (Fenwick et al., 1989).

The histological changes were accompanied by alterations in thyroid hormones. Alterations of thyroid hormones (T_3 and T_4) were investigated when rapeseed meal was used

as a protein supplement in many animal studies. Some indicated that T_3 was reduced in rapeseed meal/canola meal fed animals (Nugon-Baudon et al.,1990a and Spiegel et al., 1993) while others indicated that T_3 was increased (Kloss et al., 1994). However, T_4 was unanimously reported to be reduced in these studies. An increase in T_3 could be due to a destruction in cellular T_3 receptors by glucosinolates and impaired T_3 transfer might cause an increase in blood concentration of this hormone (Schöne et al., 1993). Increased T_3 could also result from some unidentified compensatory mechanisms which may be activated under a long period of canola meal/ rapeseed meal feeding (Opalka et al., 2001). The mechanisms may be in favor of T_3 production, since T_3 is biologically more active with one less atom of iodine in comparison with T_4. This explanation seems plausible especially in the case of iodine deficiency. T_3 level is affected by protein and energy intakes (Ingram and Evans, 1980 and Smallridge et al., 1982). This may explain why T_3 responds less consistently to the impact of glucosinolates than does T_4.

The decreased concentrations of T_3 and T_4 could be closely related to glucosinolate hydrolysis products. The thyroid is the target organ of iodine but also of glucosinolate degradation products. Normally the thyroid follicular cells are able to trap inorganic iodide at the base of the cell and transport it against an electrical gradient across the cell. Inorganic iodide is then converted to an oxidized species of iodine by a peroxidase which happens most likely at the luminal surface of the cell. The oxidized species of iodine (active iodide) are bound to thyroglobulin (Hadley, 1992). In the case of canola meal and canola seed feeding the peroxidase is involved in the oxidation of glucosinolate hydrolysis products (i.e. OZT). In this conversion process iodine will be reduced to inorganic iodide and hence active iodide

would not be available for iodination of thyroglobuline. This would interfere with the subsequent synthesis of thyroid hormones. Furthermore, OZT and other glucosinolate hydrolysis products limit the ability of peroxidase to oxidize inorganic iodide to active iodide also resulting in lowering iodization of thyroglobulin and thyroid iodine deposition (Kohler et al., 1988). In addition to the influence on peroxidase, OZT interferes with the coupling reactions necessary for T_4 formation which may lead to accumulation of thyroxine precursors in the thyroid (Akiba et al., 1976 and Elfving, 1980a).OZT may also affect the peripheral conversion of T_4 to T_3 in the liver although this has been tested in rats without conclusive results (Langer et al., 1984). The goitrogenic effects caused by OZT are not alleviated by iodine supplementation (Fenwick et al., 1989).

Thiocyanate ion, which may be derived from dietary indole glucosinolates or indirectly via the metabolism of isothiocyanates and nitrile, blocks and reduces iodine capture and may lead to goitrogenicity as an iodine competitor. The impact is only evident when iodine is deficient and iodine supplementation is effective to reduce this antithyroid phenomenon (Fenwick et al., 1989). Schöne et al. (1990) investigated the effects of glucosinolates and iodine intake via rapeseed meal diets on serum thyroid hormone levels and total iodine in the thyroid in growing pigs. It was found that the serum SCN levels were similar in the rapeseed meal diet and the rapeseed meal diet treated with copper (the low-glucosinolate diet) irrespective of iodine supplementation levels and the authors indicated that SCN may only play a minor role as a causative agent responsible for changes to iodine deposition in the thyroid. Schöne et al. (1997b) investigated the effects of glucosinolates on the alterations of thyroid hormone levels in gestating and lactating sows. The glucosinolates were provided as

rapeseed meal or rapeseed. Thiocyanate ion was significantly increased but thyroid hormone status was unchanged in sows fed rapeseed or rapeseed meal diets. This study largely discounts thiocyanate as an antithyroid factor. From these reports, the role of SCN as a goitrogenic factor still needs to be further studied.

It can be summarized that the hydrolysis products of glucosinolates are responsible for the alterations of thyroid hormones (T_3 and T_4). OZT is a potent goitrogenic compound and interferes with the synthesis of the thyroid hormones. This effect cannot be alleviated by added iodine. Thiocyanate ion affects iodine uptake and this goitrogenic effect can be overcome by iodine supplementation. The alterations in thyroid hormones can lead to change of thyroid histopathology through the a feed back mechanism at both pituitary and hypothalamic levels.

2.8 LIVER HEMORRHAGE IN THE LAYING HEN

Jackson (1969) first noticed that the main cause of increased mortality among laying hens fed rapeseed meal was liver hemorrhage and this has since been confirmed by many other researchers (Fenwick and Cutis, 1980b). Studies comparing low- and high-glucosinolate rapeseed meal and different levels of glucosinolates via combining low- and high-glucosinolate rapeseed meal indicated that hens fed higher glucosinolate content had a higher incidence of liver hemorrhage (Smith et al., 1976, Grandhi et al., 1977, Campbell, 1987a and Campbell and Slominski, 1991). However, some studies indicated liver hemorrhage was not a cause of mortality in layers (Leeson et al., 1987; and Hulan and Proudfoot, 1980). Strain differences, genetically controlled factors among commercial hybrid laying hens and varied

microflora properties of the digestive tracts may account for the discrepancies (Campbell, 1979; Yamashiro et al., 1975; March et al., 1975 and Nugon-Baudon et al., 1994).

Papas et al. (1979b) indicated that a clear cause and effect relationship between glucosinolates and liver hemorrhage was not evident but intact glucosinolates and CHB were consistently associated with liver hemorrhage but only when fed together. At the same time, intact glucosinolates fed as the major glucosinolate component of a diet did not increase the incidence of liver hemorrhage and synthetic CHB added to a control diet alone did not cause liver hemorrhage. Campbell (1987a) reported that the incidence of liver hemorrhage varied with dietary levels of intact glucosinolates and indicated that the effect was related more to the total intact glucosinolates rather than to specific intact glucosinolates. In a later experiment, the same author (1987b) fed variously treated rapeseed meals to laying hens and related the concentration of intact glucosinolates and their breakdown products to the incidence of liver hemorrhage. The results confirmed that liver hemorrhage was related to the presence of intact glucosinolates in the diet but there were no obvious correlations with their specific breakdown products.

Wight et al. (1987a) investigated four types of rapeseed meal or rapeseed cake with respect to liver hemorrhage. Untreated rapeseed cake (the seed was *B. napus*), the same rapeseed cake with glucosinolates removed by hot-water extraction, a heat treated rapeseed cake with a reduced level of total glucosinolates and an increased content of nitriles, predominantly CHB, and a commercial rapeseed meal were included in the experimental diets. Birds which died were autopsied and at the termination of the experiment period all birds were killed and subjected to a post mortem examination to determine the occurrence and

severity of liver hemorrhages. It was found that hens fed the four rapeseed meal diets had similar occurrence and severity of liver hemorrhage and a higher occurrence of liver hemorrhage than birds fed the soybean meal control diet. The nitrile rich diet (the heat-treated rapeseed cake) did not result in an increased occurrence of liver hemorrhage in comparison with other rapeseed cake or rapeseed meal diets which tends to support the previous view that nitriles are not specifically responsible for liver hemorrhage. Histologically the hemorrhages were similar after feeding extracted rapeseed cake and commercial rapeseed meal. These observations led the authors to question the role of glucosinolates in the etiology of liver hemorrhage. The researchers suggested that other factors, acting either alone or in combination with glucosinolates, may be involved in the occurrence of liver hemorrhage. In this study liver hemorrhage occurrence was examined when surviving birds were killed at the end of the experiment. The authors suggested that this post mortem analysis was a more accurate measure of hepatotoxicity than mortality per se since this latter means does not disclose the cases of non-fatal hemorrhage induced by rapeseed diets. However, others have found the incidence of non-fatal liver hemorrhage was very similar in birds fed control diets (soybean meal) and rapeseed meal diets when surviving birds were killed for examination even though high mortality rate due to liver hemorrhage was observed in rapeseed meal-fed groups (Campbell et al., unpublished data).

From the above reports, it can be seen that liver hemorrhage is positively associated with rapeseed meal feeding. Attempts were made to establish the relationship between liver hemorrhage and glucosinolates and their hydrolysis products but the information is fragmentary. Further research needs to be conducted to clearly identify the causative factors

of liver hemorrhage in rapeseed meal.

Necrosis of liver cells in man (Luke et al., 1963) and in rabbits (Arhelger et al., 1965) were observed when diets containing tannins were consumed. Tannins are one of the minor antinutrients in rapeseed meal and the role of tannins in the incidence of liver hemorrhage in laying hens is not clear. Papas et al. (1979b) suggested that the mortality rate among laying hens fed on high levels of rapeseed meal may be reduced by the addition of vitamin K. The authors speculated that it may act by inhibiting the hepatic mixed functional oxidase system, therefore reducing the interaction between chemically reactive metabolites and cellular macromolecules which is known to cause cell death or necrosis. However, this was not confirmed in a later experiment (Campbell, 1987a). Wight and Shannon (1985b) also reported a positive response of adding vitamin K in relation to liver hemorrhage and the explanation was in accordance with Papas et al. (1979b). However, negative effects of vitamin K were reported in a study done by Campbell et al. (1983a) in which a large amount of vitamin K increased liver hemorrhage. Therefore, the effects of vitamin K on liver hemorrhage in rapeseed meal fed birds are still not clear and more research is needed.

The histopathological alterations associated with liver hemorrhage have been described. Degenerative changes in hepatocytes of laying hens include cytoplasmic vacuolation, swelling of the mitochodria, and distortion of the rough endoplasmic reticulum and these changes may lead to lysis and necrosis (Bromidge et al., 1985). Yamashiro et al. (1975) claimed that hepatocyte necrosis led to the distortion of the reticulin, vascular defects and subsequent hemorrhage. Ibrahim et al. (1980a) also noticed that focal necrosis was increased in hens fed rapeseed meal but a clear relationship between histological findings and

gross hemorrhage could not be established. Martland et al. (1984) investigated the development of the lesions in the liver using a soybean meal diet along with low and high glucosinolate rapeseed meal diets. The researchers observed that reticulolysis was significantly worse in hens fed rapeseed meal diets and the severity of reticulolysis is related to the dietary glucosinolate levels. The same researchers also reported that rupture of the portal veins due to degenerative changes in the vessel wall was the major cause of hemorrhage. Wight et al. (1987b) supplemented rapeseed meal and soybean meal diets with various additives with known effects on liver metabolism and determined their influence on the incidence of liver hemorrhage. Liver hemorrhage was suggested to be caused by weakening hepatic blood vessels due to inhibited collagen synthesis caused by hypothyroidism or by lathyrogenic compounds such as nitriles. These possibilities were tested by adding the goitrogen, thioracil, and the lathyrogen, β-aminopropionitrile, to rapeseed meal diets followed by histological examination in relation to liver hemorrhage. However, none of the agents produced liver hemorrhage characteristics similar to those produced with rapeseed meal diets and the aetiology of the syndrome remains obscure.

Increases in the activities of aspartate and alanine transaminases and isocitrate and malate dehydrogenase have been detected in plasma of laying hens fed rapeseed meal (Ibrahim et al., 1980b). The enhancement of aspartate transaminases in laying hens was confirmed in later studies (Pearson et al., 1983 and Martland et al., 1984). The increased activities of lactate dehydrogenase and alkaline phosphatase in the plasma were also observed in laying hens fed rapeseed meal (Pearson et al., 1983). However, malate dehydrogenase as a marker of hemorrhage has been questioned (Martland et al., 1984). Wight and Shannon (1985b)

observed alterations of alkaline phosphatase location in the hepatocytes and in the plasma concentration of bile acids in laying hens fed rapeseed meal. These biochemical changes indicate that liver cells are affected due to rapeseed meal feeding and that various enzymes or bile acids are released into the circulation system. The mode of action of these changes is not clear. However, the biochemical changes may be useful to illustrate the hepatic toxicity of rapeseed meal feeding.

2.9 EFFECTS ON THE XENOBIOTIC METABOLIZING ENZYME SYSTEM

Detoxification enzymes metabolize a wide variety of foreign compounds and endogenous metabolic waste products to more soluble compounds which are more easily excreted. Foreign compounds are collectively known as xenobiotics. The various processes involving in xenobiotic metabolism are termed the biotransformation system. Biotransformation reactions may be divided into phase I and phase II reactions. Phase I reactions (oxidation, reduction, hydrolysis) introduce a polar group into the molecule, thereby making xenobiotics more hydrophilic and susceptible to detoxification (Reeves, 1981). Oxidation is most important among phase I reactions. Cytochrome P_{450} plays an important role in the oxidation process and is a part of an enzyme system referred to as the mixed-function oxidase system. Phase II metabolism comprises conjugation reactions which make the phase I metabolites more polar and readily excretable. Conjugation can occur with a variety of substances and the substances most frequently involved are glucuronic acid, sulfate, glycine and glutathione. Transferases catalyze these conjugation reactions, for instance, glutathione-S-transferase (GST) catalyzes the glutathione conjugation.

Glucosinolates when consumed by animals are considered as xenobiotics. The phase I and phase II enzymes and glutathione (a conjugation substrate) may be of importance as biochemical parameters to illustrate the glucosinolate effects when animals consume *Brassica* vegetables and rapeseed meal. The biotransformation system was originally thought to be primarily involved in the detoxification of xenobiotics. However, it has been shown that the conversion of inert compounds into highly reactive metabolites may also occur (Welch, 1979). Therefore, when considering the induction of this enzyme system, it is important to establish not only the specific enzymes that are being affected but also the subsequent alterations in the overall metabolism and toxicities of the compounds (McDanell et al., 1988).

An awareness of enzyme-inducing effects of indole glucosinolate hydrolysis products came from a pioneering study of Wattenberg (1971) who found considerable benzo[a]pyrene hydroxylase activity in the intestine of rats fed a diet containing *Brassica* vegetables and no benzo[a]pyrene hydroxylase activity in rats fed a purified diet. From this observation, it was suggested that compounds in *Brassica* vegetables induced the benzo[a]pyrene hydroxylase activity in the intestine. Loub et al. (1975) identified that specific inducing chemicals in the *Brassica* vegetables were indole-3-carbinol, indole-3-acetonitrile, diindolylmethane and ascorbigen. These compounds were produced from glucobrassicin hydrolysis. When given by gavage all the four compounds induced benzo[a]pyrene hydroxylase activity in rat liver and intestine.

In subsequent studies, indole-3-carbinol was investigated extensively and considered as the most potent inducer. Indole-3-acetonitrile, 3, 3'-diindolylmethane and ascorbigen (an ascorbic acid adduct of indole-3-carbinol) can also have the inducing enzyme effect. In

addition to these indole hydrolysis derivatives, goitrin was also implicated to be responsible for GST and epoxide hydrolase induction (Chang et al., 1985 and Bogaards et al.,1990). Bogaards (1990) observed that allyl isothiocyanate can enhance the activities of GST. More recently, Staack et al. (1998) compared the individual effects of four glucosinolate breakdown products from brussels sprouts (indole-3-carbinol, 1-isothiocyanato-3-(methylsulfinyl)-propane, phenylethylisothiocyanate and cyanohydroxybutene) and the collective effects of these four compounds in adult rats. It was shown that indole-3-carbinol and crambene were responsible for the majority of enhancement of hepatic cytochome P_{450}, GST, quinone reductase and glutathione reductase. A synergistic effect of indole-3-carbinol and crambene was evident on induction of GST and quinone reductase but not on glutathione reductase or cytochrome P_{450}. From these reports, it can be concluded that hydrolysis products of glucosinolates can induce the phase I and phase II enzymes and during this process highly reactive intermediates may be formed. These hydrolysis products of glucosinolates may interact with each other to produce the inducing effects.

In addition to the inducing effects of hydrolysis products of glucosinolates, the influence of cruciferous vegetable and purified individual glucosinolates found in cruciferous vegetable on enzyme activity were also investigated. McDanell et al. (1989) fed rats with diets containing freeze-dried Brussels sprouts, a glucosinolate extract from Brussels sprouts or the residue left after glucosinolate extraction. Brussels sprouts and the extract contained sinigrin, progoitrin, glucobrassicin and gluconapin. The residue had virtually none of these compounds. Both the freeze-dried Brussels sprouts and the glucosinolate extract induced mixed-function oxidase activity in small and large intestines and livers of rats within 4 to 6

hours of ingestion. The residue had no effect on enzyme activity in any of the organs tested. In the same study, the authors made the first attempt to define the inducing compounds in *Brassica* vegetables by feeding the purified individual glucosinolates (sinigrin, progoitrin, glucobrassicin, and glucotropaeolin) and observed that only the indole glucosinolate, glucobrassicin, induced mixed-function oxidase activity in the small intestine but not in the liver. Greater amounts of the pure individual glucosinolates were fed to rats than were found in Brussels sprouts, however, the inducing mixed-function oxidase activity was considerably less than that induced by the intact vegetable. Therefore, other factors, as yet unidentified, present in the vegetable may participate to some degree in the inducing effects or individual glucosinolates may be able to interact each other and have synergistic influence on mixed-function oxidase activity.

Loft et al. (1992) investigated the influence of intact and myrosinase-treated indole glucosinolates on the metabolism in vivo of metronidazole and antipyrine in the rat. The indole glucosinolates were extracted from freeze-dried broccoli and included mainly glucobrassicin and neoglucobrassicin. The intact glucosinolates had no effect on the metabolism of metronidazole and antipyrine as measured by the clearance and metabolite formation rates, however, the myrosinase-treated glucosinolates increased the metabolism of metronidazole and antipyrine. From this observation, the authors emphasized that the presence of myrosinase was crucial for the formation of biologically active products from indolyl glucosinolates. However, considering all reports, the inducing effects of myrosinase are still not clear.

A possible mechanism for indole-3-methanol as an inducer of mixed-function oxidase

was proposed by Bradfield and Bjeldanes (1987). Intraperitoneal injection of indole-3-methanol did not induce enzyme activity but when the indole was mixed with hydrochloric acid prior to injection enzyme activity was increased. When indole-3-methanol was ingested cytochrome P_{450} monoxygenase activity was induced. It was suggested that indole-3-methanol reacted with acid in the stomach and the formed condensation products were responsible for the inducing effects. The proposed active products included 3, 3'-diindolemethane and linear and/ or cyclic methyleneindole trimers and tetramers. The authors found that the inducing capacity of dietary indole correlates directly with their instability in acid solution and concluded that indole influences the levels of monoxygenase activities via a series of acid-condensation products generated in the acidic environment of the stomach.

The results of the above reports indicated that hydrolysis products of indole glucosinolates, *Brassica* vegetables and purified individual glucosinolates found in *Brassica* vegetables had inducing effects on phase I and phase II enzymes when fed to rats. Among the hydrolysis products of indole glucosinolates indole-3-carbinol was the most potent inducer. Feeding *Brassica* vegetables had a more powerful inducing effect on these enzymes than feeding pure individual glucosinolates alone indicating that individual glucosinolates can interact with each other and have synergistic inducing effects or that other factors in *Brassica* vegetables may be responsible for the inducing effects.

The alternation of the detoxification enzymes was also observed in animals fed rapeseed meal. Israel et al. (1979) reported hepatic cytochrome P_{450} was significantly increased after 3 weeks of rapeseed meal feeding in broiler chicks. Nugon-Baudon et al. (1990a) showed that hepatic microsomal P_{450} activity was decreased and GST and UDP-

glucuronyltransferase activities were increased in conventional rats fed rapeseed meal. Rabot et al. (1993b) investigated alterations of hepatic xenobiotic-metabolizing enzymes in germ-free rats fed rapeseed meal and soybean meal based diets. The specific activities of cytochrome P_{450}, GST, and UDP-glucuronyltransferase remained unchanged in both diets. From this observation, the authors indicated that intestinal microflora mediated the alterations of the xenobiotic-metabolizing enzymes. Roland et al. (1996) investigated the influence of inulin and oat fibre on the biological effects of glucosinolates in gnotobiotic rats harboring a human whole fecal flora. The experimental diets included soybean meal, rapeseed meal, rapeseed meal supplemented with inulin and rapeseed meal supplemented with oat fibre. It was observed that the rapeseed meal treatment significantly increased the concentration of cytochrome P_{450} and the specific activities of GST and UDP-glucuronyltransferase in the liver. Rapeseed meal treatment induced GST activity but reduced UDP-glucuronyltransferase activity in the small intestine. GST activity was not modified by the rapeseed treatment in the large intestine. Oat fibre and inulin modulated the alterations of these enzymes induced by rapeseed meal. These two types of fiber counter-balanced the induction of hepatic cytochrome P_{450} and lessened UDP-glucuronyltransferase in the liver but did not modify the depletion effect of UDP-glucuronyltransferase activity in the small intestine. On the other hand, they enhanced the induction of GST in the liver and large intestine but not in the small intestine. Inulin modulated these effects to a lesser extent in comparison with oat fibre. The different alterations by rapeseed meal on hepatic cytochrome P_{450} in this study and the previous study (Nugon-Baudon et al., 1990a) with conventional rats could be due to the difference of rat strain, the glucosinolate source, the composition of the diet and the experimental design

(Miller et al., 1983 and Nugon-Baudon et al., 1994). The inducing effect of hepatic GST due to rapeseed meal was consistent with other studies when either gnotobiotic rats or conventional rats were exposed to glucosinolates from rapeseed meal or other sources (i.e. *Brassica* vegetables).

Clinton and Visek (1989) and Lindeskog et al. (1988) showed that different types of fibre modified the toxicity of dietary xenobiotics and their effects on hepatic and intestinal phase I enzymes in conventional rats. Roland et al. (1996) confirmed for the first time a similar situation in gnotobiotic rats with human digestive microflora. Furthermore, the study showed that fibre may modify the effects of dietary xenobiotics on phase II enzymes. The different modulation effects of different fibers could be due to the variation in fermentative characteristics which may interfere with the bacterial metabolism of glucosinolates in the caecum. On the other hand, the metabolism of bacterial glucosinolate derivatives by the digestive xenobiotic metabolizing enzyme system could be modified through the direct action of fibre on these enzymes. The organ differences of these xenobiotic metabolizing enzyme system could be due to differences in the tissue concentrations of the inducing compound (Kore, 1993). It can be summarized that rapeseed meal feeding can increase cytochome P_{450} in chickens and the effects in rats may be different depending on the rat strain or bacterial status in rats (germ-free or conventional rats). Dietary components (e.g. fibre) can potentially alter xenobiotic detoxification by a modification of the detoxification enzymes.

Glutathione (GSH), the major non-protein thiol in most plants and animals, is a tripeptide composed of cysteine, glycine and glutamic acid (γ-glutamyl-cysteinyl-glycine). Glutathione exists both in a reduced thiol form and an oxidized disulfide form (GSSG) and

plays an important role as a substrate in the xenobiotic metabolizing enzyme system. Wallig et al. (1988a) investigated the effects of glucosinolates on GSH in a study in which 125mg 1-cyano-3,4-epithiobutane /kg body weight, a cruciferous nitrile, was given to male rats once daily by gavage for 1, 2 or 3 days. Hepatic and pancreatic GSH concentrations were increased in all treated groups. In another study (Wallig et al., 1988b) young rats were treated by gavage once daily for 4 days with 200mg CHB/kg body weight and pancreatic, hepatic and renal GSH concentrations were shown to be elevated by the CHB treatment. Wallig et al. (1992) also conducted a study with rats to determine if smaller doses could cause GSH elevation in the absence of toxicity. A single oral dose of 100mg/ kg or multiple lower doses (50 mg/kg daily for 3 days or 30mg/ kg for 6 days) caused significant and persistent increases in pancreatic GSH although hepatic levels were unchanged. Neither a single oral dose of 100mg/ kg or multiple lower doses were associated with toxicity. However, when either 100 or 50mg/kg were administered intravenously, pancreatic apoptosis was observed and elevated GSH levels were exhibited in both pancreas and liver. The results indicated that both toxicity and GSH elevation are greatly increased by administering CHB intravenously rather than orally. In the same study, it was observed that CHB initially depleted pancreatic GSH but elevated GSH at a later time period in rats fed the highest dose (100mg CHB/kg). Intravenous injection of CHB at either 100 or 50mg/kg both caused the initial loss of GSH and a following rebound in pancreas and liver. Several possible mechanisms can be considered. Firstly, a direct depletion of GSH by CHB could lead to release γ-glutamyl cysteine synthetase through the feed back mechanism and ultimately increased GSH production. A second mechanism of enhancement could be due to alterations in tissue GSH/GSSG ratios after CHB treatment

which could lead to the induction of γ-glutamyl cysteine synthetase synthesis. A third possibility is that CHB could meditate enhanced GSH levels by increasing cysteine uptake or availability for γ-glutamyl cysteine synthetase. A fourth potential mechanism of enhancement is via a direct effect of CHB or a CHB-GSH conjugate on γ-glutamyl cysteine synthetase itself. A fifth way is that the resultant ketone formed from CHB may cause an increase in the synthesis of γ-glutamyl cysteine synthetase at the transcription and translation levels and ultimately cause an increase in GSH synthesis. From these observations, it can be seen that nitriles can affect the GSH or non-protein thiol concentrations in different organs (liver, pancreas, kidney). Intravenous injection is more effective than oral ingestion with regard to the effects of nitriles on different organs. Higher doses of nitriles given either orally or intravenously can firstly decrease GSH concentrations followed by an increase in GSH concentrations and several mechanisms are possibly responsible for the phenomena.

Smith et al. (1992) conducted experiments to determine the effects of feeding canola meal (*B. campestris* and *B. napus*) on the rat hepatic glutathione detoxification system and investigated whether dietary cysteine supplements might modify such effects. It was found that canola meal feeding can cause an increase in hepatic glutathione concentration and GST activity. The elevation in hepatic glutathione concentration caused by canola meal was not an overcompensation due to initial depletion but a general hepatotoxicity. The general hepatotoxicity could impede the transport of GSH to peripheral tissues, perhaps due to reduced activity of γ-glutamyltranspeptidase and thus lead to the glutathione accumulation in the liver. Supplemental cysteine prevented the elevation in hepatic GST activity but did not overcome an increase in hepatic glutathione concentrations caused by feeding canola meal.

Supplemental cysteine may offer alternative detoxification pathways and alter hepatic utilization of glutathione for the detoxification by GST when canola meal is fed. This explanation with respect to the cysteine effect was in agreement with Bogaards et al. (1990), who also observed cysteine supplementation diminished the enhancement of GST activity due to canola meal feeding. It can be summarized that hepatic glutathione and GST concentrations are elevated due to general hepatoxicity when rapeseed meal/canola meal is included in animal diets. Other dietary factors, e.g. cysteine, can alter the elevation effects of hepatic glutathione and GST induced by rapeseed meal/canola meal feeding.

2.10 SUMMARY

Rapeseed is grown worldwide as an oilseed crop and the by-product rapeseed meal is available for use as a protein supplement in animal diets after oil extraction. The presence of glucosinolates in the meal compromises the use of the meal as a feedstuff for animals. Extensive plant selection programs over the years have resulted in a reduction in the glucosinolate content of rapeseed and low-glucosinolate cultivars (i.e. canola) are generally available worldwide. Gross effects of glucosinolates in rapeseed meal/ canola meal feeding have been reported in animal studies. Fatal liver hemorrhage in laying hens as a consequence of canola meal feeding is a unique problem in the poultry industry that has led to a less than optimum utilization of canola meal in the diet of the laying hen. Research needs to be done to evaluate effects of glucosinolate intake on the development of liver hemorrhage in the laying hen. This type of information is useful in assessing the need for dietary restriction to the use of canola meal in the laying hen diets and aid in setting priorities in plant selection

programs aimed at reducing the glucosinolate content in future canola varieties. Liver is the most important organ to detoxify foreign compounds. Glucosinolates as foreign compounds might have influence on the detoxification system in the liver. Thyroid is another target organ of glucosinolates and goitrogenicity has been reported in many species. However, the diversity of glucosinolate varieties, active properties of glucosinolate hydrolysis products and the complex medium in the digestive tracts of animals present a major hurdle in the investigation of glucosinolate effects in the liver and the thyroid of animals. In this regard, potential toxicities of glucosinolates and their hydrolysis products need to be better understood in order to assess the impact of feeding canola meal to animals with the ultimate aim of maximum utilization of this locally produced protein supplement product in livestock and poultry diets.

Chapter III

3.0 THE EFFECTS OF GLUCOSINOLATES ON PERFORMANCE PARAMETERS IN CANOLA MEAL FEEDING REGIMENS IN THE LAYING HEN

3.1 INTRODUCTION

Rapeseed meal is an excellent protein supplement rich in sulfur amino acids with a well balanced amino acid pattern. However, antinutritive effects have been noted in rapeseed meal feeding regimens among various animal species and in particular liver hemorrhage is reported to be associated with rapeseed meal feeding regimens in the laying hen. The mode of action for liver hemorrhage is not fully understood, though intact glucosinolates and their hydrolysis products have been implicated as causative agents. Glucosinolate content in rapeseed has been dramatically reduced through plant breeding. In Canada, canola cultivars contain less than 30 μmol glucosinolates per gram oil-free meal. Glucosinolate-linked symptoms are much less severe in canola meal feeding regimens as compared to the rapeseed meal regimens. However, liver hemorrhage is still noted. The effects of glucosinolates on physiological status in canola meal feeding regimens in the laying hen need to be determined so that the use of this locally available product can be expanded. Several experiments were conducted in this study to achieve this goal. The first experiment was conducted to evaluate a new canola meal produced from a canola cultivar with a low-glucosinolate content. The main purpose of the remaining experiments was to obtain data on the physiological effects of feeding diets containing various contents of glucosinolates and relate this data to incidence of liver hemorrhage and productive performance parameters. In these experiments, the productive

performance and viability of birds fed various glucosinolate-containing diets were monitored and are presented in this chapter while the physiological effects are presented in Chapter IV.

3.2 MATERIALS AND METHODS

3.2.1 Diets and birds

Experiment 1

The low-glucosinolate canola (a *Brassica campestris* yellow seeded cultivar from Agriculture and AgriFood Canada, Saskatoon) was processed in a pilot plant (Texas A & M University, college station, Texas) and the resulting meal was added as the partial or sole protein source in cereal- based diets. The inclusion rates were 10% and 20%, respectively. Two commercial canola meal diets with inclusion rates of 10% and 20% and a non-canola control diet were also used. All diets were calculated to be isonitrogenous and isoenergetic and formulated to meet nutrient requirements according to the National Research Council (1994). The composition of the experimental diets is shown in Table1. Glucosinolates were analyzed by gas-liquid chromatography according to the method of Slominski and Campbell (1987b). The glucosinolate content of the new canola cultivar and the commercial canola meal is shown in Table 2. Sixteen replicates were allotted to each treatment at random with each replicate containing16 birds for a total of 1280 birds (Dekalb SCWL). All birds were weighed at the initiation and at the end of the experiment. Diets were offered in mash form *ad libitum* for five 28-day periods. Water was available *ad libitum* throughout the experiment. Daily egg production and 28-day feed consumption data were recorded on a replicate basis. Eggs were taken on a replicate basis for three consecutive days during the third week of each 28-day

TABLE 1. Ingredient and glucosinolate content of the experimental diets used in the evaluation of low-glucosinolate canola meal in the laying hen (Experiment 1)

Ingredient	Diet type				
	Control	Low-glucosinolate CM[2]		Commercial CM[2]	
		10%	20%	10%	20%
Wheat	51.055	47.049	42.782	45.433	39.07
Barley (hulled)	20	20	20	20	20
Canola meal	0	10	20	10	20
Soybean meal	13.81	6.8	0	8.2	3.15
Meat meal	3	3	3	3	3
Tallow	0	1.23	2.5	1.45	3.07
Vegetable oil	2	2	2	2	2
Limestone	8.2	8.1	8	8.1	8
Calcium phosphate	0.39	0.29	0.2	0.29	0.2
Premix[1]	1.5	1.5	1.5	1.5	1.5
DL-methionine	0.045	0.031	0.018	0.027	0.01
Glucosinolates [2] (μmol/ g diet)	0	0.18	0.36	0.79	1.59

[1] Premix supplied the following (per kilogram of diet): vitamin A, 8,255 IU; vitamin D_3,1,000 IU; vitamin E, 5.46 IU; vitamin B_{12}, 0.0112 mg; riboflavin, 6.0 mg; Ca-pantothenate, 11 mg; niacin, 16.7 mg; choline chloride, 219.5 mg; manganese oxide, 165 mg, zinc oxide, 55 mg; iodized salt, 4.78 g; ethoxyquin, 186.6 mg.
[2] CM=canola meal; glucosinolate composition of the low-glucosinolate canola meal and commercial canola meal are shown in Table 2. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.

TABLE 2. Glucosinolate content of the low-glucosinolate canola meal used in Experiment 1 and the commercial canola meal used in Experiments 1 & 2 (μmol/g oil-free-meal)

Glucosinolate	Commercial canola meal	Low-glucosinolate canola meal
Allyl	0.1	0
3-Butenyl	1.57	0.35
4-Pentenyl	0.29	0.13
2-OH-3-Butenyl	3.5	0.47
2-OH-4-Pentenyl	0.09	0.07
4-OH-Benzyl	0.76	0.05
3-Indolymethyl	0.22	0
4-OH-3-Indolymethyl	1.41	0.73
Total	7.94	1.8
Myrosinase activity[1]	0	0.024

[1] Unit myrosinase activity equals the hydrolysis of 1 μ mole glucosinolate per minute

period throughout the experiment and average egg weights were computed. Mortality was recorded daily and a post mortem examination was carried out to note evidence of liver hemorrhage as a cause of death.

Experiment 2

Different combination ratios of soybean meal and commercial canola meal comprised five experimental diets containing graded levels of glucosinolates. The composition of the diets is shown in Table 3. Glucosinolate content of the commercial canola meal is shown in Table 2. Birds were allotted to the five diets (26 replicates/ each) at random, with 16 replicates of 16 hens each and 10 replicates of 12 hens each for a total of 1880 birds (Dekalb SCWL). All birds were weighed at the initiation and at the end of the experiment. Feed and water were available *ad libitum* for the five 28-day periods. Daily egg production and 28-day feed consumption were recorded on a replicate basis. Eggs were taken on a replicate basis for three consecutive days during the third week of each 28-day period throughout the experimental period and average egg weights were computed. Mortality was recorded daily and a post mortem examination was carried out to determine the cause of death.

Experiment 3

A maximal toxicity diet was formulated as a combination diet of canola meal (20%) and canola seed (15%). Canola seed was used as a natural source of myrosinase. In this regard, the diet contained a high content of intact glucosinolates and potentially high content of glucosinolate hydrolysis products following consumption due to the presence of active myrosinase. In addition to the maximal toxicity diet, four other diets were involved including a control diet (a soybean meal diet), two combination diets of the maximal toxicity diet and

TABLE 3. Ingredient and glucosinolate content of the experimental diets containing graded levels of glucosinolates used in a canola meal feeding regimen for laying hens (Experiment 2)

Ingredient	Diet type				
	0% CM[2]	3.2% CM	6.4% CM	12.8% CM	25.5% CM
Barley (hulless)	67.07	65.59	64.12	61.16	55.25
Soybean meal	16.3	14.26	12.23	8.15	0
Canola meal	0	3.2	6.38	12.75	25.5
Meat meal	5	5	5	5	5
Tallow	0.5	0.76	1.03	1.55	2.6
Vegetable oil	1.5	1.63	1.76	2.03	2.55
Limestone	7.8	7.76	7.73	7.65	7.5
Calcium phosphate	0.31	0.28	0.26	0.21	0.1
Premix[1]	1.5	1.5	1.5	1.5	1.5
DL-methionine	0.02	0.0175	0.015	0.01	0
Analyses [2] Glucosinolate, µmol/ g diet	0	0.25	0.51	1.02	2.02

[1] Premix: as noted in Table 1

[2] CM=commercial canola meal; glucosinolate composition of the commercial canola meal is shown in Table 2. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.

the control diet (a diet with one third of the maximal toxicity diet and two thirds of the control diet, and a diet with two thirds of the maximal toxicity diet and one third of the control diet) and a 20% commercial canola meal diet. All diets were calculated to be isonitrogenous and isoenergetic and formulated to meet nutrient requirements according to National Research Council (1994). Diet composition is shown in Table 4. Glucosinolate contents of canola seed and commercial canola meal are shown in Table 5. Birds were allotted randomly to the five experimental diets with four replicates of 18 birds each per treatment for a total of 360 birds (Bovan SCWL). All birds were weighed at the initiation and at the end of the experiment. Feed and water were offered *ad libitum* for four 28-day periods. Daily egg production and 28-day feed consumption data were recorded on a replicate basis. Group egg weights were taken on a replicate basis for three consecutive days during the last week of each of the four 28-day periods and average egg weights were computed by dividing the group egg weights by total number of eggs in each group. Mortality was recorded daily and a post mortem examination was carried out to determine the cause of death.

Experiment 4

A combination diet of 25% commercial canola meal and 2.5% canola seed was included in this experiment. The diet was similar to the maximal toxicity diet in experiment 3 with the exception that a lower percentage of canola seed was used. An *in vitro* study was conducted prior to diet formulation to ensure that the ratio of canola meal to canola seed would produce a maximal hydrolysis of glucosinolates. The ratio 10:1 (canola meal: canola seed, w/w) was found to be adequate. A control diet (a glucosinolate-free diet) and a 27.5% commercial canola meal diet were the other two experimental diets involved in this

TABLE 4. Ingredient and glucosinolate content of the experimental diets used in a canola meal feeding regimen for laying hens (Experiment 3)

Ingredient	Diet type		
	Control	20% CM[2]	20% CM+15% CS[2]
Wheat	70.94	39.3	8.95
Barley (hulled)	8	22.6	41.3
Canola meal	0	20	20
Canola seed	0	0	15
Soybean meal	6.3	0	0
Meat meal	3	2	2
Tallow	0	3.13	0
Vegetable oil	1.24	2.7	2.68
Limestone	8.07	8	7.9
Calcium phosphate	0.8	0.75	0.65
Premix[1]	1.5	1.5	1.5
Lysine	0.09	0	0
DL-methionine	0.06	0.02	0.02
Analyses [2]			
Glucosinolate, µmol/ diet	0	2.52	4.27

[1] Premix: as noted in Table 1.
[2] CM=commercial canola meal, CS=canola seed; glucosinolate composition of the commercial canola meal and canola seed are shown in Table 5. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.

TABLE 5. Glucosinolate content of the commercial canola meal and canola seed used in Experiments 3 and 4 (μmol/g oil-free-meal)

Glucosinolate	Commercial canola meal	Canola seed
3-Butenyl	2.55	2.21
4-Pentenyl	0.61	0.44
2-OH-3-Butenyl	4.9	4.62
2-OH-4-Pentenyl	0.08	0.09
4-OH-Benzyl	1.36	0
3-Indolymethyl	0.29	0.29
4-OH-3-Indolymethyl	2.79	4.05
Total	12.58	11.7

experiment. All diets were calculated to be isonitrogenous and isoenergetic and formulated to meet nutrient requirements according to National Research Council (1994). Diet composition is shown in Table 6. Twenty-six replicates of sixteen birds each were allotted to the control diet and twenty-seven replicates of sixteen birds each were allotted to the two other diets with a total of 1264 birds (Dekalb SCWL). All birds were weighed at the initiation and at the end of the experiment. The diets were offered in mash form *ad libitum* for eight 28-day periods. Water was available *ad libitum* throughout the experimental period. Daily egg production and 28-day feed consumption data were recorded on a replicate basis. Mortality was recorded daily and a post mortem examination was carried out to note evidence of liver hemorrhage as a cause of death.

3.2.2 Statistical analyses

All data collected from the four experiments were subjected to the General Linear Models procedure of SAS institute (1985) in a split-plot design using the following model: $y_{ijk}=u+d_i+p_{ij}+t_k+m_{ik}+e_{ijk}$, where y_{ijk} is the response criterion (egg production, egg weight, feed intake and feed conversion efficiency); u is the overall mean; d_i represents the effect of diets; p_{ij} represents the effect of pens within diets; t_k is the effects of different time period; m_{ik} is the effects of the interaction between diet and time period; e_{ijk} is the error term. All data collected for final body weight were subjected to the General Linear Models procedure of SAS institute (1985) in a complete randomized design using the following model: $y_{ij}=u+d_i+e_{ij}$, where y_{ij} is the response criterion; u is the overall mean; d_i is the effects of diets; e_{ij} is the error term. Tukey test was used to compare and separate means.

TABLE 6. Ingredient and glucosinolate content of experimental diets used in a canola meal feeding regimen for laying hens (Experiment 4)

Ingredient	Diet type		
	Control	27.5% CM[2]	25% CM+ 2.5% CS[2]
Wheat	43.07	26.75	30.75
Barley (hulled)	26.96	28.04	25.39
Canola meal	0	27.5	25
Canola seed	0	0	2.5
Soybean meal	15.15	0	0
Vegetable oil	3.65	7.05	5.75
Limestone	8.35	8.15	8.1
Calcium phosphate	1.25	1	1
Premix[1]	1.5	1.5	1.5
DL-methionine	0.07	0.01	0.01
Analyses [2] Glucosinolates (μmol/ g diet)	0	3.45	3.44

[1] Premix: as noted in Table 1.
[2] CM=commercial canola meal, CS=canola seed; glucosinolate composition of the commercial canola meal and canola seed are shown in Table 5. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.

3.3 RESULTS

Experiment 1

The productive performance of laying hens in the evaluation of the low-glucosinolate canola meal is shown in Table 7. Egg production of birds fed on canola meal diets was similar to that for the control-fed group irrespective of the different glucosinolate levels in the two types of canola meal. Feed intake was significantly lower in hens fed the two commercial canola meal diets and the 20% low-glucosinolate canola meal diet in comparison with that of control-fed birds ($P<0.05$). Feed intake in birds fed on the 10% low-glucosinolate canola meal diet was not different from that of the control-fed birds ($P>0.05$). Similar feed conversion efficiency was observed in birds fed on different diets ($P>0.05$). Egg weight was similar among all the treatment groups ($P>0.05$). No difference among the treatment groups was apparent for final body weight ($P>0.05$). Liver hemorrhage was not observed in birds fed the low-glucosinolate canola meal diets or the control diet. However, mortality was noted in birds fed the commercial canola meal diets with one bird dying of liver hemorrhage in each of the diets. Interaction between diets and time periods was not significant for egg production and egg weight ($P>0.05$) but was significant for feed intake ($P<0.05$). The results of analysis of variance are shown in Appendixes 1, 2 and 3.

Experiment 2

The effects of graded levels of dietary glucosinolates on laying hen performance are shown in Table 8. All treatments with graded levels of glucosinolates had a similar high rate of egg production with no consistent relationship between dietary glucosinolate content and

TABLE 7. Performance parameters of laying hens in the evaluation of low-glucosinolate canola meal[2] over eight 28-day periods (Experiment 1)

Performance parameters	Diet type					Pooled SEM
	Control	Low-glucosinolate CM[2]		Commercial CM[2]		
		10%	20%	10%	20%	
	Glucosinolate content (μmol/g diet)[2]					
	0	0.18	0.36	0.79	1.6	
Egg production, %	90.1	89.4	90.5	90.7	89.4	0.78
Feed intake, g/hen/day	102.2[a]	100.7[ab]	99.2[b]	99.9[b]	99.8[b]	0.46
Feed conversion, kg feed : kg egg	1.95	1.91	1.87	1.88	1.89	0.02
Egg weight, g	59.50	58.93	58.98	58.97	59.80	0.74
Final body weight (kg)	1.61	1.62	1.57	1.62	1.62	0.05
Mortality, LH[1]	0	0	0	1	1	

[1] Number of birds dying of liver hemorrhage

[2] CM=canola meal; glucosinolate composition of the low-glucosinolate canola meal and commercial canola meal are shown in Table 2. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.

[a-b] Means within a row with no common superscript differ significantly ($P<0.05$)

TABLE 8. Productive performance of laying hens fed canola meal [2] diets with graded levels of glucosinolates over five 28-day periods (Experiment 2)

Performance parameters	Diet type					
	Control	3.2% CM [2]	6.4% CM	12.8% CM	25.5% CM	Pooled SEM
	Glucosinolate content (μmol/ g diet)[2]					
	0	0.25	0.51	1.02	2.02	
Egg production, %	89.7	90	90.6	88.9	89.4	0.62
Feed intake, g/hen/d	99.5	99.3	98.8	98.4	97.7	0.49
Feed conversion, kg feed : kg egg	1.86	1.87	1.83	1.87	1.89	0.02
Egg weight, g	60.62[a]	59.47[ab]	59.04[b]	59.15[b]	59.04[b]	0.33
Final body weight, kg	1.64	1.62	1.63	1.63	1.61	0.05
Mortality, LH[1]	0	0	0	0	1	

[1] Number of birds dying of liver hemorrhage

[2] CM=commercial canola meal; glucosinolate composition of the commercial canola meal is shown in Table 2. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.

[a] Means within a row with no common superscript differ significantly ($P<0.05$)

egg production rate (P>0.05). Feed intake was similar among all treatment groups (P>0.05). No significant differences for feed conversion efficiency and final body weight were observed in birds fed all treatment diets (P>0.05). Egg weight of the control-fed birds was similar to that of birds fed the 3.2% canola meal diet (P>0.05) but was significantly heavier than birds fed other canola meal diets (P<0.05). Birds fed all canola meal diets had similar egg weight (P>0.05). One bird died of liver hemorrhage with the death occurring among hens fed the highest glucosinolate content (25.5 % canola meal). The interaction between diets and time periods was not significant for egg production and egg weight (P>0.05) but was significant for feed intake (P<0.05). The results of analysis of variance are shown in Appendixes 4, 5 and 6.

Experiment 3

A maximal toxicity diet formulated using a combination of canola meal and canola seed, two combination diets of this maximal toxicity diet and the control diet, and a canola meal diet were included to investigate the response of hens to glucosinolates in the canola meal feeding regimens. The productive performance of hens fed these experimental diets is shown in Table 9. Birds fed all treatment diets had a similar high rate of egg production (P>0.05). Feed intake was similar among all treatment groups (P>0.05). No significant differences for feed conversion efficiency and final body weight were observed for birds fed the treatment diets (P>0.05). Egg weights were similar in all treatment groups (P>0.05). Mortality due to liver hemorrhage was not noted in this study. There was no interaction between diets and time periods for the productive parameters, including egg production, egg weight and feed intake (P>0.05). The results of analysis of variance are shown in Appendixes

TABLE 9. Performance parameters of laying hens fed diets of varying glucosinolate content over four 28-day periods (Experiment 3)

Performance parameters	Diet type					Pooled SEM
	SBM (con.)[2]	2/3 con. 1/3 max.	1/3 con. 2/3 max.	CM/CS[3] (max.)	CM[4]	
	Glucosinolate content (μmol/ g diet)[3]					
	0	1.42	2.85	4.27	1.63	
Egg production, %	86.6	88.7	85.7	84.2	87.7	1.8
Feed intake, g/hen/day	99.9	98.9	96.5	95.6	97.0	1.3
Feed conversion, kg feed : kg egg	2.09	1.97	2.02	2.05	1.96	0.03
Egg weight, g	55.38	56.87	55.74	55.44	56.60	0.4
Final body weight, kg	1.57	1.54	1.55	1.55	1.56	0.03
Mortality, LH[1]	0	0	0	0	0	

[1] Number of birds dying of liver hemorrhage
[2] Con.=control
[3] CM/CS=20 % commercial canola meal and 15% canola seed (see Table 5 for glucosinolate composition) formulated as a maximal toxicity diet (max.). Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.
[4] CM=20% commercial canola meal (see Table 5 for glucosinolate composition)
[a-b] Means within a row with no common superscript differ significantly ($P<0.05$)

7, 8 and 9.

Experiment 4

A diet formulated using a combination of canola meal and canola seed, and a canola meal diet were involved to investigate the response of hens to glucosinolates in the canola meal feeding regimens. Performance parameters of hens fed the experimental diets are shown in Table 10. The birds fed the control diet had a significantly superior egg production in comparison with those fed both the sole canola meal diet and the combination diet of canola meal and canola seed ($P<0.05$). Feed intake was also significantly reduced by the two glucosinolate-containing diets. Final body weight of birds fed the combination diet of canola seed and canola meal was significantly lower than that of birds fed on the other two diets ($P<0.05$). Mortality due to liver hemorrhage occurred at a low rate in the eight-month feeding period. One bird died of liver hemorrhage in the sole canola meal diet and two birds died of liver hemorrhage in the combination diet of canola meal and canola seed with no mortality among control-fed birds. The interaction between diets and time periods was not significant for egg production ($P>0.05$) but was significant for feed intake ($P<0.05$). The results of analysis of variance are shown in Appendixes 10 and 11.

3.4 DISCUSSION

A low-glucosinolate canola meal was produced from a new *Brassica campestris* canola cultivar. Experiment 1 was conducted to evaluate the nutritive value of the new meal as a feedstuff in the diet of the laying hen. A high rate of egg production was maintained among hens fed the low-glucosinolate canola meal diets. The fact that there was no mortality

TABLE 10. Performance parameters of laying hens diet of varying glucosinolate content over eight 28-day periods (Experiment 4)

Performance parameters	Diet type			Pooled SEM
	Control	27.5% CM[2]	25% CM+2.5% CS[2]	
	Glucosinolate content (μmol/ g diet)[2]			
	0	3.45	3.44	
Egg production, %	85.9 [a]	82.3 [b]	83.1[b]	0.65
Feed intake, g/hen/day	102.8 [a]	99.3 [b]	98.9 [b]	0.47
Final body weight, kg	1.70 [a]	1.68 [a]	1.64 [b]	0.01
Mortality, LH[1]	0	1	2	

[1] Number of birds dying of liver hemorrhage
[2] CM=canola meal, CS=canola seed; glucosinolate composition of the commercial canola meal and canola seed are shown in Table 5. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.
[a-b] Means within a row with no common superscript differ significantly ($P<0.05$)

due to liver hemorrhage in the low-glucosinolate canola meal feeding regimens would suggest that the causative agents in the low-glucosinolate canola meal are at too low a level to cause liver hemorrhage. This is in contrast to the feeding of commercial canola meal where liver hemorrhage was evident but at a very low rate of mortality. Egg size was maintained for the low-glucosinolate canola meal feeding regimens. Differences were noted in feed intake in this experiment but the reason for the reduced feed intake among birds fed some of the canola meal diets is not readily apparent. There was a significant time period by treatment interaction for feed intake indicating inconsistency of feed intake response. However, this effect did not influence overall performance of birds fed the canola meal diets as similar egg production and egg weight were noted for all treatment groups and no interaction between time period and diets was noted for these two parameters. Similar feed conversion efficiency was observed in birds fed low-glucosinolate canola meal in comparison with birds fed soybean meal. In general, a high rate of egg production, no mortality rate due to liver hemorrhage and no detrimental influence of glucosinolates on egg weight along with uninfluenced feed efficiency in low-glucosinolate canola meal feeding regimens support the concept that the new low-glucosinolate canola meal can be used as a protein supplement based on its nutritive value. In this regard with the use of low-glucosinolate canola meal there should be no need to recommend an upper limit restriction on the use of canola meal in the diet of the laying hen.

The performance characteristics from Experiments 2, 3 and 4 are discussed collectively since the main purpose of these three experiments was to investigate the physiological effects of dietary glucosinolates rather than to evaluate the feeding value of canola meal. This aspect of rapeseed/canola feeding has been reported extensively in the

literature. A high rate of egg production was observed in these experiments with the exception that canola meal feeding had a negative impact on egg production in Experiment 4. However, a high inclusion rate of 27.5% canola meal was studied in this experiment. Reductions in egg production have been reported in previous research when high inclusion rates of canola meal or rapeseed meal of relatively high glucosinolate content were included as dietary treatments in the studies (Campbell et al., 1991 and Leslie et al., 1972). For egg weight, the other important performance characteristic, a varied response was noted in that a reduction in egg weight due to canola meal feeding was noted in Experiment 2 but no effect of treatment was evident in Experiment 3 (egg weight was not measured in Experiment 4). Factors other than diet glucosinolates, per se, would appear to be responsible for this discrepancy between experiments. This conclusion is supported by the lack of treatment effect on egg weight in Experiment 1 where diet glucosinolate level differed (i.e. low-glucosinolate canola meal vs commercial canola meal) and from literature data where inconsistent influences of rapeseed/canola meal feeding on egg size have been reported. No effects were shown by Marangos et al. (1976) and Leeson et al. (1987) as opposed to the negative effects reported by Leeson et al. (1978), Proudfoot et al. (1982) and Summers et al. (1985, 1987). Feed intake showed no significant reduction relative to controls when canola meal was fed in Experiments 2 and 3 although it was significantly reduced in birds fed the two glucosinolate-containing diets of relatively high glucosinolate content (27.5% canola meal and the combination diet of 25% canola meal and 2.5% canola seed) in Experiment 4. A significant interaction between diets and time periods was noted for feed intake in Experiments 2 and 4. However, these effects on feed intake did not influence the production performance characteristics as no

interactions between time period and diets were noted for egg production and egg weight. In addition, the lack of interaction response in egg production or egg weight over time maybe indicate that there is not an accumulative effect of dietary glucosinolate on these two production parameters. A low mortality rate due to liver hemorrhage was noted among hens receiving canola meal/seed diets in Experiments 2 and 4 and no mortality was observed in Experiment 3. The mortality was indistinguishable from that of the control-fed birds. This inconsistency with respect to liver hemorrhage in this study is in agreement with the data from the literature. Liver hemorrhage was not reported as a cause of mortality among hens fed canola meal in several studies (Leeson et al., 1987, Clandinin et al., 1986, Thomke et al., 1983 and Hulan et al., 1980) while it was listed as a cause of death in other reports (Campbell et al., 1979, Campbell, 1987ab, Summers et al., 1985 and Ibrahim et al., 1980a).

The general observation noted in the current study on laying hen performance characteristics and liver hemorrhage incidence in response to canola meal feeding are in accordance with the overall recommendations for the use of canola meal in table-egg production outlined by the Canola Council of Canada (Canola Meal Feed industry Guide, ed. D. Hickling, Canola Council of Canada, Winnipeg, MB, 2001). In this report, following a survey of the literature data relating to canola meal utilization in laying hen diets, it was concluded that canola meal feeding had no effect on number of eggs produced although a slight reduction in egg weight and feed intake were evident. At an average canola meal inclusion level of 10% it was suggested that an egg weight depression of less than 1% and a feed intake depression of about 1.5% might be expected. Caution was advised in the use of canola meal because of the association with liver hemorrhage mortality when feeding canola

meal to egg layers such that the maximum usage level was restricted to the 10% level. Optimism, however, was expressed that this restriction level could be relaxed with the eventual introduction of canola varieties of lower glucosinolate content than those currently available for use. The conclusion from the current study support the recommendations and together the information underscores the importance of the development of low-glucosinolate varieties of canola such as the one utilized in this study. As indicated the use of low-glucosinolate canola meal should be based on the nutritive worth of the meal without any consideration to an upper-limit restriction. This type of usage would result in dietary levels of canola meal exceeding the 10% level in situation where price and meal quality indicated usage of the meal as the major protein supplement in a diet.

Chapter IV

4.0 THE EFFECTS OF GLUCOSINOLATES ON PHYSIOLOGICAL PARAMETERS IN CANOLA MEAL FEEDING REGIMENS IN THE LAYING HEN

4.1 INTRODUCTION

The nutritive value of rapeseed meal/canola meal in the laying hen has been investigated in many studies. These studies focused mainly on the influence of performance characteristics in rapeseed meal/ canola meal feeding regimens. Mortality due to liver hemorrhage is reported to be a glucosinolate-linked problem in rapeseed meal/canola meal feeding regimens in the laying hen. However, the relationship between dietary glucosinolate levels and liver hemorrhage is not clear. With the reduction of glucosinolate content in canola through plant breeding, liver hemorrhage incidence in hens fed canola meal has become less severe and consequently is a less sensitive indicator to evaluate the effects of glucosinolates in canola meal feeding regimens. It is well documented that rapeseed meal/ canola meal feeding regimens result in thyroid enlargement with the effect related to dietary glucosinolate content. Measurements of thyroid enlargement coupled with the status of thyroid hormones may be used as indicators for the physiological response to glucosinolates in the laying hen. Glucosinolates as foreign compounds (xenobiotics) can cause changes in the xenobiotic metabolizing system in the liver. Measurements of liver xenobiotic metabolizing enzymes and hepatic glutathione concentration can be additional biomarkers to indicate the physiological alterations due to glucosinolates in canola meal feeding regimens. The objective of this study was to investigate the effects of glucosinolates on physiological parameters in the liver and thyroid. An attempt was made to establish the relationship between dietary glucosinolate levels and the physiological parameters.

4.2 MATERIALS AND METHODS

The performance parameters of birds for Experiments 1, 2, 3 and 4 are reported in Chapter III, and the physiological parameters of birds for these same experiments are reported in this chapter.

4.2.1 Diets and birds

Diets are described in Chapter III for the four experiments in this study. Hens were killed at the termination of the experiments to obtain data to evaluate the biological effects of glucosinolates in the laying hen. In Experiment 1, sixteen hens were selected at random from each treatment and sacrificed at the termination of the five-month feeding period. In Experiment 2, eighteen hens were selected randomly from each treatment and sacrificed at the termination of the five-month feeding period. In Experiment 3, eighteen birds were killed from each treatment at the termination of the four-month feeding period. In addition, a set of eighteen birds was chosen at random from the maximal toxicity diet at monthly intervals throughout the experiment to assess the gradual influence of glucosinolates on physiological parameters. In Experiment 4, twenty-seven birds per treatment were killed at the termination of the eight-month feeding period.

4.2.2 Sample collection

All birds selected for sample collection were weighed and anaesthetized with co_2. Blood was quickly collected by cardiac puncture using Vacutainer blood collection tubes. For each tube 340 USP units of heparin were manually added to the needles prior to blood

collection. Blood samples were cold-centrifuged at 3000g for 45 minutes and plasma aliquots were immediately (on the same day without storage) analyzed for thyroid hormone content. Livers were excised, and weighed after removal of surrounding fat. Freshly excised livers were washed with ice cold 1.15% potassium chloride solution, cut into pieces and immediately placed into liquid nitrogen prior to storage at -80°C. Thyroids were removed from each hen and subsequently weighed after the removal of adhering fat.

4.2.3 Preparation of cytosolic and microsomal fractions of the liver

The method of preparation of cytosolic and microsomal fractions of the liver was according to Guengerich (1994). All steps were carried out at 0 to 4°C. Approximately 2 grams of frozen liver were homogenized (PowerGen 700 homogenizer, Fisher Scientific) in 4ml ice-cold homogenization buffer A [0.10 M potassium chloride, 0.10 M tris-acetate, 1.0 mM ethylenediamine tetraacetic acid (EDTA) and 20 mm butylated hydroxytoluene, pH adjusted to 7.4]. The homogenized tissue was transferred to ultracentrifuge tubes and centrifuged at 105, 000 g (34,000 rpm in a Beckman 45Ti rotor, Beckman, Palo Alto, CA) for one hour at 4°C. After the complete removal of fat, the supernatant (cytosol) was stored as 1 ml aliquots in each of three vials at -80°C for later analyses. The pellet remaining at the bottom of the tubes was retained as the microsomal pellet and was homogenized in 4 ml cold buffer B (0.10 M potassium pyrophosphate, 1.0 mM EDTA and 20 mm butylated hydroxytoluene, pH adjusted to 7.4). The supernatant was discarded. The homogenate was centrifuged at 34,000 rpm (105, 000 g) for one hour at 4°C. The bottom part (microsome pellet) was homogenized in 2.0 ml cold buffer C (10 mM tris acetate, 1.0 mM EDTA and

20% glycerol, pH adjusted to 7.4). The remainder was transferred to three small vials which were placed in liquid nitrogen and subsequently stored at -80°C for later protein and enzyme assays.

4.2.4 Protein assay

Microsomal and cytosol protein concentrations of the liver were measured using a commercially available Pierce's bicinchoninic acid assay kit according to the microwell plate protocol. Microwell plates were read by a photometric microplate reader (Model 3550, Bio Rad).

4.2.5 Enzyme assays

Optimum reaction time and protein levels were determined for all enzyme assays. Blanks and samples were run in duplicate. Spectrophotometric readings were taken in a Milton Roy Spectronic 3000 spectrophotometer for all GST assays. Enzyme activity was calculated from the linear part of the activity curve. The unit of activity was defined as the amount of enzyme catalyzing the formation of one nanomole of product per minute under the conditions of the assay. Specific activity was expressed as units per mg of protein. The results for GST were expressed as nmol/min/mg protein. Spectrophotometric readings for cytochrome P_{450} were taken in a ultrospec 2000 spectrophotometer (UV/ visible, Pharmacia Biotech).

4.2.5.1 Cytochrome P_{450}. Cytochrome P_{450} concentrations were determined by the method of Guengerich (1994).

4.2.5.2 Glutathione-S-transferase. Glutathione-S-transferase was measured in three isoforms (GSTμ, π and α). GSTμ activities were determined spectrophotometrically according to Habig et al. (1974) using 3, 4 dichloronitrobenzene (DCNB) as a substrate. The assay depends on a direct change of absorbance of DCNB when conjugated with glutathione. The reaction mixture contained, in a final volume of 1.0ml: 0.1 M-KH_2PO_4/K_2HPO_4 buffer, pH 7.5, 5.0 mM-glutathione, 1.0 mM-DCNB and an appropriate amount of cytosol (diluted to a volume of 100ml). The reaction mixture was pre-incubated at 37°C for 3 minutes before the reaction was initiated by the addition of DCNB. Incubation took place in a gently shaking water bath for 3 minutes at 37°C. Enzyme activity was monitored spectrophotometrically at 345 nm. The reaction was monitored for 10 minutes with readings taken every 60 seconds.

Glutathione-S-transferase-π activities were determined spectrophotometrically according to Habig et al. (1974) using ethacrynic acid as a substrate. The assay depends on a direct change of absorbance of ethacrynic acid when conjugated with glutathione. The reaction mixture contained, in a final volume of 2.0 ml: 0.1 M-KH_2PO_4/K_2HPO_4 buffer, pH 6.5, 5.0 mM-glutathione, 0.2 mM-ethacrynic acid and an appropriate amount of cytosol (diluted to a volume of 100 ml). The reaction mixture was pre-incubated at 37°C for 3 minutes before the reaction was initiated by the addition of ethacrynic acid. Incubation took place in a gently shaking water bath for 3 minutes at 37°C. Enzyme activity was monitored spectrophotometrically at 270 nm. The reaction was monitored for 10 minutes with readings

taken every 60 seconds.

Glutathione-S-transferase-α activities were determined spectrophotometrically according to Paglia et al. (1967) and Tappel (1978). The assay measures the rate of glutathione oxidation by cumene hydroperoxide and hydrogen peroxide as catalyzed by glutathione peroxidase present in the cytosol. Rather than measuring the progressive loss of reduced glutathione, however, this substrate is maintained as a constant concentration by the addition of exogenous glutathione reductase and NADPH, which immediately convert any GSSG produced to the reduced form of glutathione. The activity of GSTα is measured indirectly by monitoring the conversion of NADPH to NADP. The reaction mixture contained in a final volume of 1.0ml: 0.043mM-KH_2PO_4/K_2HPO_4 buffer, pH 7.0, 5.0mM-glutathione, 3.76mM-NaN_3, 0.28m-NADPH, 0.0726mM-H_2O_2 or (0.0396mM-CuOOH), glutathione reductase and an appropriate amount of cytosol (diluted to a volume of 33ml). The reaction mixture was pre-incubated at 37°C for 3 minutes before the reaction was initiated by the addition of cumene hydroperoxide and hydrogen peroxide. Incubation took place in a gently shaking water bath for 3 minutes at 37°C. Enzyme activity was monitored spectrophotometrically at 340 nm. The reaction was monitored for 10 minutes with readings taken every 60 seconds.

4.2.6 Glutathione analysis

The method of glutathione analysis was a combination of Baker et al. (1990) and Vandeputte et al. (1994). Glutathione was measured as total glutathione (GS_X) and oxidized glutathione (GSSG). Five hundred milligrams of frozen liver tissues in ice cold 2.5 mls 5%

5-sulfosalicyclic acid were homogenized on ice at 1500 rpm for 60 seconds. The homogenized tissues were extracted on ice for 15 to 20 minutes and then centrifuged at 15,000 g for 10 minutes to remove the precipitated material. The supernatant was stored in 1.5 ml microcentrifuge tubes at -80°C until the time of assay. Microwell plate assays of GS_X and GSSG were monitored at 415 nm using a microplate reader (Model 3550, Bio Rad). The reaction mixture contained in a final volume of 150 ml: 0.73 mM 5, 5'-dithiobis-2-nitro-benzoic acid, 0.24 mM β-Nicotinamide adenine dinucleotide phosphate (reduced form of NADPH) and 1.2 IU/ml GSSG-reductase. Oxidized glutathione produces the identical rate of 5, 5'-dithiobis-2-nitro-benzoic acid reduction as does GS_X. Hence GSSG can only be measured by adding 2-vinylpyridine to conjugate the reduced form of glutathione prior to the GSSG assay.

4.2.7 Thyroid hormone assays

Plasma triiodothyronine (T_3) and thyroxine (T_4) concentrations were measured using coat-a count and solid-phase radioimmunoassay ^{125}I kits (DPC). A T_3 commercial kit for human plasma was used for the assay of chicken plasma with a detection limit of 3.21 nmol/L. A canine T_4 commercial kit was used for the assay of chicken plasma with a detection limit of 0.11 nmol/L.

4.2.8 Statistical analyses

All data collected at the termination of the four experiments were subjected to the General Linear Models procedure of SAS institute (1985) in completely randomized design

using the following model: $y_{ij}=u+d_i+e_{ij}$, where y is the response criterion; u is the overall mean; d_i is the effects of diets; e_{ij} is the error term. Tukey test was used to compare and separate means. In Experiment 3, data collected from monthly intervals to evaluate the gradual influence of glucosinolates on physiological parameters were subjected to the General Linear Models procedure of SAS institute (1985) in completely randomized design using the following model: $y_{ij}=u+t_i+e_{ij}$, where d is the response criterion; u is the overall mean; t_i is the effects of time periods; e_{ij} is the error term. Tukey test was used to compare and separate means.

Individual observations from groups fed canola meal diets in Experiments 1, 2, 3 and 4 were pooled to establish the relationship between diet glucosinolate levels and the physiological parameters (hepatic GS_X, hepatic GSSG, hepatic cytochrome P_{450}, plasma T_4 and plasma T_3) using regression analyses (SAS, 1985). Since absolute values of the physiological parameters varied from experiment to experiment, all data were normalized within experiments prior to the regression analyses. This was done by expressing each individual observation for each physiological parameter as a percentage of the control group average in the same experiment (control group data were not included in the final analysis). All individual normalized data were analyzed using the treatment model: $y_{ij}=u+t_i+e_{ij}$, where y_{ij} is the physiological parameter; u is the overall mean; t_i is the effects of treatment; e_{ij} is the error term. The treatments in the model were the varying levels of glucosinolates from the canola meal diets in the four experiments. The individual normalized data were also analyzed using the linear regression model: $Y_i=a+bX_i+e_i$, where Y_i is the dependent variable (a measurement on one of the physiological parameters), X_i is the independent variable (the level

of glucosinolate in the canola meal diet), e_i is the residual error. The treatment sum of squares in the treatment model was partitioned into the sum of squares due to linear regression and the sum of squares due to lack of fit. F-values of linear regression and lack of fit were obtained using the residual error term from the "treatment" model (Steel et al., 1997). If the lack of fit was significant, the non linearity was explored using higher order of polynomial regression (quadratic and cubic).

4.3 RESULTS

Experiment 1

Alterations of organ status of birds in the evaluation of low-glucosinolate canola meal are presented in Tables 11 and12. Relative liver weights were similar for birds fed the canola meal diets and the control diet ($P>0.05$). No significant differences were observed for hepatic GS_X levels among all birds fed the canola meal diets and the control diet ($P>0.05$). Hepatic GSSG concentration in birds fed the canola meal diets showed no difference in comparison with the control-fed birds, with exception that the birds fed the 20% low-glucosinolate canola meal diet had significantly lower concentration of hepatic GSSG than those fed other experimental diets ($P<0.05$). Hepatic cytochrome P_{450}, GSTα and GSTπ were similar in birds fed the canola meal diets and the control diet ($P>0.05$). Hepatic GSTμ was not detectable. The relative thyroid weight was not significantly different among birds fed the canola meal diets and the control diet ($P>0.05$). Plasma T_3 and plasma T_4 concentrations were similar among birds fed the canola meal diets and the control diet ($P>0.05$).

TABLE 11. The effects of glucosinolates on physiological parameters of the liver in the laying hen in the evaluation of low-glucosinolate canola meal over eight 28-day periods Experiment 1)

Physiological parameters	Diet type					
	Control	Low-glucosinolate CM[1]		Commercial CM[1]		Pooled SEM
		10%	20%	10%	20%	
	Glucosinolate content (μmol/ g diet)[1]					
	0	0.18	0.36	0.79	1.6	
Liver weight (g/kg BW)	18.1	18.9	19.5	19.2	18.2	0.7
GSx (μmol/g liver)	9.02	9.87	9.78	9.88	11.12	0.8
GSSG (μmol/g liver)	0.37[a]	0.32[ab]	0.25[b]	0.31[ab]	0.30[ab]	0.03
Cytochrome P_{450} (nmol/ mg protein)	19.8	19.5	18.3	20.3	18.7	1.7
GSTα (μmol/min/mg protein)	0.13	0.1	0.16	0.11	0.14	0.02
GSTπ (μmol/min/mg protein)	1.59	1.57	1.58	1.61	1.62	0.03

[1] CM=canola meal; glucosinolate composition of the low-glucosinolate canola meal and commercial canola meal are shown in Table 2. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.

[a-b] Means within a row with no common superscript differ significantly ($P<0.05$)

TABLE 12. The effects of glucosinolates on physiological parameters of the thyroid in the evaluation of low-glucosinolate canola meal in the laying hen over eight 28-day periods (Experiment 1)

Physiological parameters	Diet type					Pooled SEM
	Control	Low-glucosinolate CM[1]		Commercial CM[1]		
		10%	20%	10%	20%	
	Glucosinolate content (μmol/ g diet)[1]					
	0	0.18	0.36	0.79	1.6	
Thyroid weight (mg/ 100g BW)	8.1	8.0	8.7	9.2	9.5	0.5
Plasma T_3 (nmol/L)	1.9	1.8	1.9	1.8	1.8	0.1
Plasma T_4 (nmol/L)	9.9	8.0	9.3	10.0	9.3	0.5

[1] CM=canola meal; glucosinolate content of the low-glucosinolate and commercial canola meal are shown in Table 2. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.

Experiment 2

The influence of varying levels of dietary glucosinolates on the antinutritive effects of canola meal in target organs of laying hens are shown in Tables 13 and 14. The relative liver weight was not significantly different among birds fed the diets containing graded levels of glucosinolates ($P > 0.05$). There was no significant difference for hepatic GS_X and GSSG concentrations in birds fed the experimental diets ($P>0.05$). The relative thyroid weight of birds fed the highest glucosinolate level was significantly heavier than that of the birds fed the control diet and other glucosinolate containing diets ($P<0.05$). Both plasma thyroid hormone concentrations (T_3 and T_4) were similar in birds fed the diets varying in glucosinolate content ($P>0.05$).

Experiment 3

The organ status of birds at the termination of the experiment are shown in Tables 15 and 16. The relative liver weight was similar among birds fed the various diet treatments ($P>0.05$). The hepatic GS_X concentrations of birds fed the maximal toxicity diet and the combination diet of 2/3 maximal toxicity diet and 1/3 control diet was significantly higher as compared with those of the birds fed on the other diets ($P<0.05$). The birds fed the combination diet of 1/3 maximal toxicity diet and 2/3 control diet had similar hepatic GS_X concentration to those of the birds fed the control diet and the 20% canola meal diet ($P>0.05$). Similar hepatic GSx concentrations were observed in birds fed the 20% canola meal diet and the control diet ($P>0.05$). The birds fed the maximal toxicity diet, the combination diet of 2/3 the maximal toxicity diet and 1/3 the control diet, and the combination diet of 1/3 the maximal toxicity diet and 2/3 the control diet had significantly higher concentrations of

TABLE 13. The effects of graded levels of glucosinolates on physiological parameters of the liver in the laying hen over five 28-day periods (Experiment 2)

Physiological parameters	Diet type					
	Control	3.2% CM [1]	6.4% CM	12.8% CM	25.5% CM	Pooled SEM
	Glucosinolates content (μmol/ g diet)[1]					
	0	0.25	0.51	1.02	2.02	
Liver (mg/kg BW)	25	24.5	23.1	22.7	24	0.7
GS_X (μmol/g liver)	3.2	3.17	3	3.68	3.73	0.22
GSSG (μmol/g liver)	0.4	0.4	0.37	0.51	0.46	0.07

[1] CM=commercial canola meal; glucosinolate composition is shown in Table 2. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.

TABLE 14. The effects of graded levels of glucosinolates on physiological parameters of the thyroid in the laying hen over five 28-day periods (Experiment 2)

Physiological parameters	Diet type					
	Control	3.2% CM[1]	6.4% CM	12.8% CM	25.5% CM	Pooled SEM
	Glucosinolate content (μmol/ g diet)[1]					
	0	0.25	0.51	1.02	2.02	
Thyroid weight (mg/ 100g BW)	6.6[a]	7.3[a]	7.2[a]	7.4[a]	9.2[b]	0.5
Plasma T_3 (nmol/L)	1.5	1.4	1.1	1.2	1.1	0.1
Plasma T_4 (nmol/L)	5.5	6.3	7.6	8.2	6.7	0.7

[1] CM=commercial canola meal; glucosinolate composition of the commercial canola meal is shown in Table 2. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.

[a-b] Means within a row with no common superscript differ significantly ($P<0.05$)

TABLE 15. Physiological parameters of the liver in the laying hen fed diets of varying glucosinolate content over four 28-day periods (Experiment 3)

Physiological Parameters	Diet type					Pooled SEM
	SBM (con.)[1]	2/3 con.+ 1/3 max.	1/3 con.+ 2/3 max.	CM/CS[2] (max.)	CM [3]	
	Glucosinolate content (μmol/ g diet)[2]					
	0	1.42	2.84	4.27	2.52	
Liver (g /kg BW)	22.6	23.6	23.2	22.9	22.6	0.7
GS_X (μmol/g liver)	3.93[a]	4.57[ab]	7.30[c]	6.82[c]	4.67[ab]	0.3
GSSG (μmol/g liver)	0.67[a]	0.78[b]	1.01[bc]	1.09[c]	0.64[a]	0.06
Cytochrome P_{450} (nmol/mg protein)	0.7	0.7	1	0.9	0.9	0.1
GSTα (μmol/min/mg protein)	0.13	0.12	0.12	0.15	0.11	0.009
GSTπ (μmol/min/mg protein)	1.29	1.35	1.34	1.39	1.32	0.03

[1] Con.=control

[2] CM/CS=20 % commercial canola meal and 15% canola seed (see Table 5 for glucosinolate composition) produced as the maximal toxicity diet (max.). Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal and canola seed.

[3] CM=20% commercial canola meal (see Table 5 for glucosinolate composition)

[a-c] Means within a row with no common superscript differ significantly ($P<0.05$)

TABLE 16. Physiological parameters of the thyroid in the laying hen fed diets of varying glucosinolate content over four 28-day periods (Experiment 3)

Physiological parameters	Diet type					Pooled SEM
	SBM (con.)[1]	2/3 con.+ 1/3 max.	1/3 con.+ 2/3 max.	CM/CS[2] (max.)	CM[3]	
	Glucosinolate content (μmol/g diet)[2]					
	0	1.42	2.85	4.27	2.52	
Thyroid (mg/100gBW)	7.0[a]	9.4[ab]	12.1[bc]	13.4[c]	8.7[ab]	0.7
T_3 (nmol/L)	1.3	1.8	1.9	1.7	1.7	0.2
T_4 (nmol/L)	9.8[a]	8.2[ab]	6.4[b]	8.2[ab]	7.9[ab]	0.6

[1] Con.=control
[2] CM/CS=20 % commercial canola meal and 15% canola seed (see Table 5 for glucosinolate composition) produced as the maximal toxicity diet (max.). Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal and canola seed
[3] CM=20% commercial canola meal (see Table 5 for glucosinolate composition)
[a-c] Means within a row with no common superscript differ significantly ($P<0.05$)

hepatic GSSG than those of the control-fed birds and the birds fed the 20% canola meal diet ($P<0.05$). Hepatic GSSG concentration did not differ among birds fed the 20% canola meal diet or the control diet ($P>0.05$). Similar hepatic concentrations of cytochrome $P_{450,}$ GSTα and GSTπ were found in birds fed the various experimental diets ($P>0.05$). Hepatic GSTμ was not detectable.

The birds fed the maximal toxicity diet and the combination diet of 1/3 of the control diet and 2/3 of the maximal toxicity diet had significantly heavier thyroid weight than that of the control-fed birds ($P<0.05$). Relative thyroid weight was significantly heavier in birds fed the maximal toxicity diet than that of birds fed the 20% canola meal diet ($P<0.05$). The birds fed the various experimental diets had similar plasma T_3 concentrations ($P>0.05$). The plasma T_4 concentration of birds fed the combination diet (1/3 of the control diet and 2/3 of the maximal toxicity diet) was significantly lower than that of the control-fed birds ($P<0.05$), however, this effect was not evident for the birds fed the maximal toxicity diet.

The organ status measured in birds fed the maximal toxicity diet at three different time periods is shown in Tables 17 and 18. Relative liver weight of birds decreased over time in the experiment such that the relative liver weight of birds from the third period was significantly lower than that of the initial status birds ($P<0.05$). The concentrations of GS_X, GSSG and cytochrome P_{450} in the liver were similar for birds from the three experimental periods ($P>0.05$). The relative thyroid weight of birds from the second and third period was significantly heavier than that of the initial status birds ($P<0.05$). Plasma T_3 and plasma T_4 concentrations were similar for birds from all experimental periods ($P>0.05$).

TABLE 17. Physiological parameters in the liver of laying hens measured at monthly intervals when fed the maximal toxicity diet[1] (Experiment 3)

Physiological parameters	Period				
	Initial[2]	1st[2]	2nd[2]	3rd[2]	Pooled SEM
Liver (g/kgBW)	28.3[a]	26.6[ab]	26.6[ab]	25.1[b]	0.9
GS_X (μmol/g liver)	6.20[a]	8.57[b]	9.31[b]	9.17[b]	0.4
GSSG (μmol/g liver)	0.76	0.79	0.72	0.72	0.05
Cytochrome P_{450} (nmol/mg protein)	0.6	0.8	0.7	0.9	0.03

[1] The maximal toxicity diet was composed of 20% commercial canola meal and 15% canola seed and the glucosinolate content of diet was 4.27 μmol/ g diet.
[2] Birds were killed at the initiation of the experiment and at either 1, 2 or 3 months after the initiation of the experiment
[a-b] Means within a row with no common superscript differ significantly ($P<0.05$)

TABLE 18. Physiological parameters in the thyroid of laying hens measured at monthly intervals when fed on the maximal toxicity diet[1] (Experiment 3)

Physiological parameters	Period				
	Initial[2]	1st[2]	2nd [2]	3rd [2]	Pooled SEM
Thyroid (mg/ 100g BW)	7.6[a]	8.4[ab]	9.5[b]	12.0[c]	0.5
T_3 (nmol/L)	1.4	1.5	1.5	1.7	0.1
T_4 (nmol/L)	5.4	4.6	4.3	4.4	0.8

[1] The maximal toxicity diet was composed of 20% commercial canola meal and 15% canola seed and the glucosinolate content of diet was 4.27 μmol/ g diet.
[2] Birds were killed at the initiation of the experiment and at either 1, 2 or 3 months after the initiation of the experiment
[a-c] Means within a row with no common superscript differ significantly ($P<0.05$)

Experiment 4

Alterations of organ status of birds fed the control diet and the two glucosinolate-containing diets are shown in Tables 19 and 20. Birds fed the two glucosinolate-containing diets had significantly heavier livers than that of the control-fed birds ($P<0.05$). The hepatic concentrations of GS_X and GSSG of the birds fed the combination diet of canola meal and canola seed were significantly higher relative to those of the control-fed birds and the sole canola meal-fed birds ($P<0.05$). In contrast, the birds fed the sole canola meal diet had similar hepatic concentrations of GS_X and GSSG to those of the control-fed birds ($P>0.05$). The relative thyroid weight was significantly greater in birds consuming the glucosinolate-containing diets in comparison with that of the control-fed birds ($P<0.05$). Furthermore, the relative thyroid weight of birds fed the combination diet of canola meal and canola seed was significantly heavier than that of birds fed the sole canola meal diet ($P<0.05$). The plasma T_3 and plasma T_4 concentrations were similar in birds fed the glucosinolate-containing diets and the control diet ($P>0.05$).

In the above section, the effects of glucosinolates on physiological parameters in the liver and thyroid were reported individually for each of the four experiments. However, in an attempt to ascertain the relationship between dietary glucosinolate levels and the physiological parameters, the individual observations from the four experiments were further analyzed using regression analyses. Since the experimental period was five, five, four and eight months in Experiments 1, 2, 3, and 4, respectively, it was of importance to determine if the experimental time period would affect the physiological parameters. In this regard, in Experiment 3, the physiological parameters in the liver and thyroid were measured at three

TABLE 19. Physiological parameters of the liver in the laying hen fed canola meal as a sole protein supplement or together with canola seed over eight 28-day periods (Experiment 4)

Physiological parameters	Diet type			Pooled SEM
	Control	27.5% CM[1]	25%CM+2.5% CS[1]	
	Glucosinolate content (μmol/ g diet)[1]			
	0	3.45	3.44	
Liver (g/kg BW)	21.9[a]	24.1[b]	24.4[b]	0.5
GS_X (μmol/ g liver)	3.82[a]	4.98[a]	7.12[b]	0.32
GSSG (μmol/ g liver)	0.31[a]	0.50[a]	0.80[b]	0.05

[1] CM=commercial canola meal, CS=canola seed; glucosinolate composition of the commercial meal and canola seed are shown in Table 5. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.
[a-b] Means within a row with no common superscript differ significantly ($P<0.05$)

TABLE 20. Physiological parameters of the thyroid in the laying hen fed canola meal as a sole protein supplement or together with canola seed over eight 28-day periods (Experiment 4)

Physiological parameters	Diet Type			Pooled SEM
	Control	27.5% CM[1]	25%CM+2.5% CS[1]	
	Glucosinolate content (μmol/ g diet)[1]			
	0	3.45	3.44	
Thyroid (mg/ 100gBW)	7.8[a]	11.8[b]	17.5[c]	0.8
T_3 (nmol/L)	2.2	2.3	2.5	0.1
T_4 (nmol/L)	4	3.5	3.2	0.4

[1] CM=commercial canola meal, CS=canola seed; glucosinolate composition of the commercial meal and canola seed are shown in Table 5. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.
[a-c] Means within a row with no common superscript differ significantly ($P<0.05$)

different time periods in birds fed the maximal toxicity diet. The concentrations of hepatic GSx, hepatic GSSG, hepatic cytochrome P_{450} and thyroid hormones (plasma T_4 and T_3) were found to be similar for the three different time periods. This indicated that the effects of glucosinolates on the five parameters were not accumulative with time period, which ensures that the data for the five parameters could be pooled to do regression analysis even though the experimental time period varied among the four experiments. However, relative thyroid weight and liver weight were found to vary with time period and consequently regression analysis was not performed for these two parameters.

In Experiments 3 and 4, canola seed as a source of myrosinase, was included in the diet to maximize glucosinolate hydrolysis and thus produce a maximal toxicity diet. The maximal toxicity diet was designed to produce a maximal physiological response in the birds that would reflect the potential toxicity of canola meal diets in which intact glucosinolates could be hydrolyzed by microflora in the digestive tracts of the laying hen. In commercial practice, canola meal is fed with the absence of canola seed and consequently regression analyses were only conducted for data from the groups fed the canola meal diets in Experiments 1, 2, 3 and 4. This was done to ascertain if a relationship existed between the physiological parameters and diets of varying canola meal levels and hence the opportunity for the production of varying levels of glucosinolate hydrolysis products in the intestine of the birds. Use of the data from all four experiments allowed for consideration of a greater range of glucosinolate concentrations than that of glucosinolate concentrations in individual experiments. The results for the regression analyses are shown in Tables 21 and 22 and Figures 3-8. Significant linear regression relationship was found between dietary glucosinolate

TABLE 21. Linear regression relationship between diet glucosinolate levels and physiological parameters in the liver (GS_X, GSSG and cytochrome P_{450}) and in the thyroid (plasma T_3 and plasma T_4) in Experiments 1, 2, 3 and 4

Parameters	Linear regression coefficient [1]	Linear regression (F value)	Lack of fit (F value)	Root MSE
GSx	9.71±2.15	22.21**	2.96*	30.78
GSSG	21.06±3.15	48.69**	2.94*	44.72
Cytochrome P_{450}	12.63±7.24	3.00	0.63	56.52
Plasma T_4	-6.03±2.85	5.59*	5.98**	37.79
Plasma T_3	6.84±2.71	6.78*	2.5*	39.24

[1] Units expressed as % of average control value within an experiment per amount of glucosinolates (μmol/ g diet)

TABLE 22. Quadratic and cubic regression relationship between diet glucosinolate levels and physiological parameters in the liver (GS_X, GSSG and cytochrome P_{450}) and in the thyroid (plasma T_3 and plasma T_4) in Experiments 1, 2, 3 and 4

Parameters	Quadratic regression (Pr>t)	Cubic regression (Pr>t)
GSx	ns	ns
GSSG	**	ns
Cytochrome P450	ns	ns
Plasma T_4	ns	**
Plasma T_3	ns	ns

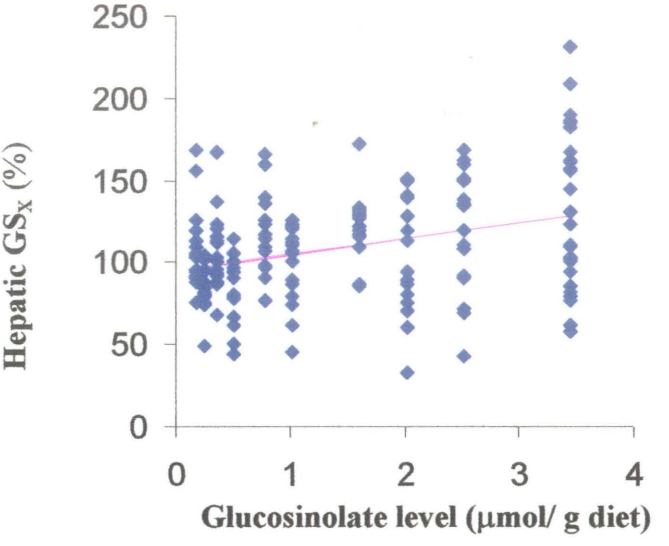

FIGURE 3. Linear relationship between hepatic GS_X and diet glucosinolate content in the laying hen.

◆ The diamond symbol represents individual observations which are expressed as a percentage of the control group average in the same experiment (control group data were not included in the final analysis) from Experiments 1, 2, 3 and 4

– The line represents predicted values

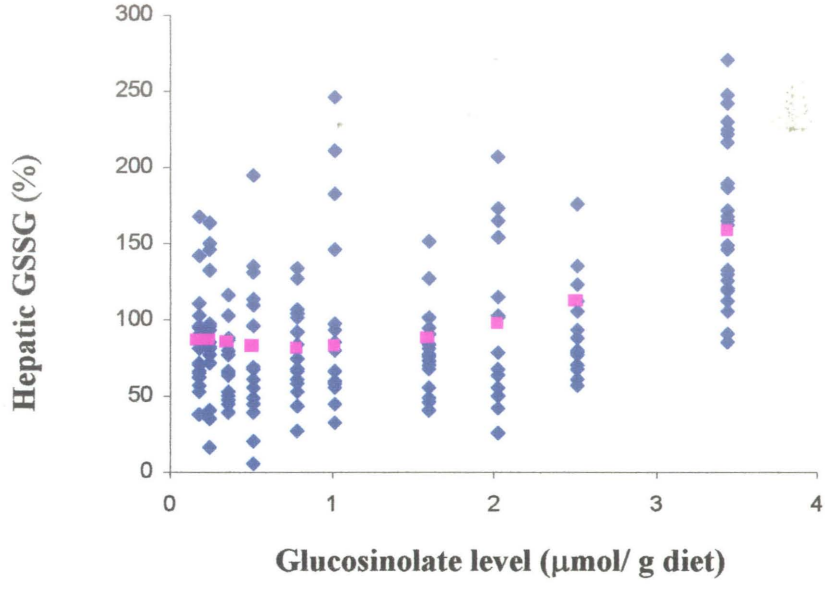

FIGURE 4. Quadratic relationship between hepatic GSSG concentration and diet glucosinolate content in the laying hen

- The diamond symbol represents individual observations which are expressed as a percentage of the control group average in the same experiment (control group data were not included in the final analysis) from Experiments 1, 2, 3 and 4
- The square symbol represents predicted values.

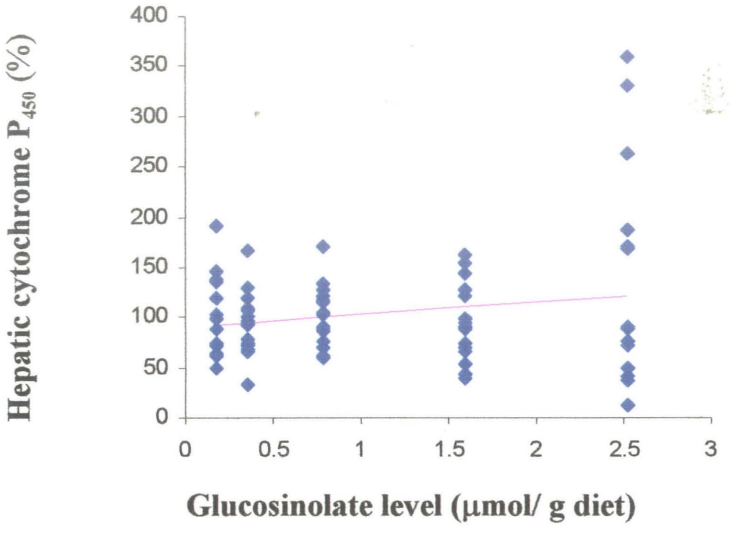

FIGURE 5. Linear relationship between hepatic cytochrome P_{450} and diet glucosinolate content in the laying hen

◆ The diamond symbol represents individual observations which are expressed as a percentage of the control group average in the same experiment (control group data were not included in the final analysis) from Experiments 1 and 3

– The line represents predicted values

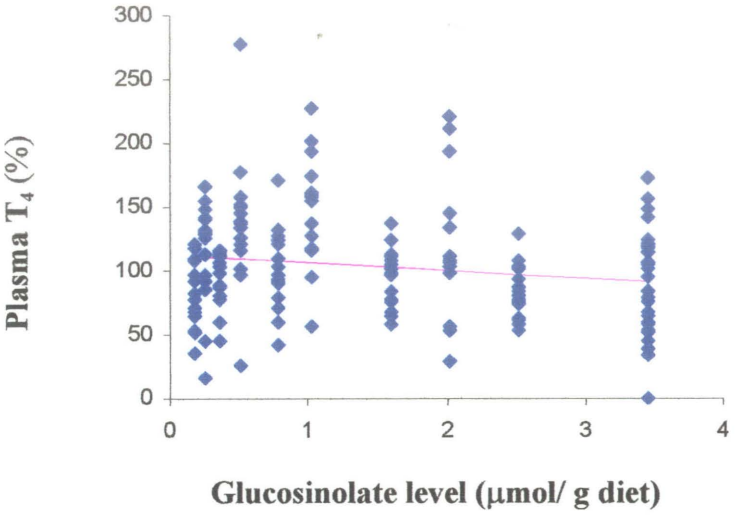

FIGURE 6. Linear relationship between plasma T_4 and diet glucosinolate content in the laying hen

◆ The diamond symbol represents individual observations which are expressed as a percentage of the control group average in the same experiment (control group data were not included in the final analysis) from Experiments 1, 2, 3 and 4

– The line represents predicted values

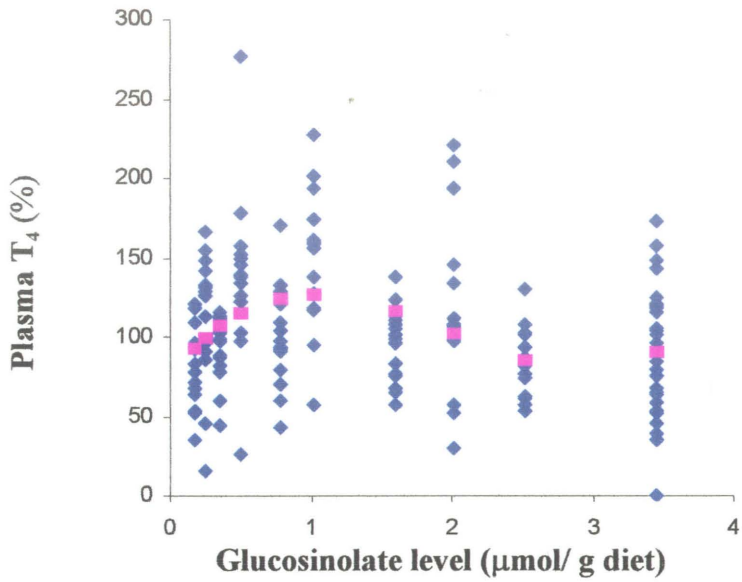

FIGURE 7. Cubic relationship between plasma T_4 and diet glucosinolate content in the laying hen

◆ The diamond symbol represents individual observations which are expressed as a percentage of the control group average in the same experiment (control group data were not included in the final analysis) from Experiments 1, 2, 3 and 4

■ The square symbol represents predicted values

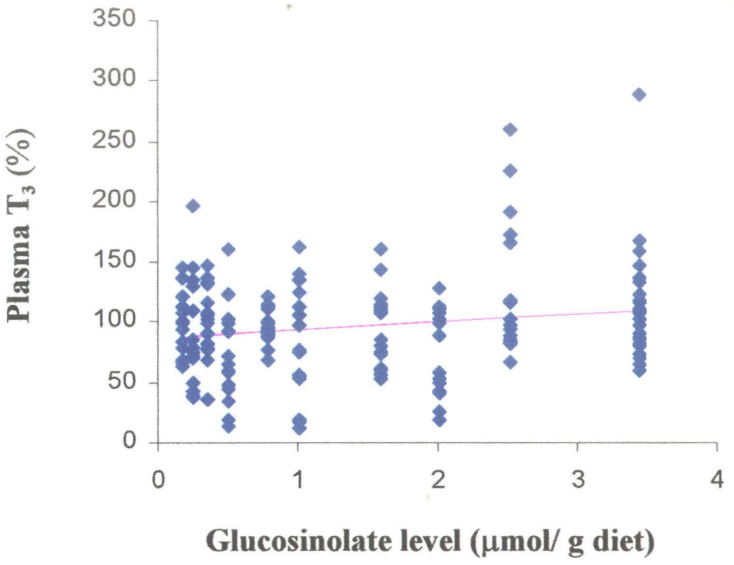

FIGURE 8. Linear relationship between plasma T_3 and diet glucosinolate content in the laying hen

◆ The diamond symbol represents individual observations which are expressed as a percentage of the control group average in the same experiment (control group data were not included in the final analysis) from Experiments 1, 2, 3 and 4

– The line represents predicted values

levels and the concentrations of hepatic GS_X and hepatic GSSG, plasma T_4 and plasma T_3 ($P<0.05$). Lack of fit (non linearity) was also significant for the relationship between diet glucosinolate levels and the concentrations of hepatic GS_X , hepatic GSSG, plasma T_4 and plasma T_3. No quadratic or cubic regression relationship was found between diet glucosinolate levels and hepatic GS_X and plasma T_3. However, a significant quadratic regression relationship was found between diet glucosinolate levels and hepatic GSSG ($P<0.05$) and a significant cubic regression relationship was noted for diet glucosinolate levels and plasma T_4 ($P<0.05$). There is no linear relationship between dietary glucosinolate levels and hepatic cytochrome P_{450} ($P>0.05$).

4.4 DISCUSSION

It is reported that glucosinolates can lead to liver hemorrhage in the rapeseed meal and canola meal feeding regimens. A low mortality due to liver hemorrhage was noted in the current study with the mortality rate ranging from 0.2-0.5% in canola meal/canola seed regimens in the laying hen. No mortality due to liver hemorrhage was observed in the control-fed birds but the mortality noted in treatment diets (glucosinolate-containing) was inconsistent in addition to being at a low rate. This inconsistency in mortality due to liver hemorrhage was in accordance with reports in the literature. From a survey of numerous literature reports, liver hemorrhage was not indicated as a cause of mortality among hens fed rapeseed/canola meal in several studies (Leeson et al., 1987, Clandinin et al., 1986, Thomke et al., 1983 and Hulan et al., 1980) while it was listed as a cause of death in other studies (Campbell et al., 1979, Campbell, 1987a, b, Summers et al., 1985 and Ibrahim et al., 1980a).

This inconsistency might be due to the different properties of microflora that are present in the gastrointestinal tract of the birds in different experiments along with other unknown factors.

Physiological parameters relating to the thyroid (relative thyroid weight, plasma T_3 and T_4)and liver (relative liver weight, glutathione as measured by GSSG and GS_X, cytochome P_{450}, GSTα, π and μ) were measured in the current study in an attempt to establish the relationship between these parameters and diet glucosinolate levels and relate this data to liver function and ultimately liver hemorrhage in the laying hen. Among the physiological parameters studied the response of the most magnitude was noted for hepatic glutathione concentration and particularly in birds fed diets designed to produce the greatest possibility for toxicity. A maximal toxicity diet (the combination diet of canola meal and canola seed), and two other combination diets of this maximal toxicity diet and the control diet were included in Experiment 3. Similarly, a maximal toxicity diet was included in Experiment 4. Sole canola meal diets were also included in these two experiments. Birds fed the combination diets of canola meal and canola seed showed a more pronounced response relative to the control-fed birds than did birds fed the sole canola meal diets with regard to liver concentrations of GS_X and GSSG. Furthermore, greater response was found in birds fed the combination diet of canola meal and canola seed than those fed the sole canola meal diet in Experiment 4 even at a similar glucosinolate content. Intact glucosinolates in the combination diets would be hydrolyzed in the digestive tracts of the birds by myrosinase present in the canola seed and by microorganism possessing myrosinase-like activity (Marangos and Hill, 1974). In birds fed canola meal diets, intact glucosinolates would only

be hydrolyzed by microorganism since myrosinase is inactivated during commercial processing and canola meal is free of active myrosinase. The different response observed between birds fed the combination diets and the sole canola meal diets indicates that glucosinolate hydrolysis products have an important influence on alterations of hepatic concentrations of GS_X and GSSG and the lesser response noted in the canola meal-fed birds is an indication that maximal glucosinolate hydrolysis was not realized in the gastrointestinal tract of birds fed the latter diets. The enhancement of GS_X indicated that the detoxification system is responding to the glucosinolate hydrolysis products in canola meal/canola seed feeding regimens and in this regard, the detoxification process in the liver is accelerated. This is supported by Wallig et al. (1990), who suggested that increased tissue glutathione could provide increased substrate for conjugation of reactive metabolites of ingested toxins in the detoxification system. Glutathione exists in two forms: the reduced form which is most often referred to as glutathione (GSH) and the oxidized form known as glutathione desulfide or GSSG. Glutathione is homeostatically controlled and a dynamic balance is maintained between the synthesis of GSH, its recycling from GSSG and its utilization (Kidd, 1997). The ratio of GSSG/GSH is very important for normal physiological status of the liver in animals (Beutler, 1989). In the current study, total glutathione or GS_X (GSH plus GSSG) and GSSG were measured. In addition to the absolute values for GS_X and GSSG, the ratio GSSG/GSH was calculated (data not shown). However, this ratio varied among diets and from experiment to experiment, precluding its use as a reliable estimate for the physiological status of liver glutathione. In this regard, GS_X is a more reliable biomarker for the effects of glucosinolates on the liver. This conclusion has been stated by other researchers (Wallig et al., 1989).

The enhancement of hepatic glutathione by glucosinolate hydrolysis products has been reported in other studies. In these studies, either total glutathione or GSH was reported. Wallig et al. (1986 and 1989) investigated the acute toxicity of CHB (a nitrile from glucosinolate hydrolysis) by oral administration to rats. The researchers found that hepatic GS_X concentration was increased. Smith et al. (1992) observed accumulation of glutathione in rats fed canola meal and the authors suggested that a general hepatotoxicity might be responsible for the enhancement of hepatic glutathione. They further postulated that the general hepatotoxicity could impede the transport of glutathione to peripheral tissues, perhaps due to reduced activity of γ-glutamyl transpeptidase, and thus lead to the glutathione accumulation in the liver. Stacck et al. (1998) evaluated the individual and collective effects of four glucosinolate derivatives (crambene, phenylethylisothiocyanate, indole-3-carbinol and iberin) from Brussels sprouts on hepatic glutathione concentration in rats. Crambene, phenylethylisothiocyanate and the mixture were found to cause a significant increase in hepatic glutathione in relation to that of control-fed rats. Indole-3-carbinol and iberin had no effect on hepatic glutathione concentration. Davis et al. (1993) hypothesized that crambene could lead to upregulation of γ-glutamyl cysteinyl ligase mRNA synthesis, which may be another mechanism responsible for glutathione elevation.

In the current study, the comparison of the differential response between birds fed the combination diets of canola meal and canola seed and those fed the sole canola meal diets indicated that glucosinolate hydrolysis products were related to the alterations of hepatic concentrations of glutathione. For birds fed the canola meal diets, the myrosinase activity of microorganism in the digestive tracts was not enough to produce the maximal hydrolysis of

glucosinolates. However, the potential toxicity in birds fed the canola meal diets could be inferred from those fed the combination diets of canola meal and canola seed. That glucosinolate hydrolysis products in gastrointestinal tracts are of importance for the physiological effects of glucosinolates in the liver observed in current study is supported by the literature reports of the enhancement in hepatic glutathione concentration due to the glucosinolate hydrolysis products. During the process of the detoxification of the glucosinolates, intermediate compounds that are toxic to the cell may be formed and result in cell necrosis. As the liver in the high producing laying hen is quite fragile an excessive amount of cell necrosis may become a focal point for hemorrhage and liver rupture. If the rupture occurs near the surface of the liver death due to excessive blood loss may occur. In other situations the blood loss may not be sufficient to result in death and the bird will survive with no apparent detrimental effects. This latter phenomenon may explain some of inconsistency in rate of liver hemorrhage within and among experiments.

In the current study, the detoxification enzymes involving cytochrome P_{450} and GST did not differ between control and treatment-fed birds. This result is not consistent with that of Israel et al. (1979), who reported hepatic cytochrome P_{450} was significantly increased after 3 weeks of rapeseed meal feeding in broiler chicks. The age difference (chicks vs layers) might be responsible for the observed difference. The lack of response in the liver detoxification enzymes noted in the current study is in contrast to literature reports of studies with rats where enzyme induction was generally noted (Aspry et al.,1983, Whitty et al., 1987 Bradfield et al., 1984, 1985 and Wortelboer et al.,1992). In a study conducted by Campbell et al. (1995), rats housed in conventional or germ-free environments were fed a rapeseed meal

diet for four weeks. The alterations of cytochrome P_{450} and GST in liver tissue were observed in conventional animals but not in germ-free counterparts. It was suggested that the absorbed glucosinolate derived products were responsible for the effects observed. Nugon-Baudon et al. (1990a) showed that hepatic microsomal P_{450} activity was decreased and GST activity was increased in conventional rats fed rapeseed meal. A more pronounced effect noted in rats than in chickens may be due to a more active microflora in the gut of rats. This view is in agreement with other researchers (Nugon-Baudon et al., 1994) who suggested that the different toxic patterns observed in different animal species are more likely to be due to differences in the native digestive microflora than to intrinsic host sensitivity toward glucosinolates. In this regard, when given a diet based on rapeseed meal, conventional rats exhibit glucosinolate-linked symptoms different from those of gnotobiotic rats harbouring either chicken or human microflora (Nugon-Baudon et al., 1988 and Rabot et al., 1993a). In another study, dietary fibre was shown to be able to modify the effects of detoxification enzymes in rats (Roland et al., 1996). It could be suggested that under specific environmental conditions significant alteration in liver detoxification enzymes might be noted in rapeseed/canola meal feeding regimens in the laying hen.

In comparison with the control-fed birds, relative thyroid weight was enlarged in birds fed the 25.5% canola meal diet in Experiment 2 and the 27.5% canola meal diet in Experiment 4. Relative thyroid weight of birds fed the maximal toxicity diet was significantly heavier than that of birds fed the sole canola meal diet even at a similar glucosinolate level in Experiment 4. These results indicate that glucosinolate hydrolysis products have an important influence on the physiological response in the thyroid. As discussed above, the maximal hydrolysis of

glucosinolates in the intestine of the birds is not realized by microflora alone but the potential toxicity of glucosinolates in birds fed the canola meal diets can be inferred from the response of those birds fed the combination diets of canola meal and canola seed. The physiological response in the thyroid corroborate the liver glutathione data and give further evidence that glucosinolate hydrolysis products play an important role in the potential toxicity of glucosinolates in canola meal feeding regimens.

No significant response was observed in birds fed the sole canola meal diets relative to the control-fed birds with regard to hepatic GS_X and GSSG (with one exception in Experiment 1). In order to obtain a greater range of dietary glucosinolate levels than that studied in the four individual experiments, regression analyses were conducted for all individual observations for hepatic GS_X and GSSG from the canola meal diets. In this analysis, significant linear relationship was found between diet glucosinolate levels and hepatic concentrations of GS_X and GSSG (Table 21 and Figures 3-8). Lack of fit for these two parameters was also significant. From the comparison between the F ratio of linear regression and lack of fit, linear regression was shown to be the major component, indicating that the linear model is an appropriate one to explain the relationship between diet glucosinolate levels and hepatic glutathione. Non linearity relationships (quadratic and cubic regression analyses) were done between the two parameters and diet glucosinolate levels. No quadratic or cubic regression was found between hepatic GS_X and diet glucosinolate levels, indicating that the relationship between hepatic GS_X and diet glucosinolate levels is mainly linear. The significant linear relationship indicated that hepatic GS_X can be enhanced even at very low glucosinolate levels and in this regard, in order to overcome the enhancement of hepatic GS_X, dietary

glucosinolate levels would need to be maintained at as low a level as possible. A significant quadratic relationship was found between diet glucosinolate levels and hepatic GSSG (Figure 4). The quadratic relationship showed that hepatic GSSG can be increased slowly when diet glucosinolate level is low and increased at a higher rate when diet glucosinolate levels are high. However, inferences drawn from this quadratic relationship between diet glucosinolate levels and hepatic GSSG should be done with caution. As discussed above, the ratio of the oxidized form to the reduced form of glutathione (GSSG/GSH) was not consistent in the current study and it was concluded that total glutathione or GS_X (GSSG+GSH) is a better measure of liver detoxification activity. In a previous study conducted in this laboratory, liver total glutathione (GS_X) was used as a measure to estimate the diet glucosinolate levels below which little or no physiological response to glucosinolates was evident (Campbell et al., 1991). In that study a "no-effect" diet glucosinolate level of 0.7-1.4 μ mol/ g diet was suggested. The data from the current experiments support and extend this conclusion and it can recommended that the glucosinolate level in future varieties of canola meal should be maintained at low levels, below those that are present in current varieties. Use of current varieties of canola meal in the diet of the laying hen at a 10% level (about ½ the maximum usage level to supply protein needed by the laying hen) would result in a glucosinolate level of about 1.2 μ mol/ g diet.

The observed goitrogenicity in the current study was consistent with other reports (Bell et al., 1965, Srivastava et al., 1975 and Papas et al., 1979a, Goh et al., 1985 and Nassar et al., 1986). Greater response was observed in birds fed the combination diets of canola meal and canola seed in relation to those fed the sole canola meal diets indicating that the extent

of goitrogenicity depends on the amount of glucosinolate hydrolysis products present. There are several explanations regarding the goitrogenicity in rapeseed meal/canola meal feeding regimens. Normally, the thyroid follicular cells are able to trap inorganic iodide at the base of the cell and transport it against an electrical gradient across the cell. Inorganic iodide is then converted to an oxidized species of iodine by a peroxidase. The oxidized species of iodine (active iodide) are bound to thyroglobulin (Hadley, 1992). In rapeseed meal/canola meal feeding, the peroxidase is involved in the oxidation of glucosinolate hydrolysis products (i.e. OZT). In this conversion process iodine will be reduced to inorganic iodide and hence active iodide would not be available for iodination of thyroglobulin. This would interfere with the subsequent synthesis of thyroid hormones. In addition, the coupling reactions necessary for T_4 formation may be interfered with by OZT, leading to accumulation of thyroxine precursors in the thyroid (Akiba et al., 1976 and Elfving, 1980a). The peripheral conversion of T_4 to T_3 in the liver may also be affected by OZT, although this has been tested in rats without conclusive results (Langer et al., 1984). In addition to the physiological effects of OZT on the thyroid, thiocyanate ion which may be derived from dietary indole glucosinolates or indirectly via the metabolism of isothiocyanates and nitriles blocks and reduces iodine capture and acts as an iodine competitor. All these mechanisms can contribute to goitrogenicity in rapeseed meal/canola meal feeding regimens and substantiate the conclusion that glucosinolate hydrolysis product formation is integral to the physiological response of laying hens to dietary glucosinolates.

Plasma T_4 and plasma T_3 showed little response to diet glucosinolate levels in each of the four experiments conducted in the current study. This result indicating that plasma T_3 and

plasma T_4 can be maintained at a normal level via the compensation of the enlarged thyroid is consistent with the data reported by others (Papas et al., 1979 and Schone et al., 1997b). In our studies, when considering data from all four experiments, significant linear regression relationships were found between diet glucosinolate levels and plasma T_4 and plasma T_3 (Figures 6 and 8). Plasma T_4 decreased and plasma T_3 increased with increasing diet glucosinolate levels. Lack of fit was also significant for these two parameters. From the comparison between the F-ratio due to the linear regression and lack of fit, it can be suggested that the linear relationship between diet glucosinolate levels and plasma T_4 and plasma T_3 only explains a small portion of the variance. Higher order polynomial regression relationships between diet glucosinolate levels and plasma T_4 (Figure 7) and plasma T_3 were attempted but offered little explanatory value. Notwithstanding the high variance due to other factors, the observation of decreasing plasma T_4 and increasing plasma T_3 with response to glucosinolates is consistent with data reported by Rognoni et al. (1982) and Kloss et al. (1994). It is well known that T_4, the main hormone released by the thyroid gland, is converted to the biologically active form, T_3, in target cells. Rapeseed meal/canola meal feeding may activate some unidentified compensatory mechanisms under the influence of low concentrations of glucosinolates, leading to the production of T_3 rather than T_4, which spares one atom of iodine (Schöne et al., 1997a and Opalka et al., 2001). The increased T_3 could also be due to a destruction of cellular T_3 receptors by glucosinolates and the impaired T_3 transfer might cause an increase of this hormone concentration in blood (Schöne et al., 1993).

Chapter V

5.0 THE EFFECTS OF CANOLA MEAL FEEDING WITH TWO ADDED LEVELS OF IODINE ON REPRODUCTIVE PARAMETERS AND A STUDY OF STRAIN EFFECTS IN RESPONSE TO CANOLA MEAL FEEDING REGIMENS IN THE LAYING HEN

5.1 INTRODUCTION

The nutritional value of rapeseed meal has been thoroughly investigated for table egg production in the laying hen. Few studies, however, have investigated the effects of rapeseed/canola meal feeding regimens in breeder laying hens. It was reported in some studies that rapeseed meal/canola meal feeding regimens had no influence on fertility and hatchability in breeder laying hens. However, reduced body weights and enlarged thyroids were noted in day-old chicks in these experiments. Alleged negative effects associated with canola meal feeding have been reported in breeder laying hens in commercial broiler breeder production units. Hence, it is not clear whether commercial canola meal feeding regimens have an influence on fertility and hatchability in breeder laying hens. Iodine supplementation in rapeseed/canola meal feeding regimens in lactating swine has been used to alleviate antithyroid effects in piglets but there is little data available to indicate if a similar response would occur in breeder laying hens. The objectives of this study were to investigate if canola meal feeding regimens had effects on fertility and hatchability and to ascertain if iodine supplementation could counteract any possible effects of canola meal feeding on fertility and hatchability in breeder laying hens.

In previous studies in this laboratory, incidence of liver hemorrhage among hens fed canola meal diets tended to be higher than that noted in the current study (Campbell, unpublished results). One possible difference could be that many of the earlier experiments conducted in this laboratory utilized Shaver laying hens. Consequently a secondary objective of this experiment was to determine if Shaver laying hens showed a different response to

canola meal feeding in comparison to another commercial SCWL strain (Hyline).

5.2 MATERIALS AND METHODS

A hatchability study to investigate effects of canola meal feeding regimens was done with laying hens of a table egg strain (Shaver SCWL). Artificial insemination was performed to study the fertility and hatchability. The effects of canola meal feeding with two added dietary levels of iodine were studied. In addition, as a secondary component of the study, two SCWL strains (Shaver and Hyline) were used to investigate a possible strain difference in the response of laying hens with regard to liver hemorrhage incidence when fed diets of relatively high glucosinolate content.

5.2.1 Diets and birds

Experiment 5

Two canola meal diets with two levels of supplemental iodine (0.04 ppm and 0.4 ppm) and a control diet with one level of supplemental iodine (0.04 ppm) were included in this study. The canola meal diets were maximal toxicity diets composed of 25 % canola meal and 2.5% canola seed and similar to that used in Experiment 4. The composition of the experimental diets is shown in Table 23. Glucosinolate composition is shown in Table 5. Iodine was added in the form of calcium iodate. All diets were calculated to be isonitrogenous and isoenergetic and formulated to meet the nutrient requirements according to National Research Council (1994). Six replicates were allotted at random to the control diet and seven replicates to the canola meal diets (with two supplemental iodine levels of 0.04ppm and 0.4

TABLE 23. Ingredient and glucosinolate content in the experimental diets used in canola meal feeding with two added levels of iodine for laying hens from Shaver and Hyline strains (Experiment 5)

Ingredient	Diet type		
	Control (0.04ppm I)	CM/CS[3] (0.04 ppm I)[4]	CM/CS (0.4 ppm I)
Wheat	42	27.3	27.3
Hulled barley	25.99	28.62	28.62
Soybean meal	17	0	0
Canola meal	0	25	25
Canola seed	0	2.5	2.5
Limestone	8.35	8.1	8.1
Calcium phosphate	1.25	1	1
Vegetable oil	3.84	5.95	5.95
Premix[1, 2]	1.5	1.5	1.5
DL-Methionine	0.07	0.03	0.03
Analyses[3] Glucosinolates (µmol/ diet)	0	3.45	3.44

[1] Premix: as noted in Table 1.
[2] White salt was used instead of iodized salt
[3] CM=canola meal; CS=canola seed; CM:CS is 10:1; glucosinolate composition of CM and CS are shown in Table 5. Diet glucosinolate levels were calculated from the analyzed glucosinolate content of canola meal.
[4] 0.04 ppm and 0.4 ppm: levels of supplemental iodine as calcium iodate in experimental diets

ppm, respectively) for each SCWL strain (Shaver and Hyline). Each replicate contained 16 birds with a total of 640 birds. All birds were weighed at the initiation and at the end of the experiment. Diets were offered in mash form *ad libitum* for eight 28-day periods and water was available *ad libitum* throughout the experiment. Daily egg production and 28-day feed consumption data were recorded on a replicate basis. Group egg weights were taken on a replicate basis for three consecutive days during the last week of each of the eight 28-day periods. Mortality was recorded daily and a post mortem examination was carried out to note evidence of liver hemorrhage as a cause of death.

After eight-month feeding period 320 of the Shaver strain hens were used for the hatchability study at the age of sixty-three weeks. Twenty roosters obtained from the University poultry flock were used for semen collection. During the experimental period semen was collected twice within a three-day interval from all roosters. Prior to semen collection, all roosters were trained to get used to the collection and handling procedures. Roosters were fed a wheat-soybean meal based maintenance diet. Feed was removed four hours prior to semen collection to avoid fecal contamination. Freshly collected and pooled semen (0.05 cc) was artificially inseminated in each hen using a syringe equipped with a flexible tube and a glass tip. All hens were inseminated twice within a three-day interval to get maximum fertility. Eggs were collected for four consecutive days following the second insemination. All eggs were sorted by replicates for the three experimental diets.

The collected eggs, after cooling to room temperature, were stored at 16°C . The day before the eggs were set for incubation, all the eggs were removed from the storage room and warmed to room temperature overnight. All eggs were placed on incubator trays with the

treatments being distributed randomly throughout the incubator (Robbins IH-A, Colorado). Candling was done at Day 7 to establish fertility. Hatchability was computed in two ways: by dividing the number of hatched chicks by the number of fertile eggs or by the total numbers of set eggs. Newly hatched chicks were selected randomly from the three treatments and weighed in groups of 10 chicks each. Average body weight of chicks was calculated by dividing the total weight by the total number of chicks in each group.

5.2.2 Sample collection

Eggs laid by the Shaver strain birds were collected on one day during the last experimental period. The collected eggs of each replicate from the three experimental diets were broken together into one container. The liquid portions were homogenized, freeze-dried and stored for later analyses of iodine and SCN. At hatching, the randomly selected chicks were anaesthetized with co_2 after weighing. Blood was quickly collected by cardiac puncture using vacationer blood collection tubes (for each tube 340 USP units of heparin were manually added to the needles prior to blood collection). Blood samples were cold-centrifuged at 3000g for 45 minutes and plasma aliquots were immediately (on the same day without storage) analyzed for thyroid hormone content. Thyroids that were obtained from the Shaver birds at the end of the experiment were stored at -20°C for later iodine analysis.

For the strain comparison study, twelve hens of each strain were chosen at random from each treatment at the termination of the eight-month feeding experiment. The hens were weighed and anaesthetized with carbon dioxide. Blood was quickly collected by cardiac

puncture using Vacationer blood collection tubes (for each tube 340 USP units of heparin were manually added to the needles prior to blood collection). Blood samples were cold-centrifuged at 3000g for 45 minutes and plasma aliquots were immediately (on the same day without storage) analyzed for thyroid hormone content. Livers were excised, and weighed after removal of surrounding fat. Thyroids were removed from each bird and subsequently weighed after the removal of adhering fat.

5.2.3 Thyroid hormone assays

Plasma triiodothyronine (T_3) and tetraiodothyronine (T_4) concentrations were measured as indicated in Chapter IV.

5.2.4 Thiocyanate ion analysis

Intact glucosinolates present in rapeseed meal can be hydrolyzed by supplemental myrosinase and thiocyanate ion will be released from this hydrolysis process. In addition, free SCN is also present in rapeseed meal. Both total SCN (free SCN and SCN released from the hydrolysis of intact glucosinolates) and free SCN were analyzed in feed samples. Egg SCN content was analyzed as free SCN as it is assumed that no glucosinolates are present in eggs. Thiocyanate ion analysis was carried out by a method modified from Johnson and Jones (1965), in which SCN was determined by treating a trichloroacetic acid filtrate with a solution of ferric nitrate. Potassium thiocyanate was used to prepare a standard curve.

For total SCN content, feed samples (0.5 g) were heat treated with 7 ml of boiling

water for 3 min. Three milliliters of myrosinase solution (10mg mL^{-1}) were added after the samples were cooled. Following incubation for 2 h during which time the samples were shaken continuously, 5 ml of a 20% trichloroacetic acid solution was added to each tube and the solution was centrifuged at 3000 g for 30 minutes. Three milliliters of supernatant in triplicate lots were mixed with 3 ml of 0.4 M ferric nitrate in 1 N nitric acid. Two drops of a 5% mercuric chloride solution were added to one of the tubes. Mercuric chloride destroys the yellowish coloration of the ferric nitrate reagent with phenolic compounds present in feed samples since this would interfere with the color of the ferric thiocyanate complex. The readings from these tubes with added mercuric chloride were used as a control. Readings were taken spectrophotometrically within 1-2 min at 460 nm in an ultrospec 2000 spectrophotometer (UV/ visible, Pharmacia Biotech).

Free SCN content was analyzed slightly different for feed and egg samples. For feed samples, 5 ml of a 20% trichloroacetic acid solution was added to each ground feed sample (0.5 g) and allowed to stand for approximately 20 minutes to inactivate endogenous myrosinase. Ten ml of water was added to extract SCN for one hour during which time the samples were shaken continuously. Following extraction, the solutions were centrifuged at 3000 g for 15 minutes. The readings were taken spectrophotometrically as described for total SCN. For egg samples,10 ml of water were added to each ground freeze-dried egg samples (2.5 g) to extract SCN and the samples were shaken continuously during extraction for one hour. Five milliliters of a 20% trichloroacetic acid solution were added to the samples after extraction, and the solution was centrifuged at 3000g for 30 minutes. The readings were taken spectrophotometrically as described for total SCN.

5.2.5 Iodine analysis

The method of iodine analysis was adapted from Pino et al. (1996) and the Hycel procedure (Hycel Inc.).

5.2.5.1 Sample preparation. Wet thyroid tissue samples were homogenized in 1 ml deionized water. Freeze-dried egg samples were extracted with hexane for 8 hours to remove interfering substances prior to iodine analyses.

5.2.5.2 Chemicals. Perchloric acid (70%), ammonium metavanadate, arsenic trioxide, concentrated sulfuric acid (98%), sodium chloride, potassium iodate, ceric ammonium sulfate [$(NH_4)_4Ce(SO_4)_4.2H_2O$] were purchased from Sigma Chemical Co. (St. Louis, MO). Deionized water was used for preparations of all reagents and dilution procedures. Solutions of potassium iodate were used to establish a calibration curve.

5.2.5.3 Assay procedure. Two ml of digestion reagent (0.05% ammonium metavanadate in 70% perchloric acid) were added to18x150mm tubes containing the same volume of each standard (0.1, 0.2, 0.3, 0.4 and 0.5 mg/ mL iodine) or sample. The tubes were placed, at 5 second intervals, in a heating block at 180°C for digestion. The digestion time for thyroid and egg samples was 30 minutes and 60 minutes, respectively. After digestion, the tubes were removed, at 5 second intervals, from the heating block and allowed to cool down completely to room temperature. Two milliliters of arsenious acid (0.0253 mol/L), 1 ml of 1.25 M

sulfuric acid and 1 ml of deionized water were added to standards and samples in order. After vortexing, the tubes were put into a 20°C water bath for 10 minutes to equilibrate. The reaction was started by adding 0.5 ml of ceric ammonium sulfate (0.0158 mol/L) to each tube, which was incubated precisely for 10 minutes in the 20°C water bath. Readings were taken spectrophotometrically at 420 nm against deionized water (Spectronic 601, Milton Roy Company). Iodine assays were very sensitive to temperature changes and a set of standard calibrations was run with each set of samples.

5.2.6 Statistical analyses

All data in the hatchability study were subjected to the General Linear Models procedure of SAS institute (1985) in completely randomized design using the following model: $y_{ij}=u+d_i+e_{ij}$, where y_{ij} is the response criterion (fertility, hatchability, day-old chick weight, chick plasma T_3, chick plasma T_4, SCN and iodine content); d_i is the effects of diets; u is the overall mean, e_{ij} is the error term. Tukey test was used to compare and separate means.

Data collected for comparing strain effects were subjected to the General Linear Models procedure of SAS (Steel et al., 1997) in completely randomized design using the following model: $y_{ij}=u+t_i+e_{ij}$, where y_{ij} is the response criterion; u is the overall mean; t_i is the effects of diets; e_{ij} is the error term. Tukey test was used to compare and separate means. Contrasts were made for the following comparisons using the GLM procedure of SAS (Steel et al., 1997): the average performance of the Shaver birds vs the average performance of the

Hyline birds in canola meal feeding; the average performance of birds fed 0.04 ppm iodine level vs the average performance of birds fed 0.4 ppm iodine level in canola meal feeding; the difference in performance between the control diet and the canola meal diet (0.04 ppm iodine) for Shaver birds vs the difference for Hyline birds; the average biological response of the Shaver birds vs the average biological responses of the Hyline birds in canola meal feeding; the average biological response of birds fed 0.04 ppm iodine level vs the average biological responses of birds fed 0.4 ppm iodine level in canola meal feeding; the difference in biological responses between the control diet and the canola meal diet (0.04 ppm iodine) for Shaver birds vs the difference for Hyline birds.

5.3 RESULTS

The effects of canola meal feeding with two levels of iodine supplementation on reproductive characteristics in the laying hens of Shaver strain are shown in Table 24. Fertility and hatchability were similar for hens fed the three experimental diets ($P>0.05$). Chicks hatched from the eggs laid by hens fed the canola meal diets were significantly lighter at hatching than those hatched from eggs laid by the control-fed birds ($P<0.05$).

The effects of canola meal feeding with two added levels of iodine on thyroid hormone levels in day-old chicks are shown in Table 24. Triiodothyronine was similar among chicks hatched from eggs laid by hens fed the three experimental diets. Chicks hatched from eggs laid by hens fed the canola meal diet with 0.04 ppm supplemental iodine had significantly lower T_4 than the chicks hatched from eggs laid by hens fed the other two diets ($P<0.05$).

TABLE 24. The effects of canola meal feeding regimens with two added levels of iodine on fertility and hatchability in Shaver laying hens and on day-old weight and thyroid hormone levels of hatched chicks (Experiment 5)

Parameters	Diet type			
	Control (0.04 ppm I)	CM/CS[1] (0.04 ppm I)[2]	CM/CS (0.40ppm I)	Pooled SEM
Fertility, %	98.8	96.4	97.6	3.1
Hatchability of fertile eggs, %	72.6	76.2	78.6	3.6
Hatchability of total eggs, %	67.8	69.8	73	3.3
Day-old chick weight, g	43.2[a]	42.3[b]	41.2[b]	0.5
T_3, nmol/L	2.7	2.8	2.5	0.2
T_4, nmol/L	7.1[a]	6.4[b]	7.9[a]	0.4

[1] CM=canola meal; CS=canola seed; CM:CS is 10:1; glucosinolate composition of CM and CS are shown in Table 5 and the dietary glucosinolate content of all diets is noted in Table 23.

[2] 0.04 ppm and 0.4 ppm: levels of supplemental iodine as calcium iodate in experimental diets

[a-b] Means within a row with no common superscript differ significantly ($P<0.05$)

Tetraiodothyronine was similar for chicks hatched from eggs laid by hens fed the control diet and the canola meal diet with 0.4 ppm supplemental iodine ($P>0.05$).

Contents of total SCN and free SCN of the three experimental diets are presented in Table 25. The effects of canola meal feeding with two added levels of iodine on SCN content of eggs are also presented in Table 25. The SCN content in eggs laid by the control-fed birds was significantly lower than that in eggs laid by birds fed the canola meal diets ($P<0.05$). The effects of canola meal feeding with two levels of iodine supplementation on iodine contents of eggs and thyroids are shown in Table 26. The iodine content was significantly reduced in eggs laid by birds fed the canola meal diets in comparison with that of the eggs laid by the control-fed birds ($P<0.05$). Thyroid iodine content of birds fed the canola meal diets was also significantly reduced in contrast to that of the control-fed birds ($P<0.05$).

The effects of strain (Shaver vs Hyline) and of interactions between strain and diets on performance characteristics in canola meal feeding with two added iodine levels in the laying hen were analyzed using contrasts. Results of the contrasts are shown in Table 27. Birds responded similarly to canola meal feeding with regard to egg production, feed consumption and feed conversion efficiency for both strains ($P>0.05$). Egg weight of the Hyline birds fed canola meal diets was significantly heavier than that of the Shaver birds ($P<0.05$). Final body weight of the Hyline birds was significantly greater than that of the Shaver birds in canola meal feeding regimens ($P<0.05$). Birds responded similarly to the two supplemental iodine levels in canola meal feeding with regard to egg production, feed consumption and feed conversion efficiency regardless of strain ($P>0.05$). A low mortality due to liver hemorrhage was noted in birds fed canola meal diets and the effects were not

TABLE 25. The contents of free thiocyanate ion (SCN)and total SCN in experimental diets and the effects of canola meal feeding regimens with two levels of iodine supplementation on SCN content of eggs in the laying hen (Experiment 5)

SCN content	Control (0.04 ppm I)	CM/CS[3] (0.04 ppm I)[4]	CM/CS (0.4 ppm I)
SCN in diets [1]	undetectable	0.73	0.79
Total SCN in diets	undetectable	1.61	1.69
Free SCN in eggs [2]	$0.007^{a}\pm0.0075$	$0.09^{b}\pm0.0065$	$0.09^{b}\pm0.007$

[1] SCN content of diets is calculated in μmol/g diet

[2] SCN content of egg is calculated as in μmol/g dry egg (without shell)

[3] CM=canola meal; CS=canola seed; CM:CS is 10:1; glucosinolate composition of CM and CS are shown in Table 5 and the dietary glucosinolate content of all diets is noted in Table 23.

[4] 0.04 ppm and 0.4 ppm: levels of supplemental iodine as calcium iodate in experimental diets

[a-b] Means within a row with no common superscript differ significantly ($P<0.05$)

TABLE 26. The effects of canola feeding regimens with two levels of iodine supplementation on the iodine content of eggs and thyroids in the laying hen (Experiment 5)

Parameters	Diet Type			
	Control (0.04 ppm I)	CM/CS[3] (0.04 ppm I)[4]	CM/ CS (0.4 ppm I)	Pooled SEM
Eggs[1]	2.1[a]	1.2[b]	1.6[b]	0.1
Thyroids[2]	693.4[a]	313.4[b]	372.2[b]	23.8

[1] Iodine concentration of thyroid is calculated in μg/g wet thyroid tissue
[2] Iodine concentration of egg is calculated as in μg/g dry egg (without shell)
[3] CM=canola meal; CS=canola seed; CM:CS is 10:1; glucosinolate composition of CM and CS are shown in Table 5 and the dietary glucosinolate content of all diets is noted in Table 24.
[4] 0.04ppm and 0.4ppm: levels of supplemental iodine as calcium iodate in experimental diets
[a-b] Means within a row with no common superscript differ significantly ($P<0.05$)

TABLE 27. Performance parameters of laying hens from Shaver and Hyline strains in canola meal feeding with two levels of supplemental iodine over an eight-month feeding period (Experiment 5)

Diet		Egg production, %	Feed intake, g/hen/day	Egg weight, g	Feed conversion, kg feed : kg egg	Final body weight, kg	Mortality, LH[4]
Control (0.04 ppm I)[1]	Hyline	81.8[ab]	103.2[a]	61.8[a]	1.85	1.7	0
	Shaver	84.6[a]	101.7[ac]	61.6[a]	1.84	1.71	0
CM/CS [2] (0.04ppm))	Hyline	81.4[ab]	98.8[bc]	61.1[a]	1.85	1.69	1
	Shaver	80.9[b]	98.8[b]	60.0[b]	1.87	1.65	1
CM/CS (0.4 ppm I)	Hyline	80.4[b]	98.9[bc]	61.5[a]	1.83	1.68	0
	Shaver	81.4[b]	97.1[b]	60.5[b]	1.81	1.65	1
Pooled SEM		0.8	0.7	0.2	0.02	0.02	
Contrasts		P-value					
Shaver vs Hyline in CM feeding		0.27	0.19	<0.05	0.95	0.04	
0.04 ppm I vs 0.4 ppm I in CM feeding		0.26	0.25	0.04	0.11	0.82	
The difference between control and CM (0.04 ppm) in Hyline vs that in Shaver		0.04	0.26	0.04	0.55	0.15	

[1] 0.04ppm and 0.4ppm: supplemental iodine level in experimental diets

[2] CM=canola meal; CS=canola seed; CM:CS is 10:1; glucosinolate composition of CM and CS are shown in Table 5 and the dietary glucosinolate content of all diets is noted in Table 23.

[3] Single degree of freedom orthogonal contrasts were performed comparing the effects of the strains (Shaver and Hyline) in CM feeding and two levels of supplemental iodine as calcium iodate (0.04 ppm and 0.4 ppm) in CM feeding; the difference between control and CM (0.04 ppm) for Shaver birds vs that for Hyline birds

[4] Birds dying of liver hemorrhage

[a-b] Means within a column with no common superscript differ significantly (P<0.05)

different from that of the control-fed birds. Birds fed the canola meal diet with 0.04 ppm supplemental iodine had significantly lower egg weight than those fed the canola meal diet with 0.4 ppm iodine regardless of strain ($P<0.05$). The differences in egg production and egg weight between birds fed the control diet and the canola meal diet (0.04 ppm iodine) were greater in the Shaver birds than that of the Hyline birds ($P<0.05$). The difference in feed intake, feed conversion efficiency and final body weight between birds fed the control diet and the canola meal diet (0.04 ppm iodine) was similar in both strains. The Hyline birds had greater final body weight than the Shaver birds for canola meal diets ($P<0.05$). However, the difference in final body weight between birds fed the control diet and the canola meal diet (0.04 ppm I) was similar in both strains.

The effects of strain (Shaver vs Hyline) and of interactions between strain and diets on physiological characteristics in canola meal feeding with two added iodine levels in the laying hen were analyzed using contrasts. The results of contrasts are shown in Table 28. Birds of both strains responded similarly to canola meal feeding with regard to relative thyroid weight, plasma T_3 and plasma T_4 ($P>0.05$). Relative liver weight of the Hyline birds when fed canola meal diets was significantly heavier than that of the Shaver birds. Birds responded similarly to two levels of supplemental iodine in canola meal feeding with regard to relative thyroid weight, plasma T_3, plasma T_4 and relative liver weight. The difference in relative thyroid weight, plasma T_3, plasma T_4 and relative liver weight between birds fed the control diet and the canola meal diet (0.04 ppm iodine) was similar for both strains.

TABLE 28. Biological effects of laying hens from Shaver and Hyline strains in canola meal feeding with two added levels of iodine over an eight-month feeding period (Experiment 5)

Diet	Strain	T_3 (nmol/L)	T_4 (nmol/L)	Thyroid (mg/ 100g BW)	Liver (g/ kg BW)
Control (0.04 ppm I)[1]	Hyline	1.0	4.9	6.5^b	24.2^b
	Shaver	0.9	5.0	6.3^b	17.8^a
CM/CS [2] (0.04 ppm I)	Hyline	0.9	5.5	11.8^a	24.5^b
	Shaver	1.0	5.2	11.6^a	19.8^a
CM/ CS (0.4 ppm I)	Hyline	1.2	6.3	12.3^a	23.2^b
	Shaver	1.3	4.2	14.5^a	19.2^a
Pooled SEM		0.1	0.5	1	0.8
Contrasts [3]		P-value			
Shaver vs Hyline in CM feeding		0.45	0.08	0.36	<0.01
0.04 ppm I vs 0.4 ppm I in CM feeding		0.09	0.81	0.11	0.2
The difference between control and CM (0.04 ppm I) in Shaver vs in Hyline		0.46	0.6	0.98	0.25

[1] 0.04ppm and 0.4ppm: supplemental iodine level in experimental diets
[2] CM=canola meal; CS=canola seed; CM:CS is 10:1; glucosinolate composition of CM and CS are shown in Table 5 and the dietary glucosinolate content of all diets is noted in Table 24.
[3] Single degree of freedom orthogonal contrasts were performed comparing the effects of the strains (Shaver and Hyline) in CM feeding and two levels of supplemental iodine as calcium iodate (0.04 ppm and 0.4 ppm) in CM feeding;
[a-b] Means within a column with no common superscript differ significantly ($P<0.05$)

5.4 DISCUSSION

In the current 8-month study, hatchability and fertility were maintained at normal levels even when breeder laying hens were fed a maximum toxicity diet with a potential for maximum production of glucosinolate hydrolysis products. These data indicated that canola meal can be used as a protein supplement without any compromise in reproductive performance of breeder laying hens. This conclusion is in agreement with other research reports. March et al. (1972) determined fertility and hatchability of eggs laid by hens from a SCWL strain in rapeseed meal feeding regimens in which the inclusion rates for rapeseed meal were 10.5% and 19.6%. The hatchability trials were done in four periods from the eggs laid by the hens at ages from 25 to 89 weeks. No adverse effects of rapeseed meal feeding regimens on fertility and hatchability were observed for the four different periods. Nassar et al. (1985) found canola meal feeding regimens did not affect fertility and hatchability adversely when canola meal partially or fully replaced soybean meal in experimental diets for hens of a SCWL strain. Kiiskinen et al. (1989) found no negative effects of canola meal feeding regimens (low-glucosinolate canola meal and high-glucosinolate canola meal both at inclusion rates of 5% and 10%) on fertility and hatchability in hens of a SCWL strain. The decreased average weight of day-old chicks hatched from eggs laid by hens fed the canola meal diets in the current study is in agreement with other reports (March et al., 1972 and Kiiskinen et al., 1989). A difference between the current study and the studies reported in the literature is that a maximal toxicity diet was involved in the current study. No adverse effects on fertility and hatchability were observed even when the maximal toxicity diet was used, which adds credence to the statement that canola meal can be used effectively as a protein

supplement in breeder laying hen diets.

In the current study the hatchability values were relatively low for all treatments and in this regard a low hatchability had been expected since the eggs for the hatchability trial came from laying hens of sixty-three weeks of age. In addition, hatchability could have been influenced by experimental procedures since chicks were removed from the hatchery for weighing and blood sampling at hatching. The door of the hatching facility was opened frequently to conduct this procedure and may have caused a loss of moisture in the hatchery which would have contributed to the observed low hatchability. It can be seen however that the general effects were similar regardless of treatment.

The increased plasmaT_4 concentration in chicks hatched from the eggs laid by the hens fed the canola meal diet with 0.4 ppm supplemental iodine and similar plasma T_3 concentration in chicks from eggs laid by hens from the three experimental treatments were in accordance with the research done by Schöne et al. (1997b). These researchers noted that serum T_4 was increased in piglets when dams were supplied with iodine in rapeseed meal and rapeseed diets while the iodine supplementation had no influence on serumT_3 in piglets. The increased plasma T_4 could also be due to a small amount of goitrogenic agents being transferred to the eggs which could have affected the thyroid hormone status of the chicks. In this regard, Elfving (1980b) reported that OZT at a low dose of 10 to 50 mg daily i.p. increased serum T_4 levels in rats.

The increased concentration of SCN in the eggs laid by the hens in canola meal feeding regimens is parallel to the studies done by Papas et al. (1979c), Laarveld et al. (1981)

and Schöne et al. (1997b) who showed that canola meal or rapeseed meal feeding increased SCN concentration in the milk of cows and sows. The source of thiocyanate ion in eggs noted in this current study was most likely of dietary origin as indicated by the analyzed SCN contents in the experimental diets.

An increase in egg SCN content and a reduction in the iodine contents of eggs and thyroids were noted in hens fed canola meal in the current study. This effect was likely due to the action of SCN as a competitive inhibitor of iodine transfer in biological systems. The reduction of iodine content in the eggs in the canola meal and canola seed feeding regimens agrees with the study done by Papas et al. (1979a) who observed a reduction in iodine content of eggs laid by hens in rapeseed meal feeding regimens. In the same study, the feeding of methyl thiocyanate (synthetic organic thiocyanate) at the rate of .33% of the diet caused a decrease in egg iodine content relative to values for a control diet. The authors surmised from these observations that the reduced iodine content in egg could result from competitive inhibition of iodine transfer into the egg by SCN. Laarveld et al. (1981) and Papas et al. (1979c) also reported that a lower iodine content in milk was associated with a higher SCN concentration in milk. Radio-tracer studies on egg iodine transfer to eggs demonstrated that rapeseed meal in breeder diets depressed egg iodine content, with high-glucosinolate rapeseed meal causing a more severe effect than a low-glucosinolate counterpart (Roos et al., 1975 and Goh et al., 1977).

The reduced iodine deposition in the thyroid of birds in canola meal feeding regimens is in agreement with the studies done in pigs (Schöne et al., 1990, 1991 and 1997a). However, the similar iodine content in the thyroid of hens fed the two canola meal diets with

two added iodine levels was in contrast to the results of the pig experiments, in which it was reported that supplemental iodine increased iodine content in the thyroid. Thiocyanate ion, derived from dietary indole glucosinolate or indirectly via the metabolism of isothiocyanates and nitriles, is known to block and reduce iodine capture by the thyroid, and thus may lead to the reduced iodine content noted in this organ.

It can be seen that there were no statistically significant changes in thyroid hormone levels (plasma T_4 and plasma T_3) of laying hens. However, a reduction in plasma T_4 was noted in day-old chicks. The thyroid was enlarged in laying hens when fed canola meal/canola seed. In addition, the results described in the previous study showed that plasma T_3 and plasma T_4 responded additively to diet glucosinolate levels indicating possible alterations of thyroid hormone levels of laying hens. These alterations of thyroid status in the laying hen may affect chick thyroid status, resulting in the observed reduction of T_4 in day-old chicks. Although canola meal feeding regimens caused a decrease in iodine content and an increase in SCN content in the eggs, these alterations did not affect reproductive performance of the laying hen. The observed thyroid enlargement in the hens did not affect fertility and hatchability of the eggs laid by these hens. In this regard, a potential problem with hatchability in breeder laying hens should not be a reason for a constraint in the dietary usage level for canola meal in breeder laying hen diets. However, more research is needed to investigate if the reduction in day-old chicks can influence future performance.

In the strain comparison component of the study canola meal feeding had a similar effect on egg production for both strains (Shaver vs Hyline). However, a difference in egg production was greater between the control diet and the canola meal diet (0.04 ppm I) in the

Shaver birds than that in the Hyline birds, indicating that an interaction between diet and strain was involved. This interaction indicated that the Shaver birds may be more sensitive to dietary glucosinolate than Hyline birds. The low mortality rate due to liver hemorrhage and reduced feed intake in both strains were consistent with the results from the previous four experiments conducted in this study. No interaction between diet and strain was involved in final body weight. However, a strain effect existed in final body weight with the Hyline birds being slightly heavier than the Shaver birds. Both the effects of interaction between diet and strain and supplemental iodine showed a significant effect on egg weight. The observed strain difference for egg weight is in agreement with a previous study of Hulan et al. (1980), in which a genotype difference was noted with regard to egg weight in rapeseed meal feeding regimens. The suggested positive effect of iodine supplementation on egg weight in canola meal feeding regimens is worthy of further study.

Birds of both strains responded to canola meal feeding in a similar way with regard to thyroid weight, plasma T_3 and plasmaT_4, but a strain effect was shown for relative liver weight. No interaction between diet and strain was evident in all the physiological parameters measured. The significant difference in relative liver weight indicated that one genotype had heavier liver than the other (Hyline> Shaver).

Overall, canola meal feeding had a similar effect on productive performance and physiological response in hens from both strains and differences noted in some parameters could be explained by inherit genetic factors. These data indicate that consideration of a strain effect is not needed when canola meal is included in laying hen diets.

Chapter VI

6.0 GENERAL DISCUSSION AND CONCLUSIONS

Experiments were conducted to evaluate the feeding value of low-glucosinolate canola meal, to elucidate and monitor physiological responses in the liver and thyroid of laying hens in relation to diet glucosinolate content and to assess the reproductive performance of breeder laying hens fed canola meal. Conventional diets comprised of canola meal and low-glucosinolate canola meal allowed for evaluation of responses to varying diet glucosinolate content and the use of non-conventional diets comprised of a combination of canola meal and canola seed as a source of active myrosinase enzyme investigated responses in the laying hen at which a maximal toxic effect of dietary glucosinolates was expected. In addition to conventional laying hen production parameters and liver hemorrhage incidence response criterion, measures of thyroid status (organ weight and plasma hormone levels) and liver detoxification activities (glutathione levels and xenobiotic enzyme levels) were included.

The overall results of four experiments indicated that the physiological responses to diet glucosinolates in the liver and thyroid of the laying hen were very dependent on the production of glucosinolate hydrolysis products in the gastrointestinal tracts of hens. Liver glutathione concentration was enhanced in response to elevated glucosinolate hydrolysis product formation and this indicated that the liver detoxification was accelerated since

glutathione is a major substrate of the liver detoxification system. During detoxification of the glucosinolate hydrolysis products active intermediate compounds may be produced which could result in cell necrosis. As the liver in the high producing laying hen is quite fragile an excessive amount of cell necrosis may become a focal point for hemorrhage and liver rupture. In this regard, liver hemorrhage could be a secondary effect of the detoxification process and hence total liver glutathione concentration can be used as a reliable predictor for liver hemorrhage development.

The physiological data along with liver hemorrhage incidence data and production performance characteristics obtained in the current study are in agreement with the recommendation of Canola Council of Canada that the maximum usage level should be restricted to the 10% level regarding the utilization of canola meal in commercial laying hen production. In addition, the positive production performance characteristics noted among birds fed the new low-glucosinolate canola meal corroborate and extend the suggestion made by the Canola Council of Canada that future varieties of canola should have a low glucosinolate content such that canola meal could be used in the diet of laying hens without the need for an upper-limit constraint. The significant linear relationship between liver total glutathione concentration and diet glucosinolate levels for the birds fed canola meal in the four experiments indicated that liver total glutathione responded additively to diet glucosinolate levels, which gives further indication that diet glucosinolate levels should be maintained at a low level. This information together with literature data suggesting that responses to dietary glucosinolates are evident at levels in the range of 0.7-1.4 μmol/g and above can be taken as a strong recommendation to the canola industry that an attempt should

be made to produce future canola varieties with lower glucosinolate content than that currently present in existing varieties. This would allow for the unconditional promotion of the use of canola meal in the diet of the laying hen at a maximum level rather than the current upper-limit recommendation of 10% of the diet.

Thyroid enlargement observed in this study is in agreement with the suggestions of reports in the literature that the goitrogenic effects observed in rapeseed/canola meal feeding regimens in various species are a consequence of the excessive intake of glucosinolate hydrolysis products. The linear relationship between diet glucosinolate levels and plasma T_3 and plasma T_4 noted in the current study indicate an increase in T_3 and a decrease in T_4 in response to increasing levels of dietary glucosinolates. These data supported the theory that in animals consuming glucosinolates the more active biological hormone form, T_3, rather than T_4 is produced in a compensatory mechanism to spare one atom of iodine.

In the study with breeder laying hens canola meal feeding resulted in an increased consumption of SCN due to the dietary contribution of glucosinolate derived SCN. An increase in egg SCN content and a reduction in the iodine contents of eggs and thyroids were noted in hens fed canola meal in the current study. This effect was likely due to the action of SCN as a competitive inhibitor of iodine transfer in biological systems. Hatchability and fertility were maintained at a normal level in the breeder laying hens fed canola meal diets despite the SCN induced responses. However, reduction in body weight and plasma T_4 of day-old chicks hatched from the eggs laid by hens fed canola meal were noted with the former effects counteracted by iodine supplementation. It can be argued from the data that canola meal can be effectively used as a protein supplement in the diet of breeder laying hens.

Chapter VII

7.0 REFERENCES

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Chapter VIII

8.0 APPENDIXES

Appendix 1. ANOVA for productive parameters in the evaluation of low-glucosinolate canola meal in the laying hen (Experiment 1)

Egg production

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	96	4593.7559	47.851624	7.55	<.0001
Error	303	1920.2085	6.337322		
Corrected Total	399	6513.9644			

Source	DF	Type III SS	Mean Square	F-Value	Pr > F
Diet	4	101.983176	25.495794	4.02	0.0034
Pen(Diet)	72	3491.7265	48.496201	7.65	<.0001
Period	4	896.79065	224.197662	35.38	<.0001
Diet*Period	16	97.27385	6.079616	0.96	0.5016

Tests of hypotheses using the Type III MS for Pen(Diet) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	4	101.9831759	25.495794	0.53	0.7171

Appendix 2. ANOVA for productive parameters in the evaluation of low-glucosinolate canola meal in the laying hen (Experiment 1)

Feed intake

Source	DF	Sum of Squares	Mean Square	F Value	Pr >F
Model	96	2488.344975	25.92026	10.77	<0.0001
Error	303	729.411	2.407297		
Corrected Total	399	3217.755975			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	4	428.183297	107.045824	44.47	<.0001
Pen(Diet)	72	1184.114625	16.446036	6.83	<.0001
Period	4	788.70585	197.176463	81.91	<.0001
Diet*Period	16	78.15615	4.884759	2.03	0.0115

Tests of hypotheses using the Type III MS for Pen(Diet) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	4	428.183297	107.0458242	6.51	0.0002

Appendix 3. ANOVA for productive parameters in the evaluation of low-glucosinolate canola meal in the laying hen (Experiment 1)

Egg weight

Source	DF	Sum of Squares	Mean Square	F Value	Pr >F
Model	57	940.995525	16.508693	24.8	<0.0001
Error	142	94.54317	0.665797		
Corrected Total	199	1035.538696			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	4	27.9047321	6.976183	10.48	<.0001
Pen(Diet)	33	699.6710675	21.2021536	31.84	<.0001
Period	4	214.130608	53.532652	80.4	<.0001
Diet*Period	16	2.487432	0.1554645	0.23	0.9991

Tests of hypotheses using the Type III MS for Pen(Diet) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	4	27.90473206	6.97618301	0.33	0.8564

Appendix 4. The result of ANOVA of productive parameters of laying hens fed canola meal diets with graded levels of glucosinolates over five 28-day periods (Experiment 2)

Egg production

Source	DF	Sum of Squares	Mean Square	F Value	Pr >F
Model	124	7269.67467	58.62641	5.69	<0.001
Error	525	5409.62379	10.30405		
Corrected Total	649	12679.29846			

Source	DF	Type III SS	Mean Square	F-Value	Pr > F
Diet	4	224.859441	56.21486	5.46	0.0003
Pen(Diet)	100	4665.310051	46.653101	4.53	<.0001
Period	4	2307.820308	576.955077	55.99	<.0001
Diet*Period	16	95.827231	5.989202	0.58	0.8987

Tests of hypotheses using the Type III MS for Pen(Diet) as an error term

Source	DF	Type III SS	Mean Square	F-value	Pr > F
Diet	4	224.859441	56.2148602	1.2	0.3135

Appendix 5. The result of ANOVA of productive parameters of laying hens fed canola meal diets with graded levels of glucosinolates over five 28-day periods (Experiment 2)

Feed intake

Source	DF	Sum of Squares	Mean Square	F Value	Pr >F
Model	124	5698.707451	45.957318	7.13	<0.001
Error	525	3385.571333	6.448707		
Corrected Total	649	9084.278785			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	4	323.199644	80.799911	12.53	<.0001
Pen(Diet)	100	2928.809051	29.288091	4.54	<.0001
Period	4	2138.389785	534.597446	82.9	<.0001
Diet*Period	16	349.574215	21.848388	3.39	<.0001

Tests of hypotheses using the Type III MS for Pen(Diet) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	4	323.1996436	80.7999109	2.76	0.0318

Appendix 6. The result of ANOVA of productive parameters of laying hens fed canola meal diets with graded levels of glucosinolates over five 28-day periods (Experiment 2)

Egg weight

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	55	820.925622	14.9259204	28.03	<.0001
Error	144	76.68205	0.5325142		
Corrected Total	199	897.607672			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	4	81.642406	20.4106015	38.33	<.0001
Pen(Diet)	31	138.866725	4.4795718	8.41	<.0001
Period	4	603.812927	150.9532318	283.47	<.0001
Diet*Period	16	6.034253	0.3771408	0.71	0.7822

Tests of hypotheses using the Type III MS for Pen(Ration) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	4	81.64240601	20.4106015	4.56	0.0052

Appendix 7. The result of ANOVA of productive parameters of laying hens fed diets containing graded levels of glucosinolates over four 28-day periods (Experiment 3)

Egg production

Source	DF	Sum of Squares	Mean Square	F Value	Pr >F
Model	34	0.16305513	0.00479574	9.96	<0.001
Error	45	0.02166163	0.00048137		
Corrected Total	79	0.18471675			

Source	DF	Type III SS	Mean Square	F-Value	Pr > F
Diet	4	0.01914337	0.00478584	9.94	<.0001
Pen(Diet)	15	0.07503888	0.00500259	10.39	<.0001
Period	3	0.06162605	0.02054202	42.67	<.0001
Diet*Period	12	0.00724682	0.0006039	1.25	0.2782

Tests of hypotheses using the Type III MS for Pen(Ration) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	4	0.01914337	0.00478584	0.96	0.4592

Appendix 8. The result of ANOVA of productive parameters of laying hens fed diets containing graded levels of glucosinolates over four 28-day periods (Experiment 3)

Egg weight

Source	DF	Sum of Squares	Mean Square	F Value	Pr>F
Model	34	116.1300923	3.415591	4.85	<.0001
Error	45	31.6932682	0.7042948		
Corrected Total	79	147.8233605			

Source	DF	Type III SS	Mean Square	F-Value	Pr > F
Diet	4	30.16286818	7.54071704	10.71	<.0001
Pen(Diet)	15	37.88498806	2.52566587	3.59	0.0004
Period	3	41.18781374	13.72927125	19.49	<.0001
Diet*Period	12	6.89442233	0.57453519	0.82	0.6333

Tests of hypotheses using the Type III MS for Pen(Diet) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	4	30.16286818	7.54071704	2.99	0.0535

Appendix 9. The result of ANOVA of productive parameters of laying hens fed diets containing graded levels of glucosinolates over four 28-day periods (Experiment 3)

Feed intake

Source	DF	Squares	Mean Square	F Value	Pr >F
Model	34	1072.798455	31.552896	4.62	<0.001
Error	45	307.2709	6.828242		
Corrected Total	79	1380.069355			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	4	207.177805	51.7944512	7.59	<.0001
Pen(Diet)	15	387.72595	25.8483967	3.79	0.0003
Period	3	373.134505	124.3781683	18.22	<.0001
Diet*Period	12	104.760195	8.7300162	1.28	0.2641

Tests of hypotheses using the Type III MS for Pen(Diet) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	4	207.177805	51.7944512	2	0.1457

Appendix 10. ANOVA of productive parameters of laying hens fed diets containing varying glucosinolate content over eight 28-day periods (Experiment 4)

Egg production

Source	DF	Sum of Squares	Mean Square	F Value	Pr >F
Model	81	1.60856751	0.01985886	12.88	
Error	558	0.86065943	0.0015424		
Corrected Total	639	2.46922693			

Source	DF	Type III SS	Mean Square	F-Value	Pr > F
Diet	2	0.14869091	0.07434545	48.2	<.0001
Pen(Diet)	58	0.47464746	0.00818358	5.31	<.0001
Period	7	0.93983956	0.13426279	87.05	<.0001
Diet*Period	14	0.03219378	0.00229956	1.49	0.1093

Tests of hypotheses using the Type III MS for Pen(Diet) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	2	0.14869091	0.07434545	9.08	0.0004

Appendix 11. ANOVA of productive parameters of laying hens fed diets containing varying glucosinolate content over eight 28-day periods (Experiment 4)

Feed intake

Source	DF	Sum of Squares	Mean Square	F Value	Pr >F
Model	81	8769.10805	108.26059	14.41	
Error	558	4193.01754	7.51437		
Corrected Total	639	12962.12559			

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	2	1728.709727	864.354864	115.03	<.0001
Pen(Diet)	58	2483.088602	42.811872	5.7	<.0001
Period	7	3577.420417	511.06006	68.01	<.0001
Diet*Period	14	630.940927	45.067209	6	<.0001

Tests of hypotheses using the Type III MS for Pen(Diet) as an error term

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Diet	2	1728.709727	864.354864	20.19	<.0001