Chapter 6.

Effects of Gramine and Hordenine on Broiler Chickens

6.1. Introduction

In the previous experiment (see Chapter 5), mice were provided with feed containing different levels of gramine and hordenine. The feed containing 500 ppm gramine was consumed the least and this was significantly different from the group given standard feed. However, the body weight means were not significantly different between the treatment groups. In the experiment described in Chapter 6, broiler chickens were given feed containing the alkaloids gramine and hordenine (same concentrations as for the previous experiment). The objective of this experiment was to examine the effect of gramine and hordenine on broiler chickens with the expectation that they would provide a better model for the study of the role of allelochemicals as self-defence agents in barley.

6.2. Materials and Methods

6.2.1. Experimental Design

There were five experimental groups and six replications in a Completely Randomised Design which was conducted from December 1994 to January 1995 at the animal house complex of the University of New England. Six chickens were placed in each cage, making 36 chickens per treatment and a total of 180 chickens for the experiment. The treatments were different concentrations (w/w) of gramine and hordenine which were incorporated into feed as follows:

A = feed containing 50 ppm hordenine
B = feed containing 500 ppm hordenine
C = feed containing 50 ppm gramine
D = feed containing 500 ppm gramine
E = standard feed as a control group

Two hundred one-day-old broiler chickens, both male and female, were reared in the brooder (see Section 3.4.2). Only 180 chickens were used for the experiment (see Section 3.4.2).

6.2.2. Data Collection

Body weight and feed intake were recorded every week. Chickens were carefully caught by holding their legs and placing them in a small bucket, on a scale. Feed intake was recorded as the amount of feed taken during the whole week by subtracting the leftover feed from the total amount of feed given in the feeder for the week. General observations on the chickens and the cages were made every day. At the end of the experiment (seven weeks of age), liver tissue was collected from chickens as described in Section 3.5. The determination of gramine and hordenine in the liver was undertaken using the procedure of Hoult and Lovett (1993) as described in Section 3.6. Twelve livers from each treatment group were subjected to HPLC analysis.

6.2.3. Histological Examination

Four chickens were selected to represent each treatment group according to their body weight. Those which had the lowest and highest body weight, both male and female, were subjected for histological examination following the procedure described in Section 3.7.

6.3. Results

6.3.1. Body Weight

Body weights of broiler chickens increased significantly over the experimental period ($P < 0.0001$) in all treatment groups (Figure 6.1, Table 6.1). Although there were no significant differences between treatment groups, there was a significant interaction between treatment and age (P = 0.001). The mean weekly body weights are presented in Figure 6.1.

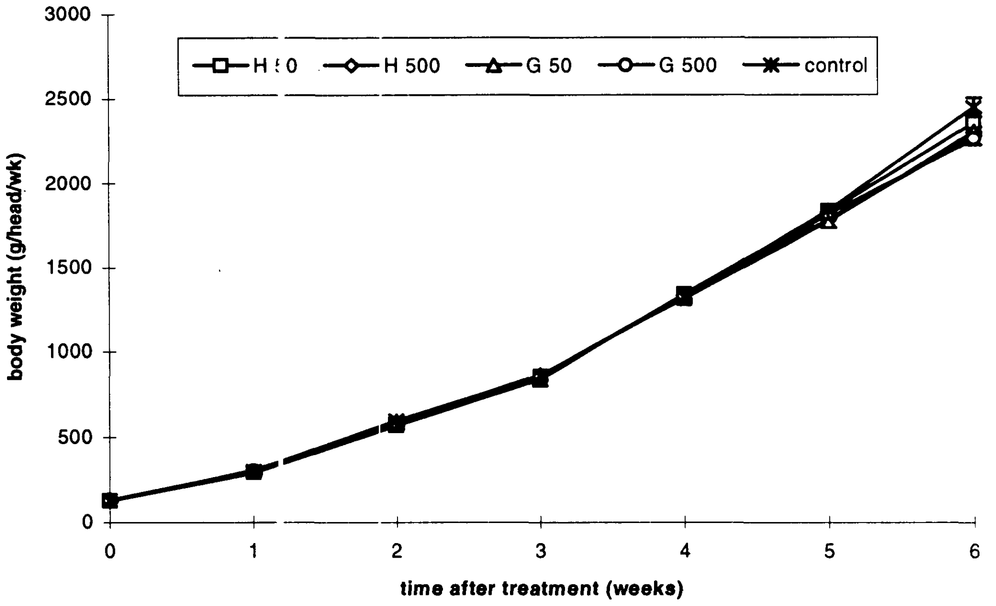


Figure 6.1. Body weight of broiler chickens over seven weeks of experimental period

(Values are Mean ± SEM, H 50 and H 500 = feed containing 50 and 500 ppm hordenine respectively, G 50 and G 500 = feed containing 50 and 500 ppm gramine respectively, control = no gramine and hordenine)

The group of chickens receiving feed containing 500 ppm gramine tended to achieve the lowest final body weight whereas the group receiving control feed had a tendency to be highest at the end of the experiment. However, all treatment groups tended to have very similar body weights throughout the experimental period, except for the last time of data collection. By the end of the experimental period there was a significant difference (P = 0.048) between the control group and the group receiving feed containing 500 ppm gramine. Data for weekly body weight of each treatment group are presented in Table 6..

Table 6.1. Body weights of chickens (g) receiving different levels of alkaloids

Group	Week after treatment						
	0	1	2	3	4	5	6
H 50	125.9^{a} ± 5.42	293.6^{a} ± 14.9	568.2^{a} ± 22.3	838.0^{a} ± 35.0	1340.1^{a} ± 34.4	1832.1^{a} ± 39.3	2356.8^{ab} ± 56.0
H 500	129.5^{a} ± 5.48	306.1^{a} ± 13.1	589.3^{a} ± 18.7	863.2^{a} ± 20.8	1332.5^{a} ± 16.4	1786.6^{a} ± 17.4	2276.9^{ab} ± 36.3
G 50	127.8^{a} ± 4.91	305.8^{a} ± 13.4	591.1^{a} ± 17.9	867.9^{a} ± 27.9	1314.7^{a} ± 27.5	1769.8^{a} ± 33.1	2305.0^{ab} ± 27.1
G 500	127.7^{a} ± 4.81	301.7^{a} ± 13.6	581.0^{a} ± 22.2	858.6^{a} ± 28.5	1326.2^{a} ± 34.5	1816.4^{a} ± 31.6	2260.0^{b} ± 42.4
Control	126.1^{a} ± 4.69	302.6^{a} ± 11.8	597.1^{a} ± 17.7	859.1^{a} ± 27.8	1325.2^{a} ± 33.8	1840.9^{a} ± 31.5	2446.6^{a} ± 56.3

(Values are Mean ± SEM, Values within a column with different superscripts are significantly different from one another. H 50 and H 500 = feed containing 50 and 500 ppm hordenine respectively, G 50 and G 500 = feed containing 50 and 500 ppm gramine respectively, control = no hordenine and gramine)

6.3.2. Feed Intake

The means for total feed intake of every chicken for each treatment group are presented in Figure 6.2 and Appendix M. Feed intake was not significantly different between treatment groups and there was no significant interaction between treatment and age. However, feed intakes differed significantly over the experimental period ($P < 0.0001$).

Feed intakes of all treatment groups fluctuated throughout the experimental period. Consistent with body weight values, feed containing 500 ppm gramine tended to be consumed the least.

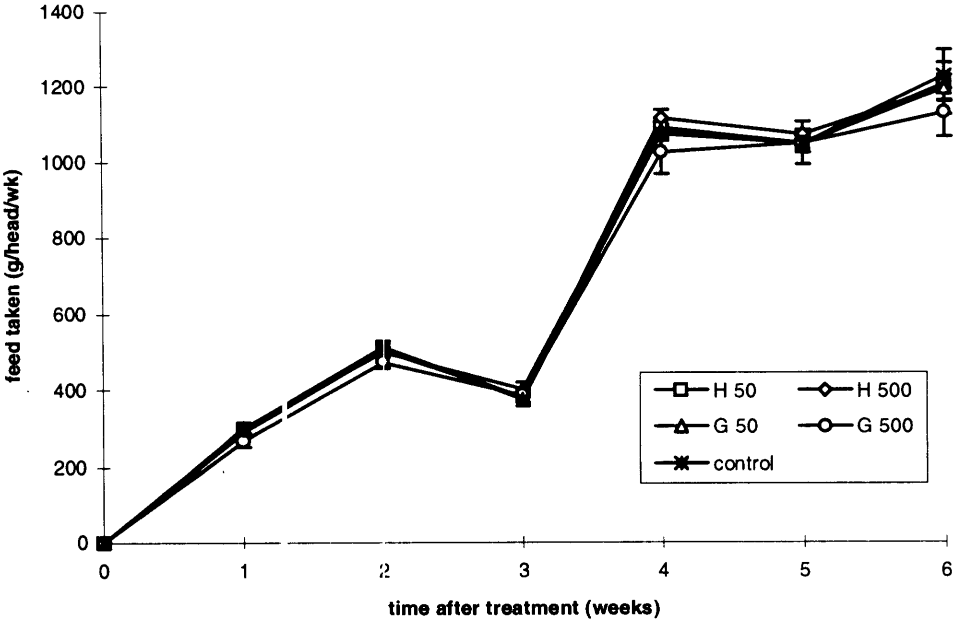


Figure 6.2. Chicken feed ingested over seven weeks of age

(Values are Mean ± SEM, H 50 and H 500 = feed containing 50 and 500 ppm hordenine respectively, G 50 and G 500 = feed containing 50 and 500 ppm gramine respectively, control = no gramine and hordenine)

6.3.3. Liver Weight

The mean values for liver weight of chickens receiving different levels of gramine and hordenine incorporated into feed are presented in Figure 6.3 and Appendix K. There was no significant difference between treatment groups, although liver weight tended to be lowest in the 500 ppm hordenine group.

6.3.4. HPLC Analysis of Liver

The mean content of gramine and hordenine in livers, recovered by HPLC analysis, are presented in Table 6.2. For each treatment group, the number of livers in which alkaloid was detected, out of 12 livers tested, is indicated in brackets after the mean.

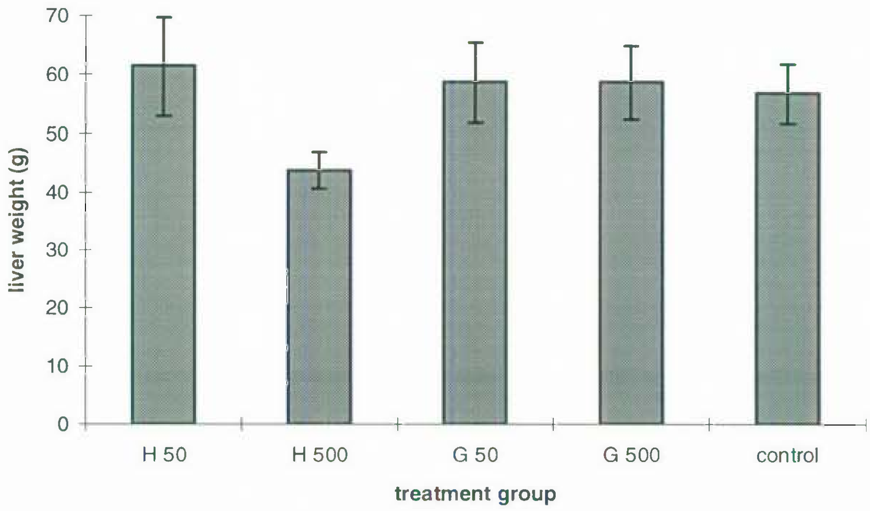


Figure 6.3. Liver weight of chickens at seven weeks of age

(Values are Mean ± SEM, H 50 and H 500 = feed containing 50 and 500 ppm hordenine respectively, G 50 and G 500 = feed containing 50 and 500 ppm gramine respectively, control = no gramine and hordenine)

All treatment groups receiving alkaloid containing feed resulted in the recovery of both alkaloids through HPLC analysis in at least some livers. Levels of gramine and hordenine in the liver varied between treatment groups. The group receiving 500 ppm hordenine demonstrated the highest level of recovered alkaloid from the livers.

6.3.5. Feed Conversion Ratio

Feed conversion ratio is the ratio of total feed ingested over the body weight gained. Different concentrations of the alkaloids gramine and hordenine presented to the chickens of this experiment did not significantly affect the feed conversion ratio (P = 0.330). The means of feed conversion ratio are presented in Figure 6.4 and Appendix N. There was a tendency for chickens receiving feed containing 500 ppm gramine to have the lowest feed conversion ratio value (1.94) whereas chickens given control feed tended to be highest (2.05).

Table 6.2. Gramine and Hordenine Content of Chicken Liver (μg/g fresh wt.)

Treatment Group	Mean (n)* ± SEM
Feed containing hordenine 50 ppm	2.82 (n=7) ± 0.42
Feed containing hordenine 500 ppm	3.09 (n=11) ± 0.81
Feed containing gramine 50 ppm	0.28 (n=2) ± 0.05
Feed containing gramine 500 ppm	2.89 (n=8) ± 0.85
Standard feed	Not Detectable

* n = number of livers showing detectable levels

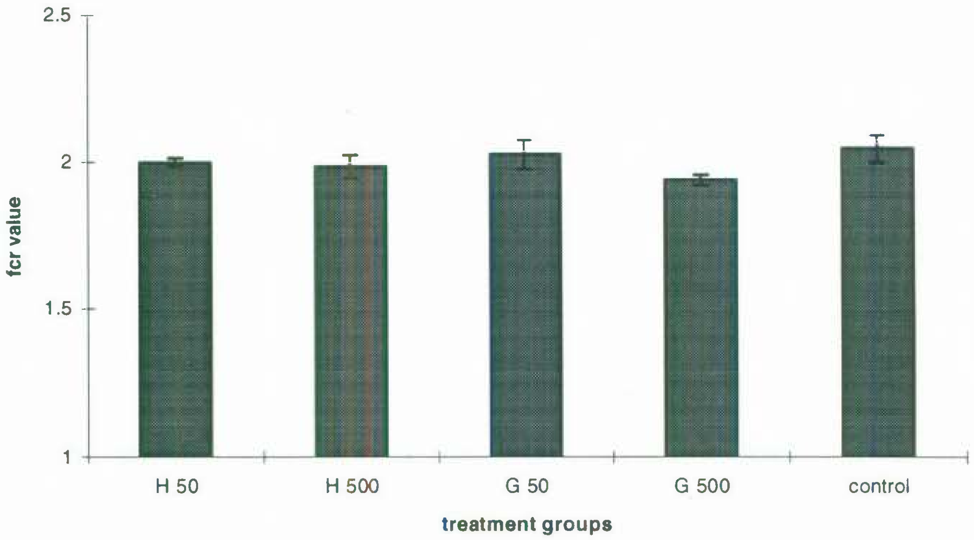


Figure 6.4. Feed conversion ratio of chickens receiving different levels of alkaloids

(Values are Mean ± SEM, H 50 and H 500 = feed containing 50 and 500 ppm hordenine respectively, G 50 and G 500 = feed containing 50 and 500 ppm gramine respectively, control = no gramine and hordenine)

6.3.6. Histological Examination

The livers of chickens exposed to low concentration (50 ppm) of gramine and hordenine did not show any abnormalities in the liver tissues. However, there was a difference between normal tissue from the group given standard feed and tissue from groups receiving high concentrations (500 ppm) of gramine or hordenine. Most of the samples of tissue taken from these groups showed vacuolation of the cells of the liver. Micrographs of liver tissue are presented in Plate 6.1.

6.3.7. Additional Observation

The only visible difference betwen treatment groups of both chicken experiment was that the chicken receiving 500 ppm gramine in the feed showed "scab-like" lesions on their feet during approximately weeks 4 and 5 of the experiment (Plate 6.2).

6.4. Discussion

Even though the body weight of chickens increased significantly with age in all treatment groups it was not significantly affected by alkaloids incorporated into the feed. At the end of the experiment (seven weeks of age), however, the body weight tended to be different (P = 0.048) between the group receiving standard feed (2446.6 g) and the group receiving feed containing 500 ppm gramine (2260.0 g). This finding agreed with other work that demonstrated the effects of plant secondary metabolites on the growth of chicks.

For example, Vohra *et al.* (1966) showed that gain in weight of chickens decreased as the tannic acid content of the diet was increased up to 4% level. Similarly, when grain sorghum was used in the ration of growing chickens at levels of 28 - 63%, a growth depression up to 50% occurred due to tannin content of sorghum (Chang and Fuller 1964). Other workers demonstrated that the herbaceous legume *Indigofera spicata* Forsk markedly depressed growth and induced symptoms of toxication which was shown as paralysis of legs and wings, or both (Rosenberg and Zoebisch 1952) and this was closely associated with amount of 3-nitropropanoic acid in the plant (Britten, Palafox, Frodyma and Lynd 1963).

The non significant effect of gramine and hordenine on the growth of chicks might be due to a very low concentration of both alkaloids incorporated into diet. Therefore, it is assumed that the levels given were not enough to depress chicken growth rate. In addition, the effects of alkaloids are dependent upon the age and state of health of an animal and the length of time that the animal has been exposed to the alkaloids. Fuller, Chang and Potter (1967) found that the growth of chickens was depressed significantly at the 1.0% level of tannic acid. Another study in which the alkaloid coniine was administered to chickens showed that morbidity in chickens dosed at 50 mg coniine/kg body weight was 90% (Frank and Reed 1990).

In terms of feed intake, the concentration of alkaloids might not be enough to affect the amount of feed taken by the chickens, although the treatment group receiving 500 ppm gramine tended to consume the least. In addition, the treatments given in this experiment did not affect feed conversion ratio. Feed consumption of chickens decreased as the level of tannic acid increased in their diets (Vohra *et al.* 1966). Similarly, at a level of 2% supplementation to poultry rations, gallotannins depressed chicken feed intake (Chang and Fuller 1964). Potter and Fuller (1968) reported that 1% of tannic acid in diet was equivalent to that occurring in the high tannin grain sorghum. However, a level of 5% tannic acid caused chick mortality of about 70% between 7 and 11 days on the experiment (Vohra *et al.* 1966).

As shown in Figure 6.2, the feed intake of all chickens increased up to the end of week 2, then decreased slightly by the end of week 3. This pattern of feed intake is probably related to the fact that chickens were moved from the large brooder to the metabolism cages at the end of week 2. The large change in feed intake between weeks 3 and 4 is associated with the change of feed at the end of week 3 from chick starter feed to broiler finisher feed. Over the last two weeks of the experiment, feed intake increased only slightly.

Plate 6.1. Micrographs of livers from chickens receiving different levels of gramine and hordenine incorporated into feed. Magnification 400x.

A = 50 ppm gramine
B = 50 ppm hordenine
C = 500 ppm gramine
D = 500 ppm hordenine
E = control

Arrow heads indicate some vacuoles

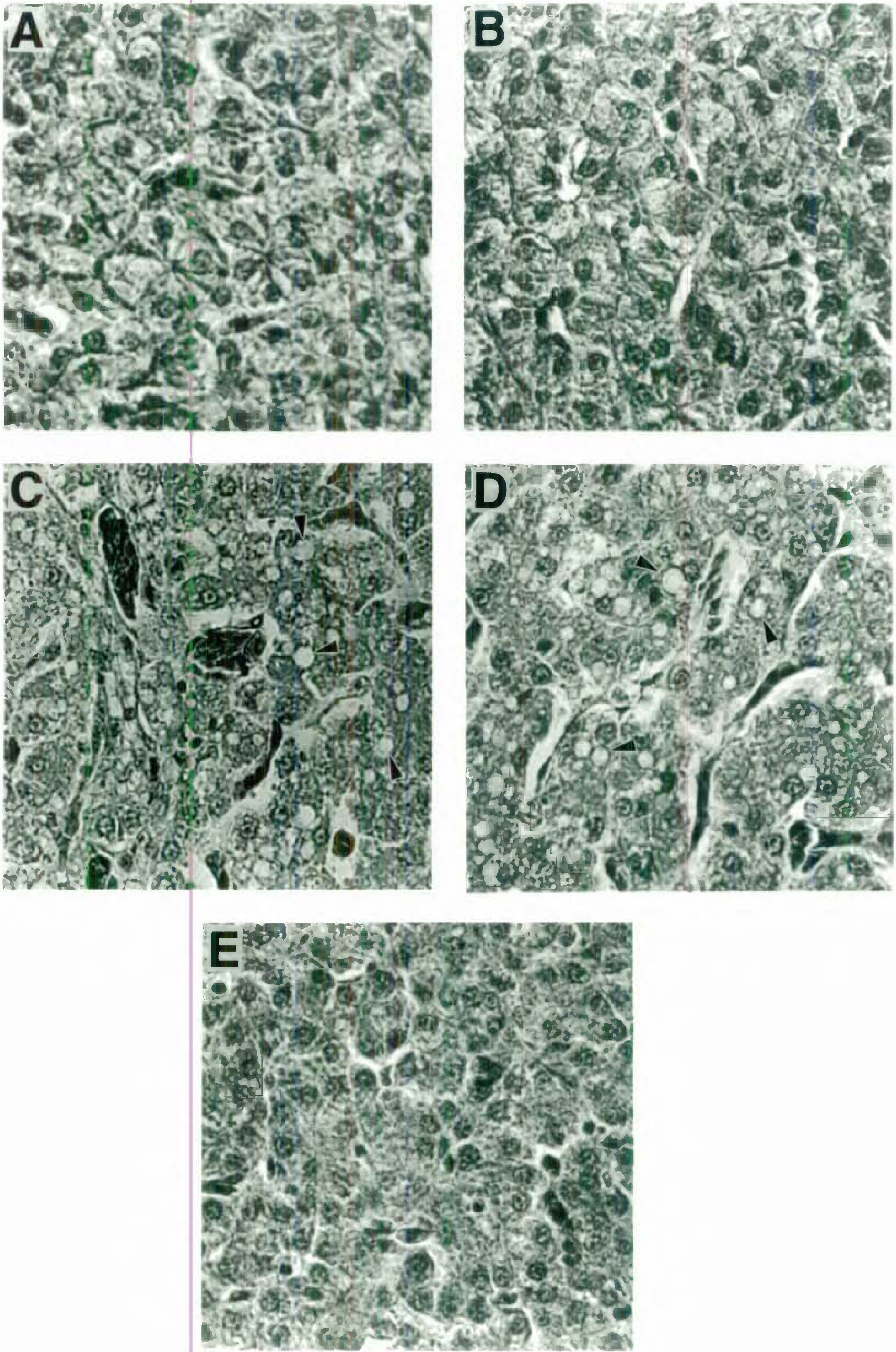

A
B
C
D
E

Plate 6.2. Lesions on the feet of chickens receiving 500 ppm gramine in feed during weeks 4 and 5 of the experiment (experiment described in both Chapter 6 and 7).

Liver weight was not significantly different between treatment groups although the mean liver weight was lowest for the 500 ppm hordenine group. However, the amount of alkaloids recovered were associated with the treatment concentrations. The HPLC analysis indicates that the higher the concentration of alkaloids in feed, the higher the content of the livers. Histological examination showed that high concentrations of alkaloids caused liver damage as indicated by many vacuoles in the liver tissue. This finding shows that both gramine and hordenine at high concentration affected broiler chickens as indicated by liver abnormalities.

Microscopic examination of liver tissue clearly showed an abundance of vacuoles distributed evenly. This might be true neutral fat (Smith and Jones 1958) and shown as empty, clear, or almost clear, unstained, round spaces in ordinary tissue section. This phenomenon is called fatty degeneration and the fat appears as droplets in the cytoplasm of the epithelial cells.

This finding agreed with the work of Lovett *et al.* (1989) who demonstrated the effects of the allelochemical benzylamine on linseed (*Linum usitatissimum* L.). These workers found that an increase of benzylamine concentration caused increases in number and size of vacuoles in linseed root tips. Levitt, Lovett and Garlick (1984) showed similar effects of plant secondary metabolites of *Datura stramonium* on root tip cells of *Helianthus annuus*.

Similarly, indospicine which was isolated from seed and leaf of *Indigofera spicata* Forsk, produced characteristic liver lesions in mice (Hegarty and Pound 1968). Indospicine caused accumulation of fat in the liver (Hegarty and Pound 1968) and liver degeneration (Hutton, Windrum and Kratzing 1958). In contrast, canavanine and arginine were also isolated from *I. spicata* Forsk but did not cause fat accumulation in the liver. Hepatic damages due to pyrrolizidine alkaloid was demonstrated by Peterson and Jago (1984) who found that 0.06% of the alkaloid caused a 70% mortality in young rats. They concluded that this death was due to acute haemorrhagic necrosis and chronic liver damage.

Consistent with the finding of the previous experiment (described in Chapter 5), the experiment using broiler chickens resulted in a tendency for lowest feed intake and body weight to be found in the treatment group receiving 500 ppm gramine.

Germinating barley produces both gramine and hordenine. Therefore it is important to examine any possible synergistic effects of the two alkaloids as part of plant defence against animals. The study in which combinations of gramine and hordenine were incorporated into feed and presented to broiler chickens will be discussed in Chapter 7.

Chapter 7

Synergistic Effects of Gramine and Hordenine on Broiler Chickens

7.1. Introduction

As outlined in Chapter 2, chemicals may be more effective as crop protectant agents if used in combination rather than separately. This synergistic effect has been described for some allelochemicals (Pellessier 1993). It was decided to investigate possible synergistic effects of the two allelochemicals gramine and hordenine. Growing broiler chickens were used as a model for this investigation with the expectation that it could provide information on the use of these allelochemicals as self-defence agents in barley.

7.2. Materials and Methods

The Completely Randomised Design, which incorporated five experimental groups and six replications was conducted from May to June 1995 at the animal house complex of the University of New England. Four chickens were placed in every cage, making 24 chickens per treatment and a total of 120 chickens for all treatments. The treatments were combinations of different concentrations of gramine and hordenine in the feed as follows:

A = standard feed as a control group
B = feed containing 500 ppm gramine
C = feed containing 500 ppm hordenine
D = feed containing 500 ppm hordenine + 50 ppm gramine
E = feed containing 500 ppm gramine + 50 ppm hordenine

One hundred and sixty one-day-old broiler chickens, both male and female, were reared in a brooder (see Section 3.4.2). The procedure of feed preparation was conducted as described in Section 3.3.2 and chickens were maintained as described in Section 3.4.2.

The collection of data for body weight and feed intake was conducted every week, following the same procedure as described in Section 6.2.2. At the end of the experiment, all chickens were euthanased with CO_2 gas and the livers removed. The livers were then subjected to HPLC analysis for the detection of alkaloids and histological examination to assess any changes (as described in Sections 6.2.2 and 6.2.3).

7.3. Results

7.3.1. Body Weight

Statistical analysis of data indicated that the body weight of chickens increased significantly over the experimental period ($P < 0.0001$) in all treatment groups (Figure 7.1). However, there was no significant difference between treatment groups and no significant interaction between treatment and age. The group of chickens receiving feed containing 500 ppm gramine had a tendency to achieve the lowest body weight whereas chickens from the control group tended to be highest at the end of the experiment (see Appendix O).

7.3.2. Feed Intake

Feed intake changed significantly over the experimental period in all treatment groups ($P < 0.0001$). However, there was no significant difference between treatment groups and there was no significant interaction between treatment and age. The mean weekly feed intakes of chickens for each treatment group are presented in Figure 7.2.

The chickens from the control group tended to consume the highest amount of feed whereas the group receiving feed containing 500 ppm gramine tended to consume the least (4756.1 g and 4502.3 g respectively) (see Appendix P).

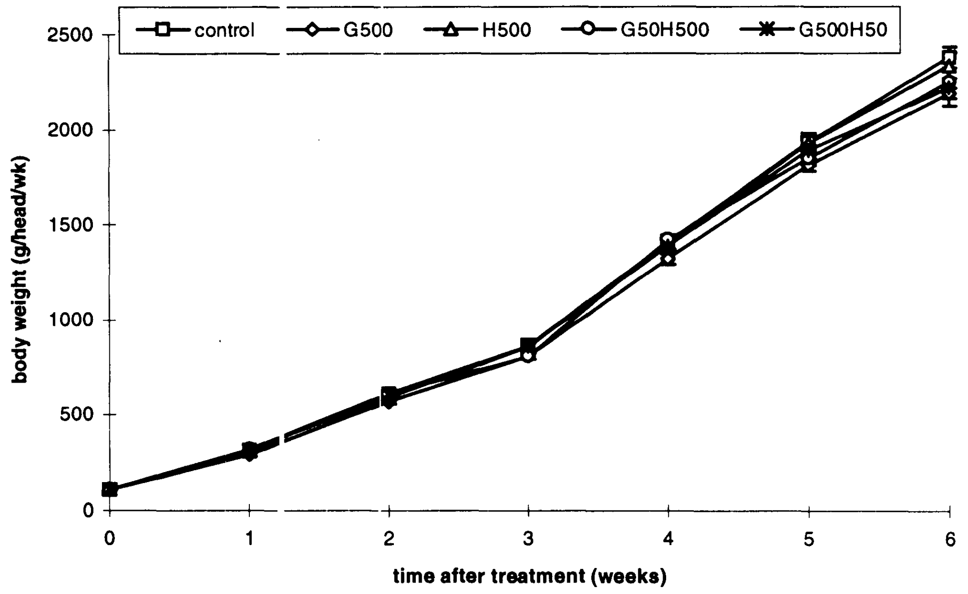


Figure 7.1. Body weight of broiler chickens over seven weeks of age

(Values are Mean ± SEM, G 500 and H 500 = feed containing 500 ppm gramine and hordenine respectively, G50H500 = feed containing combination of 50 ppm gramine and 500 ppm hordenine, G500H50 = feed containing combination of 500 ppm gramine and 50 ppm hordenine control = no gramine and hordenine)

7.3.3. Liver Weight

There were significant differences in liver weights between the treatment groups (P = 0.002) (Figure 7.3, Table 7.1). The chickens receiving the combination of 500 ppm gramine and 50 ppm hordenine had significantly lower liver weights than all other treatment groups.

7.3.4. HPLC Analysis of Liver

Table 7.2 demonstrates the mean content of gramine and hordenine in chicken livers after receiving alkaloid-treated feed for 6 weeks. For each treatment group, the number of livers in which alkaloid was detected, out of 12 livers tested, is indicated in brackets after the mean. All groups receiving alkaloid-containing feed showed the

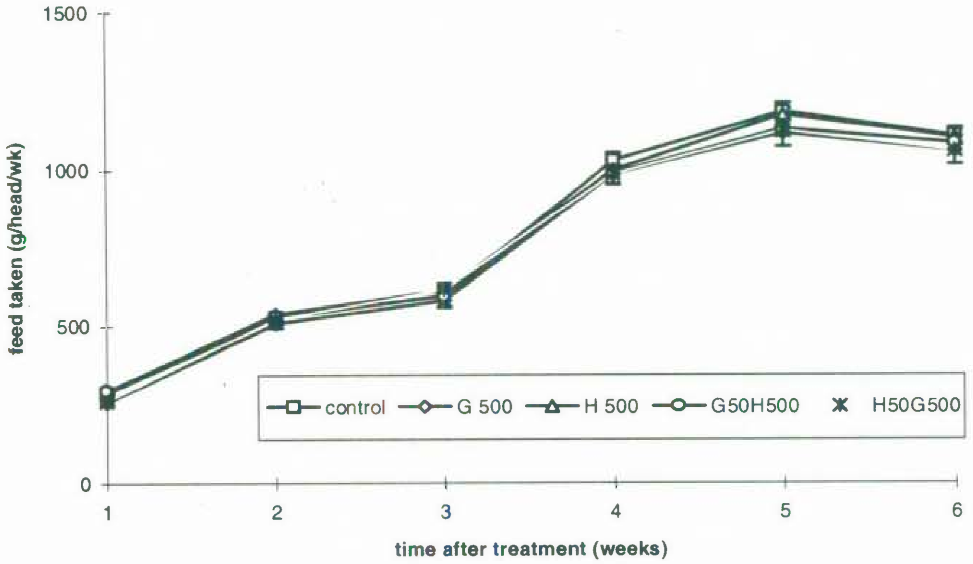


Figure 7.2. Chicken feed intake over the experimental period

(Values are Mean ± SEM, G 500 and H 500 = feed containing 500 ppm gramine and hordenine respectively, G50H500 = feed containing combination of 50 ppm gramine and 500 ppm hordenine, H50G500 = feed containing combination of 50 ppm hordenine and 500 ppm gramine, control = no gramine and hordenine)

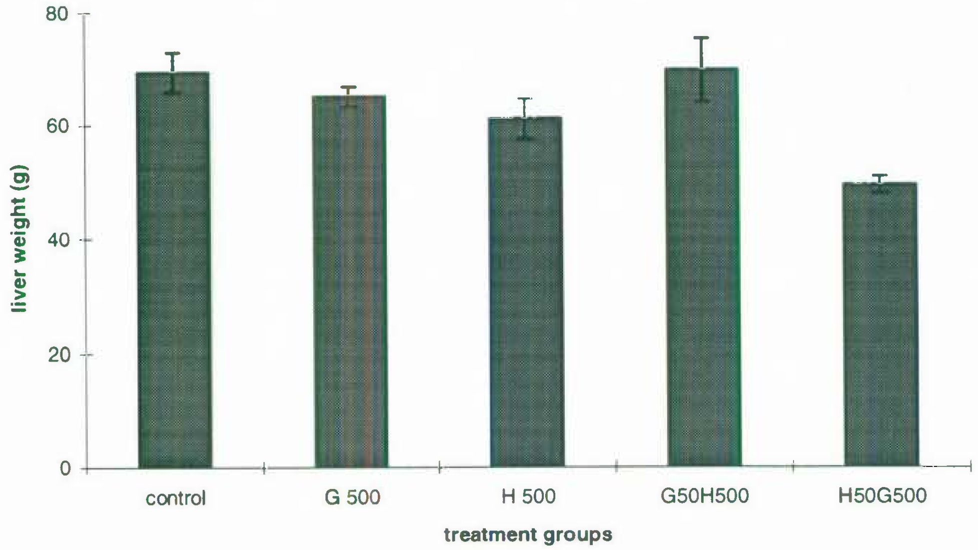


Figure 7.3 Liver weight of chickens at seven weeks of age

(Values are Mean ± SEM, G 500 and H 500 = feed containing 500 ppm gramine and hordenine respectively, G50H500 = feed containing combination of 50 ppm gramine and 500 ppm hordenine, H50G500 = feed containing combination of 50 ppm hordenine and 500 ppm gramine, control = no gramine and hordenine)

Table 7.1. Liver weights (g) of broiler chickens at seven weeks of age

Treatment group				
Control	G 500	H 500	G50H500	H50G500
69.30^a ± 3.51	64.93^a ± 1.85	60.90^a ± 3.56	69.54^a ± 5.70	49.21^b ± 1.54

(Values are Mean ± SEM, Values with the same letters are not significantly different one another, G 500 and H 500 = feed containing 500 ppm gramine and hordenine respectively, G50H500 = feed containing combination of 50 ppm gramine and 500 ppm hordenine, H50G500 = feed containing combination of 50 ppm hordenine and 500 ppm gramine, control = no gramine and hordenine)

recovery of either alkaloid through HPLC analysis in at least some livers. For the group receiving 500 ppm gramine plus 50 ppm hordenine, levels of hordenine in the livers were undetectable. Similarly, gramine was not detected in the livers from the 500 ppm hordenine plus 50 ppm gramine group.

Table 7.2. Gramine and Hordenine Content of Chicken Liver (µg/g fresh wt.)

Treatment Group	Mean (n)* ± SEM
Gramine 500 ppm	4.25 (n=11) ± 0.63
Hordenine 500 ppm	3.27 (n=6) ± 0.58
Hordenine 500 ppm +Gramine 50 ppm (hordenine content)	4.92 (n=12) ± 0.52
Gramine 500 ppm + Hordenine 50 ppm (gramine content)	3.61 (n=9) ± 0.79
Control	Not Detectable

* n = number of livers showing detectable levels

7.3.5. Feed Conversion Ratio

Feed conversion ratio was not significantly different between treatment groups. The mean feed conversion ratios are presented in Figure 7.4 and Appendix Q.

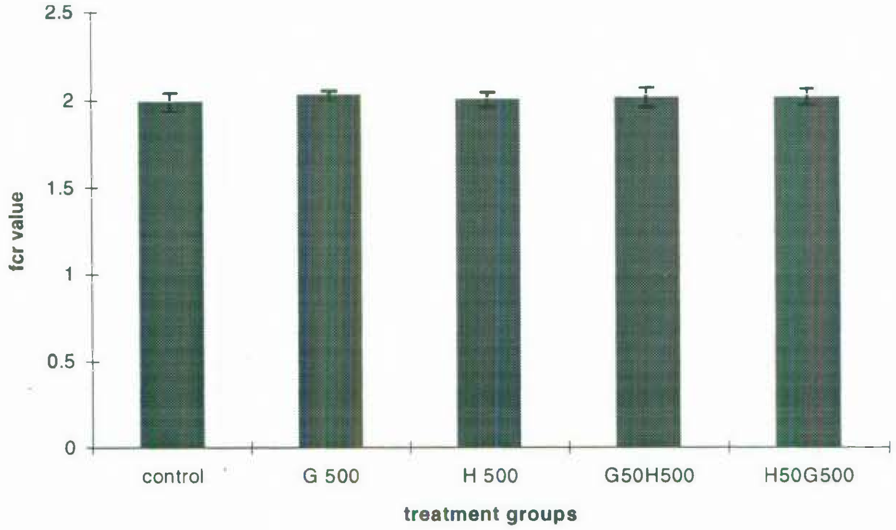


Figure 7.4. Feed conversion ratio of chickens exposed to combinations of different levels of alkaloids gramine and hordenine

(Values are Mean ± SEM, G 500 and H 500 = feed containing 500 ppm gramine and hordenine respectively, G50H500 = feed containing combination of 50 ppm gramine and 500 ppm hordenine, H50G500 = feed containing combination of 50 ppm hordenine and 500 ppm gramine, control = no gramine and hordenine)

7.3.6. Histological Examination

Groups receiving feed containing alkaloids gramine and hordenine showed abnormalities in liver tissue. All samples from these groups showed vacuolation of the liver cells which were different from the livers of the control group. Micrographs of liver tissue are presented in Plate 7.1.

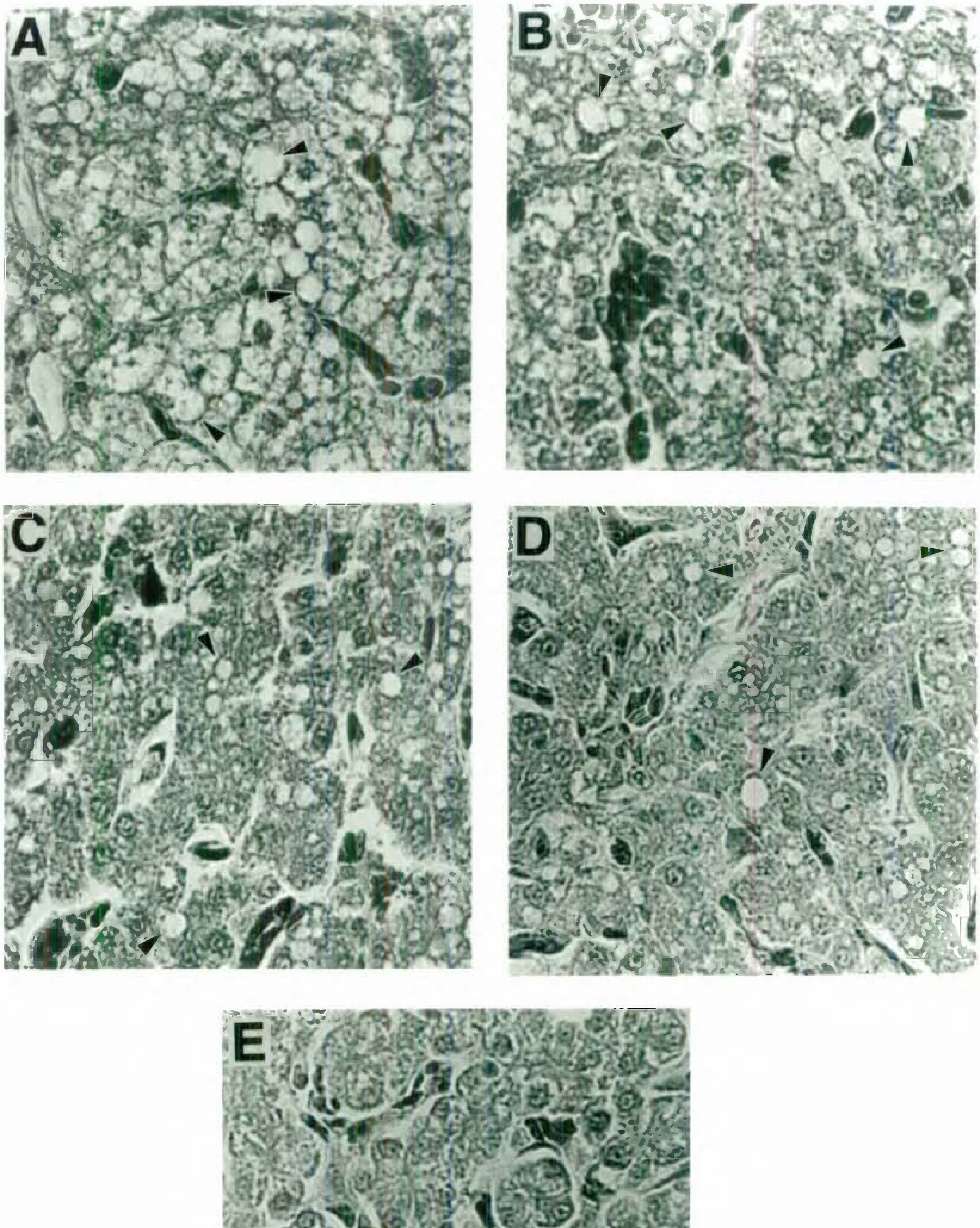
7.4. Discussion

Combinations of the alkaloids gramine and hordenine had no significant effect on the body weights of broiler chickens. However, the group receiving standard feed achieved the highest mean body weight which was 8.5% higher than the mean body weight of the 500 ppm gramine group (lowest).

Plate 7.1. Micrographs of livers from chickens receiving combinations of different levels of gramine and hordenine incorporated into feed. Magnification 400x.

A = 500 ppm gramine
B = 500 ppm hordenine
C = 500 ppm gramine + 50 ppm hordenine
D = 500 ppm hordenine + 50 ppm gramine
E = control

Arrow heads indicate some vacuoles


A
B
C
D
E

In other work, synergistic effects of a variety of compounds on a variety of organisms have been shown. For example, working on Colorado potato beetle [*Leptinotarsa decemlineata* (Say)] larvae, Mahdavi *et al.* (1991) found that a mixture of the insecticide fenvelerate and piperonyl butoxide, a synthetic synergist, was highly toxic and reduced LD_{50}s for the beetle. A similar phenomenon occurred when oat seedlings were exposed to combinations of ferulic acid and atrazine, a herbicide, (Einhellig 1987). A mixture of ferulic acid at 0.25mM with either of 1 and 10 ppb atrazine caused more inhibition to seedling growth than did either treatment alone. Other workers found that the growth rate of rats was significantly reduced when cobalt and ethanol were mixed in their drinking fluid (Derr *et al.* 1970). The inhibition happened more from the combination treatment than from single treatments.

With barley alkaloids, Liu and Lovett (1993a) demonstrated that the combination of gramine and hordenine had a synergistic effect on white mustard root tips. They found that the mixture of 50 ppm gramine and 50 ppm hordenine reduced radicle length of white mustard by 22%.

Feed intake of the chickens was not affected by combinations of gramine and hordenine incorporated into feed. There was an increase in feed intake until week 5 of the treatment after which it decreased slightly. Interestingly, there was a large change in feed intake between week 3 when the chickens were consuming chick starter feed and week 4 when the birds were first exposed to broiler finisher feed. Therefore, it is assumed that the concentration of gramine and hordenine given to the chickens were not enough to suppress their feeding intake.

However, feed conversion ratio was not significantly affected by combinations of gramine and hordenine. Bernays (1991) demonstrated that gramine had a deterrent effect on feeding behaviour of grasshopper, *Schistocerca americana,* even though gramine did not significantly reduce growth rate and efficiency of conversion of food to body mass of sixth instar grasshopper nymphs.

In terms of liver weights there was a significant difference between treatment groups. The group receiving feed containing 500 ppm gramine + 50 ppm hordenine had the lowest liver weight, which was significantly different from all other groups. This finding indicated that the combination of a high concentration of gramine and a low concentration of hordenine affected chicken liver weight although it did not significantly affect body weight. This agreed with the work of Derr *et al.* (1970) which showed that synergism between cobalt and ethanol significantly decreased organ weight of rats. Dry weight of heart was lowest from the combination group and caused 32% reduction of heart weight compare to a group given water as a control. The HPLC analysis of livers indicated that alkaloid was recovered from at least some livers. When either alkaloid was incorporated in feed at the lower concentration (50 ppm) it was not detected in the livers.

Histological examination indicated that there were liver abnormalities in groups receiving alkaloid-treated feed. All livers tested from these groups showed vacuolation of the cells of the livers which was clearly shown in micrographs of liver tissues. This is consistent with the finding reported in Chapter 6, that the higher (500 ppm) concentration of the alkaloids in feed led to changes in the livers.

Pass *et al.* (1979) showed that pyrrolizidine alkaloids of *Heliotropium europaeum* caused enlargement of hepatocytes and fatty change of hepatocytes. Pyrrolozidine alkaloids from tansy ragwort, *Senecio jacobaea*, caused terminal hepatopathy in either a chronic pattern or a chronic-delayed pattern in calves. In addition, the level of serum liver enzymes (glutamate dehydrogenase, γ-glutamyl transferase and alkaline phosphatase) increased.

The conclusion which could be drawn from this experiment is that the concentrations of gramine and hordenine used did not show any significant synergistic effect. Although the chickens were generally not affected as indicated by their growth rates and feed conversion ratios, the alkaloids caused some liver damage.

Chapter 8

General Discussion

8.1. General Discusson

Agricultural practices are basically aimed at fulfilling human needs, most particularly to feed the ever-growing population. Pressures on agriculture such as those posed by pests, diseases and weeds urge people to find proper means of maximising agricultural productivity while minimising damage to the environment caused, mainly, by agricultural chemicals. Naturally-occurring products provide an attractive, potential solution to this problem. It has been widely documented that plant secondary metabolites, which have been isolated from a wide range of plant species, play an important role in maintaining ecological equilibrium.

Barley, one of the major grain crops, produces the plant secondary metabolites gramine and hordenine in the early stages of its growth (Bowden and Marion 1951; Leete *et al.* 1952; Overland 1966; Lovett and Liu 1987; Liu and Lovett 1990). Both alkaloids help barley to suppress the growth of different species of plants such as *Stellaria media* (Overland 1966), *Abutilon theophrasti* (Brown 1974), *Ageratum repens* (Scragg and McKelvie 1976) and *Sinapis alba* (Liu and Lovett 1990). The suppression occurs at different stages of plant growth such as inhibition of germination or later growth of the neighbouring plants. An interesting finding was reported by Liu and Lovett (1990) who demonstrated that damage of cell walls and disorganisation of organelles occurred in *S. alba* radicle tips exposed to gramine and hordenine.

Lovett and Hoult (1993) reported that gramine and hordenine had significant inhibitory effects on the growth of a fungal pathogen *Drechslera teres*. The work of Sepulveda and Corcuera (1990) suggested that gramine and hordenine had the ability to increase the resistance of barley to the bacterium *Pseudomonas syringae*. In addition, the gramine content in the leaves of barley was negatively correlated with the population growth rate of the aphid *Rhopalosiphum padi* (Zuniga and Corcuera 1986), feeding behaviour of *R. padi* and *Schizaphis graminum* (Zuniga *et al.* 1988) and reproduction of *Metopolophium dirhodum* and *R. maidis* (Corcuera *et al.* 1992).

Another insect species affected by gramine and hordenine was the migratory grasshopper [*Melanoplus sanguinipes* (F.)]. The suppression occurred through decreased survival and mean weight of the nymphs (Wescott *et al.* 1992). The growth of armyworm larvae (*Mythimna convecta*) was suppressed by the presence of gramine and hordenine (Lovett and Hoult 1993).

A pasture grass, *Phalaris arundinacea* L., has been reported to produce alkaloids such as gramine, hordenine and tryptamine alkaloids (Gallagher *et al.* 1964). Neurological damage in livestock known as *Phalaris* staggers has been demonstrated to be due to consumption of forage grasses of *Phalaris* spp. (Kennedy *et al.* 1986). At least nine alkaloids, including gramine and hordenine, have been isolated from *P. arundinacea* L., which causes diarrhoea in grazing steers and lambs (Marten *et al.* 1976). The palatability of the grass to ruminant animals is negatively correlated with total alkaloid content (Marten *et al.* 1973).

The effects of the alkaloids gramine and hordenine, addressed above, indicate their potential contribution to the self defence of barley against different plant and animal species. These facts form the basis for exploring the use of these naturally occurring secondary metabolites in biological control. In turn, the use of these metabolites would be expected to reduce the use of synthetic biocides, leading to the better development of ecologically sound agricultural practices.

Research has been conducted to examine the possibility of using gramine and hordenine as self defence agents against vertebrates. In the first experiment, laboratory mice were provided with a feeding choice of standard feed or feed containing different levels, 50 and 500 ppm, of one or other of the alkaloids. There was no significant difference in body weight gained or feed intake between treatment groups. However, there was a trace amount of both alkaloids recovered from the livers of high level treatment groups.

The second experiment was conducted using the same levels of alkaloids incorporated into standard feed except that mice were allocated to one of five treatment groups (control, 50 ppm gramine, 500 ppm gramine, 50 ppm hordenine, 500 ppm hordenine) and there was no feeding choice. The results indicated that there was a difference in feed intake between treatment groups and that the group receiving 500 ppm gramine consumed the least amount of feed. However, there were no significant differences in other data collected between the treatment groups.

In the third experiment, broiler chickens were exposed to the same levels of alkaloids as those of experiment 2. Overall, there was no significant effect of gramine and hordenine on broiler chickens. However, at the end of the experiment, 6 weeks after treatment commenced, the body weights of the group receiving 500 ppm gramine in feed differed significantly from those of the control group. Histological examination indicated an abnormality in the liver tissues. Cell vacuolation occurred in the groups consuming the high (500 ppm) levels of both gramine and hordenine but not in the group receiving the lower alkaloid level (50 ppm).

The last experiment was conducted to examine any possible synergistic effect of both alkaloids on broiler chickens. Combinations of different levels of gramine and hordenine were given to the chickens (control, 500 ppm gramine, 500 ppm hordenine, 500 ppm gramine + 50 ppm hordenine, 500 ppm hordenine + 50 ppm gramine). Consistent with the results of third experiment, histological examination of liver tissues of alkaloid treated groups showed tissue abnormalities.

Mice, rabbits and chickens have all been utilised to provide a rapid and inexpensive biological assay for toxicological research. At the first stage of the experiments, laboratory mice were used as a model to study the effect of the alkaloids gramine and hordenine on vertebrate species. However, mice are relatively slow-growing and it is not possible to expose them to the treatment until they have been weaned onto laboratory feed. This weaning process takes about three weeks after birth. By the time the experiment was commenced, the mice had already achieved approximately 89% of their adult body weight. Therefore, it was considered that laboratory mice were not the best animal model for this study.

Broiler chickens were considered to be superior to laboratory mice because they have a very fast growth rate. Besides, they are able to seek food soon after hatching. Consequently, it was decided to use one-day-old broiler chickens as an animal model for studying the effects of the alkaloids gramine and hordenine. It was expected that the faster growth rate of the chickens would provide more information concerning the effects of the alkaloids on vertebrate animals.

The effects of both gramine and hordenine on the growth of the animals used in the present study is, probably, due to the low concentration of alkaloid given which, in most cases, was not sufficient to interfere with the growth rate of the animals. Goelz *et al.* (1980), for instance, demonstrated that approximately one-third of meadow voles died when were fed either 0.25 or 0.50% gramine in diets, concentrations which were much higher than these used in the present study.

Plants have evolved a variety of defence strategies, based mainly on secondary metabolites, which allow them to avoid overgrazing by herbivores (Harborne 1990). However, the toxicity of plant secondary metabolites is always relative, dependent upon the dose taken in a given time (Harborne 1988; Duffus and Duffus 1991; Freeland 1991), the concentration present in the food (Duffus and Duffus 1991), the age and state of health of animal, the mechanism of absorption and mode of excretion (Harborne 1988). Furthermore, Illius and Jessop (1995) indicated that the nutrient absorption rate affects the tolerance of the animal to the concentration of absorbable plant secondary metabolites in food.

In addition, the presence of secondary metabolites is not the only determining factor of animal feeding behaviour. Factors such as the abundance and distribution of plants, their nutrient content, the digestive capability and capacity of the herbivore, the presence of predators and learned behaviours can significantly influence feeding strategy (McArthur, Hagerman and Robbins 1991).

Animals are equipped with an ability to detoxify foreign compounds with which they come in contact, including plant secondary metabolites, through various enzymatic processes (Jakoby 1980). For example, oxidative reactions, which play a very important role in the metabolism of these compounds, are carried out in numerous tissues of the body and involve enzyme such as oxidases and dehydrogenases (Scheline 1978; Hassal 1990). However, enzyme activity in cattle was disrupted by secondary metabolites contained in the seed of *Castanospermum australe*. The activity of α-glucosidase was inhibited when cattle were fed the seeds of this plant (Reichmann *et al.* 1989). Lymphocyte alpha-glucosidase activity was reduced by at least 90% within 8 h of dosing and returned to normal after several weeks. In addition, cattle developed haemorrhagic gastroenteritis (McKenzie *et al.* 1988b). It was concluded that the indolizidine alkaloid of *C. australe*, castanospermin, in the seeds caused Pompe's disease which is characterised by an absence of lysosomal alpha-glucosidase.

Hassal (1990) noted that enzymes which metabolise foreign compounds serve two related functions. Firstly, the metabolic changes which alter the molecular structure to be less harmful than the original substance. Secondly, these same changes render many substances more soluble in water and more polar, thereby increasing the rate of elimination from the body.

However, the proportion of plant secondary metabolites which are potentially toxic to mammals, fungi or microorganisms and their biochemical mechanisms are not well understood (Feeny 1976). It is likely, therefore, that the small amounts of the secondary compounds present in plants have little or no effect on the growth or fitness of adapted generalist mammals, although tissues containing large amounts of compounds are likely to be avoided (Freeland and Janzen 1974).

Histological examination of chicken livers indicated that cell vacuolation occurred due to alkaloid treatments. The vacuoles were distributed evenly in the liver tissue of high alkaloid treatments (Plate 6.1 and 7.1). This finding agreed with previous studies that demonstrated that alkaloids caused liver damage. For example, Pass *et al.* (1979) reported hepatic lesions in broiler chickens due to pyrrolizidine alkaloids of *Heliotropium europaeum.* There was marked midzonal fatty change of hepatocytes and marked irregularity in the shape of hepatic cords. In all livers, the size of hepatocytes varied from smaller to larger than normal. Lessard *et al.* (1986) reported that, in 14 of 15 horses, hepatic megalocytosis was the most consistent histological lesion which was accompanied by necrosis of hepatic cells. Terminal liver disease and vacuolar changes occurred in the liver of calves fed tansy ragwort (*Senecio jacobaea*)-contaminated pellets (Craig *et al.* 1991). Tansy ragwort has been known for its toxicity due to the pyrrolizidine alkaloids. Furthermore, Winter *et al.* (1990) found that pyrrolizidine alkaloids caused hepatic lesions in yaks (*Bos grunniens*).

Dalvi (1985) showed that an alkaloid sanguinarine, presents in the seeds of *Argemone mexicana*, was responsible for the hepatotoxic potential of this plant in rats. Microscopic examination of the liver tissue showed progressive cellular degeneration and necrosis. Furthermore, rats exhibited considerable loss of body weight and liver weight and slightly enlarged livers with fibrinous material. Levin (1976) reported liver damage in several mammals, caused by coumarins, and high concentrations of coumarins were lethal to the animals.

Hutton *et al.* (1958) reported that another alkaloid, indospicine, was responsible for liver degeneration in rabbits. These workers found that liver damage could be produced by feeding green leaf, dried leaf or seed of *Indigofera endecaphylla.* Furthermore, the animals had a decreased feed intake (which was almost certainly a symptom of the progressive liver disease). One species of grain crops, grain sorghum

(*Sorghum bicolor* L.), has been reported causing reduction in growth rate and slightly elevated liver lipids of chickens (Chang and Fuller 1964).

In the present study, feed intake and body weight gained of both mice and chickens tended to be lowest when the feed contained gramine 500 ppm. This agreed with the work of Goelz *et al.* (1980) who demonstrated that gramine significantly reduced weight gains of meadow voles when fed either 0.25 or 0.50% gramine in diets. However, Goelz *et al.* (1980) found that feed intake from the 0.50% gramine group was higher than that of the control group, which is in contrast to the present study in which animals from the control group ingested more feed than other treatment groups.

The ingestion of the higher concentration in feed (500 ppm) of both gramine and hordenine was associated with the occurrence of vacuolated cells in the livers of chickens. Therefore, it appears that the higher concentrations of both alkaloids in feed are sufficient to induce changes in liver structure. However, these changes were not necessarily associated with reduced feed intake or growth rate of the animals. It has been reported that gramine was toxic to meadow voles by an impairment of kidney function (Goelz *et al.* 1980).

Pang (1983) stated that liver is the major organ for biotransformation of most endogenous compounds. The liver also regulates intermediary metabolism and is primarily responsible for the metabolism of exogenously administered compounds such as gramine and hordenine.

The visible effects of 500 ppm gramine on chickens, Plate 6.2, were "scab-like" lesions on their feet during approximately weeks 4 and 5 of the experiment. Similarly, a toxic phenol, hypericin, induced swelling, extreme itching oedema and cracking of the skin of livestock (Levin 1976). Winter *et al.* (1990) reported unusual skin lesions in yaks *(B. grunniens)* which were consistently associated with pyrrolizidine alkaloids but have so far not been reported in other animals. The skin lesions in yaks were shown to be a thickening of the epithelial layer and enlargement of the keratinized epithelial cells. Therefore, it is possible that gramine had a similar effect on the feet of broiler chickens.

Workers have found that cell vacuolation occurred in plant tissue due to alkaloid treatments. For example, the work of Levitt *et al.* (1984) showed that root tip cells of *Helianthus annuus* contained greater quantities of lipid than the control cells and vacuolation was more pronounced in one alkaloid treatment group. Linseed, *Linum usitatissimum* L., root tips showed similar responses to benzylamine treatment (Lovett *et al.* 1989). Increasing concentrations of allelochemicals caused an increase in the number and size of vacuoles and there was evidence of phagocytosis and disorganization of organelles.

Hordenine and gramine applied to root tips of white mustard caused damage to cell walls, increase in both number and size of vacuoles, autophagy and disorganization of organelles (Liu and Lovett 1990; Liu and Lovett 1993a). Both gramine and hordenine reduced the radicle length, health and vigour of root tips of white mustard (Liu and Lovett 1993a).

8.2. Conclusion

The main finding of these experiments on mice and chickens was that gramine, at high concentration (500 ppm), had a deterrent effect on mice but not broiler chickens in terms of reducing feed intake. Hordenine had fewer effects than gramine. This supports the observation that hordenine has a relatively low toxicity (Henry 1939; Watt and Breyer-Brandwijk 1962; Goelz *et al.* 1980). Both gramine and hordenine at 500 ppm in feed were associated with liver abnormalities. Broiler chickens provided a better model than laboratory mice in this study. This was due to their fast growth rate and the fact that they could be exposed to the alkaloid-treated feed when they were one day old.

The present study and previous investigations on a number of different species indicate that barley has the potential for self defence against other organisms through its biologically active secondary metabolites, gramine and hordenine. As the production of gramine and hordenine may vary due to genetic and environmental factors (Hanson *et al.* 1981, 1983; Lovett and Hoult 1992), further investigation of the effects of different varieties of barley, on vertebrates, should be carried out.

A long term research objective could be a plant breeding program to produce a barley cultivar containing levels of alkaloid adequate to provide an enhanced self defence capability. Such a development could contribute to a reduced dependence on synthetic biocides.