Chapter 3

Materials and Methods

3.1. Introduction

It has been addressed in the previous chapter that allelochemicals affect growth and survival of different species (Miles *et al.* 1991; Westcott *et al.* 1992; and Cheeke 1995). Gramine and hordenine also play a role in self-defence mechanisms of barley against pathogen and herbivore attack. Therefore, it was decided to study the possibility that the alkaloids gramine and hordenine act as self-defence agents against vertebrates. The purpose of the experiments described here was to study the effects of gramine and hordenine, incorporated into diets, on the growth of laboratory mice and broiler chickens.

3.2. Animals

Outbred Albino mice (*Mus musculus*), both male and female, were obtained from the Animal House of the University of New England, Armidale, NSW. Three three-week-old mice were placed in each box (240 mm length, 120 mm width, 105 mm height). Mice were grouped according to their date of birth and were fed with standard laboratory feed for one week prior to the commencement of the experiment.

Broiler strain chickens (*Gallus domesticus*) of both sexes were obtained from a commercial hatchery (BAIADA) at Kootingal, NSW. The chickens were obtained at one-day-old, both male and female, and kept in an electrically-heated brooder at 35°C.

Birds, like mammals, have evolved the ability to regulate their rates of heat production and heat dissipation thereby maintaining a relatively constant deep body temperature over a wide range of environmental conditions. They are, therefore, called "homeotherms". Day-old chickens are normally regarded as heterotherms (body temperature being dependent on the environmental temperature) and homeothermy is fully developed within a week to a fortnight (Romijn and Lokhorst 1955 as cited by Freeman 1971).

3.3. Preparation of Feed

3.3.1. Mouse Feed

Standard laboratory mouse feed (Custom Mix, Fielders Agricultural Products Tamworth, NSW) was used (feed composition is presented in Appendix A). Both gramine (Sigma Chemical Co. USA) and hordenine hemisulphate (Sigma Chemical Co., USA) were weighed as required and determined as ppm (parts per million) of feed (w/w). Either gramine or hordenine was dissolved in 200 ml 95% ethanol and was then diluted into distilled water to make 1000 ml solution for each treatment. The same amount of ethanol and distilled water was mixed into feed without either gramine or hordenine added to produce the control feed.

Ten kg of mouse feed for each treatment was ground in a hammer mill (Jas Smith Pty Ltd, Ballarat) to make fine flour which was then sprayed with the prepared solution and stirred evenly. The mixture was then repelletted in a pelleting machine (R. A. Lister & Co. Ltd., Dursley, England). The pellets were air-dried at room temperature overnight and put into a labelled bag the following day.

3.3.2. Chicken Feed

Chick Starter Feed (Fielders Agricultural Products, Tamworth, NSW) and Broiler Finisher Feed (Fielders Agricultural Products, Tamworth, NSW) were used as the chicken diets (see Appendix B for feed composition). Ten kg of feed for each treatment was placed into a cement mixer (Lightburn & Co. Ltd., Australia) and the prepared solution was sprayed onto the feed while the mixer was turning. Having finished the solution, the mixer was being kept turning for a further 10 minutes to maximise the mixing process. The feed was then air-dried overnight and put into labelled plastic bags. Because of the very large amount of feed required by the chickens, the mixing process was conducted every week during the experimental period.

3.4. Housing, Care and Maintenance

All experiments were conducted at the Animal House the University of New England, under approval from the Animal Care and Ethics Committee. Water and feed were provided *ad libitum* and animals were checked twice per day.

3.4.1. Mice

The treatments were commenced when the mice were 4 weeks of age. Room temperature was kept at 23 ± ^{o}C and 12/12 hour light cycle onset at 7 am; relative humidity was 60%. Mice were kept in boxes and fibre-sorb [Fibre-Sorb (Australia) Pty. Ltd., Summer Hill] was used as bedding material in the box. Water and fibre-sorb were replaced every week when the boxes were changed for clean ones. During the experiment water was added if there was a lack of water in the bottle. Maximum cleanliness of the room was maintained at all times.

Mice and feed were weighed every two days to record data on growth rate and feed intake.

3.4.2. Chickens

One-day-old broiler chickens were placed in the brooder (Multiple Electric Brooder, Multiplo Incubator & Brooder Pty. Ltd., Sydney, 100 chickens for each level) for one week. The brooder temperature was maintained at 35^{o}C. All chickens were given chick starter feed at this stage. At one week of age, chickens were weighed and fitted with wing tags. Only those chickens which were within an average body weight range were used for the experiment (127.39 ± 12.37g for experiment discussed in Chapter 6 and 107.33 ± 6.21g for experiment discussed in Chapter 7). Feed was added into the feeder every two days and water was changed every two days. Light was provided continuously.

At one week of age chickens were grouped according to treatment groups and began receiving chemically treated feed. Sub-groups of 4 or 6 chickens were placed

into individual compartments (72 cm length, 42 cm width and 26 cm height) of a bigger brooder (Superior Quality Equ pment, F. Rynan Pty. Ltd., Austral, NSW) until they were three weeks of age. At his stage, sub-groups were transferred to metabolism cages (75 cm length, 75 cm width and 35 cm height) which were placed in a different room. Chickens were still given chick starter feed for another week (until they were four weeks of age) after which the chick starter feed was replaced by broiler finisher feed.

Room and cages were maintained at maximum cleanliness. Litter was removed regularly for sanitary reasons and the floor was cleaned every day.

3.5. Sacrifice of Animals and Collection of Tissue

At the end of all experiments, animals were euthanased with an overdose of CO_2 gas. All mouse livers were collected and weighed before placing into small vials. The livers were then maintained in a freezer at -20°C for later HPLC analysis of gramine and hordenine.

Small portions of chicken liver (right lobe) were fixed in neutral-buffered formalin solution (see Appendix C) for histological examination. The remainder of the livers were frozen for later analysis of alkaloids, as for the mouse livers. The carcases were disposed of through the UNE Animal House disposal unit.

3.6. Determination of Gramine and Hordenine

Hordenine and gramine content in the livers was determined following the procedure of Hoult and Lovett (1993) and was conducted at the Analytical Laboratory, Department of Agronomy and Soil Science, University of New England. Frozen liver was thawed at room temperature and approximately 1.5 g was ground in a mortar with 30 ml 0.01% acetic acid. After standing for 24 h at room temperature, the extracts were filtered through glass wool. The filtrate was adjusted to pH 9.15 before being centrifuged at 3000 rpm for 5 min. Ten ml aliquot of the extracts and the standard

solution of authentic gramine and hordenine hemisulphate, obtained from Sigma Chemicals, were taken and applied to prepared Sep-Pak C_{18} cartridges (Waters Associates) which were rinsed with 2 ml ACN, followed by 2 ml 0.001 M KH_2PO_4 before aliquot application. All types of solution, which flowed through the cartridges under gravitational force, were collected in disposable containers except the final eluates which were collected in small glass vials. For detail flow diagrams, see Figure 3.1 and Figure 3.2. AR or HPLC grade chemicals and solvent were used throughout.

The final eluates were evaporated to dryness [under a stream of nitrogen at 40°C for the first two experiments and using Refrigerator Vapor Trap (Savant instruments, Inc. Farmingdale, N.Y. USA) for the last two experiments] and taken up in 1 mL mobile phase before being injected into an HPLC system. The mobile phase was 0.025 M KH_2PO_4 + 0.1% TEA (triethylamine), pH 7.15/ACN (2:1 for hordenine) and (6:4 for gramine). See Appendix D for the preparation of mobile phase.

Figure 3.1. The flow diagram of purification for hordenine:

ACN 2 mL

⇩

0.001 M KH_2PO_4 pH 7 2 mL

⇩

aliquot applied 10 mL

⇩

0.05 M KH_2PO_4 pH 9.5/isopropanol (70:30) 2 mL

⇩

0.05 M KH_2PO_4 pH 2.3/isopropanol (70:30) 1.5 mL

Figure 3.2. The flow diagram of purification for gramine:

ACN 2 mL

⇩

0.001 M KH_2PO_4 pH 7 2 mL

⇩

aliquot applied 10 mL

⇩

0.05 M KH_2PO_4 pH 9.5/isopropanol (70:30) 2 mL

⇩

0.05 M KH_2PO_4 pH 9.5/isopropanol (95:5) 2 mL

⇩

0.05 M KH_2PO_4 pH 2.3/isopropanol (70:30) 1.5 mL

Ten microlitre aliquots of each purified standard and liver samples were injected into a Waters HPLC system consisting of a M40 pump (flow rate 2 ml/min); U6K injector; UV/VIS spectrophotometer (wavelength 221 nm); and the results recorded as peak areas using a Waters 745 data module. A Water's μBondapak Phenyl 10 μ (3.9 mm x 30 cm) was used as the column and duplicate injections were made for each sample. Identification of hordenine and gramine in liver samples, expressed as ppm of fresh weight, was by retention time relative to authentic compound, following the formula, *viz.*:

$$\frac{\text{area of sample x concentration of standard sol. x 30}}{\text{area of standard x sample weight}}$$

3.7. Histological Examination

Histological examination of chicken livers was conducted at the Histology Laboratory, Department of Physiology, University of New England. Soon after animals were euthanased, livers were removed from the body and weighed. Small portions of the liver (right lobe) were taken and placed into 10% neutral buffered formalin in small vials. The livers were then cut into 5 mm sections and placed into labelled histological cassettes. All cassettes were then placed into a jar containing 10% buffered formalin solution, ready to be processed in the histokinette (Thomas Optical & Scientific Co., Melbourne).

The histokinette, which can process 40 tissue cassettes at a time, had to be warmed up at least a day before the processing to stabilise the temperature. This process, dehydration, is meant to remove all water from the tissue which was achieved by immersing the tissue in a series of solution of ethanol in water, with gradually increasing percentages of alcohol and xylene solutions (Humason 1972) (see Appendix E). The tissues were then ready to be embedded in paraffin wax.

At the end of processing, after 19 hours, the histokinette was stopped and the cassettes were removed. They were then placed in a cup filled with molten wax and placed in a vacuum chamber for about 30 minutes. This process removed any air bubbles imprisoned within the tissue and in the wax.

The cassettes were then placed on a hot plate and opened. The tissue was taken from each cassette using warmed forceps and then placed into a metallic mould containing molten wax. The labelled parts of the cassettes were deposited on top of the corresponding tissue in the moulds and the wax was allowed to solidify. Once the wax had solidified the mould was removed, leaving the liver tissue as a block of embedded tissue. The blocks were then trimmed to fit properly on the microtome (Ernst Leitz, Germany). The tissue block was then cut into 5 µm sections which were placed on glass slides, 3 sections on each slide. The slides were air-dried over-night, ready to go through the staining process.

The staining process was conducted following the procedure of Ehrlich's Haemotoxylin and Eosin (Humason 1972). The slides were placed into a staining basket that could hold 25 slides and then put into an oven at 60°C for 20 minutes to allow wax in the sections to melt. The basket was then ready to go into a series of solutions for the staining process (see Appendix F). After staining, the samples were mounted by using Eukitt (O. Kindler, West Germany) and were ready to be examined under a microscope.

3.8. Experimental Design

A Completely Randomised Design was used for every experiment. All data were subjected to analysis of variance (ANOVA) by using Minitab Release 8.2 (Minitab Inc.,USA) at the 95% level of confidence. A repeated measures analysis of variance was used to analyse the data for body weight and feed intake. One factor analysis of variance was employed to analyse liver weights and feed conversion ratio of chicken experiments. Concentration of gramine and hordenine in liver tissue was expressed as mean values ± SEM. When significant differences were detected, further analysis of multiple comparisons was conducted according to Duncan's Multiple Range Test (Steel and Torrie 1980).

Chapter 4

Preliminary Assessment of the Effects of Gramine and Hordenine on Vertebrates

4.1. Introduction

Herbivores, both insects and mammals, can experience adverse effects when they ingest secondary metabolites which are produced by plants. As discussed in Chapter 2, gramine and hordenine are found in shoots and roots of germinating barley and may have adverse effects on plant, fungal and insect (invertebrate) species. In this experiment, the effects of these compounds on mice were examined to provide complementary data on a vertebrate. Mice are considered a pest species which may consume young barley plants. As has been suggested for other species (Leather and Dixon 1981; Lovett *et al.* 1989; Reidel 1986; Kanehisa, Tsumuki, Kawada and Rustamani 1990; Liu and Lovett 1993b) barley alkaloids may play a role in "self defence".

Due to a lack of information as to the likely effects of gramine and hordenine on mice, the animals were given feeding choice. Furthermore, in deference to the Animal Care and Ethics Committee of the University of New England, mice were fed both standard laboratory feed and treated feed at the same time to diminish concerns as to animal welfare.

4.2. Materials and Methods

The experiment was conducted for 8 weeks (October to December 1993); the treatment was commenced when mice were 4 weeks of age. Room temperature was maintained at 23 $\pm$ 1^{o}C and 12:12 hour light cycle, onset at 7 am. Relative humidity was 60%. Water and feed were provided *ad libitum*. Animals were checked twice a day. Both standard feed and feed containing alkaloids were placed in the feed compartment in the lid of the mouse cage. The two types of feed were divided by a metal plate which was placed in the feeder.

Mice used in the experiment were allocated evenly to the treatment groups based on their date of birth. Mice were kept in boxes and fibre-sorb was used as bedding (as described in Section 3.4.1).

The Completely Randomised Design had 4 treatments and 5 replications, with 3 mice in every box to make a total of 60 mice. The treatments were different levels (w/w) of gramine and hordenine in the feed i.e.:

A = standard feed + feed containing 50 ppm hordenine

B = standard feed + feed containing 500 ppm hordenine

C = standard feed + feed containing 50 ppm gramine

D = standard feed + feed containing 500 ppm gramine

The feed was mixed with gramine and hordenine following the procedure described in Section 3.3.1.

Mice were weighed every two days at approximately the same time in the morning. From these data growth rates were calculated. A total of 120 g feed was supplied per box every second day. This quantity of feed is the maximum capacity of the feed compartment. Feed taken was determined by using the formula:

feed taken (g) = 120 (g) - feed left (g) - feed on bedding (g)

where: 120 (g) = feed given (60 g of treatment + 60 g of standard ration)

feed left = leftover on the feeder (g)

feed on bedding = unchewed feed left on bedding (g)

Feed on bedding was collected by carefully separating the feed from the bedding material and then weighing the feed. Feed was weighed on the same day as body weight was determined. Feed remaining was weighed to determine intake. Data are presented on a weekly basis.

At the end of experiment, liver tissue was collected from mice as described in Section 3.5 for later analysis as described in Section 3.6.

4.3. Results

4.3.1. Body Weight

The body weights of mice in each treatment group, over the course of the experiment, are presented in Figure 4.1, Appendix G and the body weights as a percentage of initial body weight are presented in Figure 4.2. There was a significant effect of age ($P < 0.0001$) , with animals in all treatment groups increasing in weight over the course of the experiment. Although there was no significant effect of treatment on body weight, there was a significant interaction between age and treatment ($P = 0.007$).

The group given feed containing 50 ppm gramine plus standard feed achieved the highest mean body weight. However, the group receiving 500 ppm hordenine feed plus standard feed reached the highest final body weight as a percentage of initial body weight. The treatment with 500 ppm gramine had a tendency to have the lowest growth rate throughout the experiment. After the first week of the treatment, this group had the lowest mean body weight, although body weight had increased by the end of week two.

4.3.2. Feed Intake

Mouse feed intake was not significantly different between treatment groups but changed significantly with time ($P < 0.0001$). There was no significant interaction between treatment and age. The mean weekly feed intakes are presented in Figure 4.3 and Appendix H. The 500 ppm gramine treatment group tended to consume more feed than any other treatment group. For the first two weeks of this treatment there was an increase in feed ingested which remained almost steady for the next four weeks before declining. The 50 ppm hordenine treatment group, in contrast, had the lowest mean feed consumption. Feed intake in the remaining treatments was relatively uniform throughout.

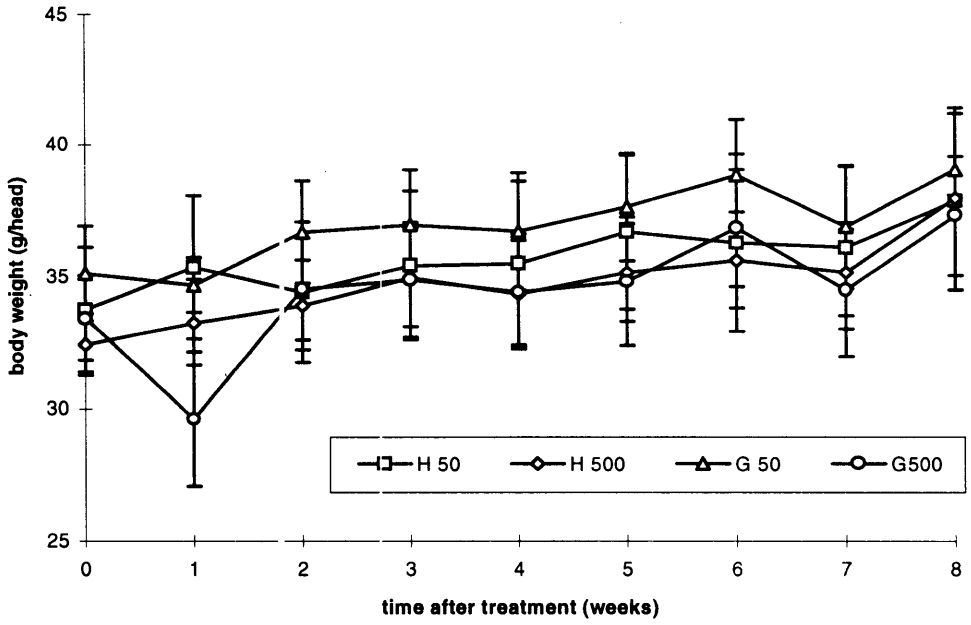

Figure 4.1. Body weight of mice for eight weeks of treatment

(Values are Means ± SEM, H 50 = standard feed + feed containing 50 ppm hordenine, H 500 = standard feed + feed containing 500 ppm hordenine, G 50 = standard feed + feed containing 50 ppm gramine and G 500 = standard feed + feed containing 500 ppm gramine.)

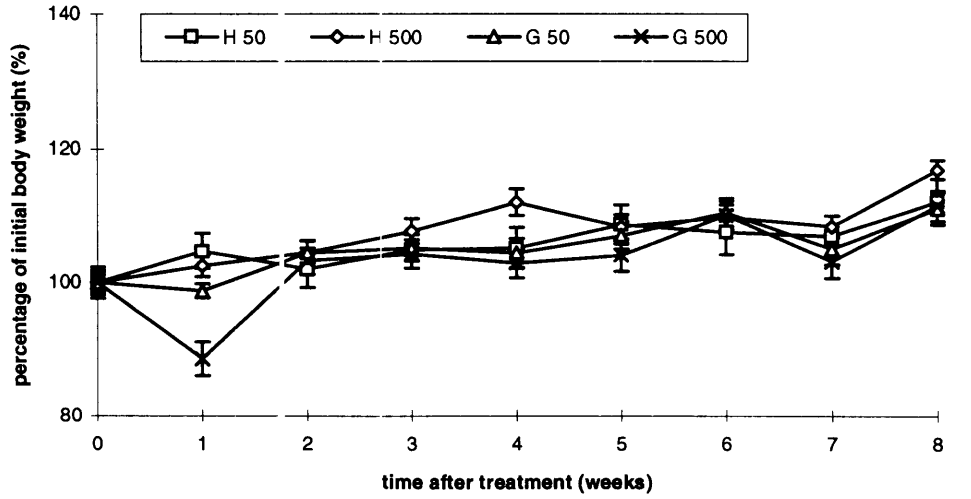

Figure 4.2. Body weight of mice as a percentage of initial body weight

(Values are Means ± SEM, H 50 = standard feed + feed containing 50 ppm hordenine, H 500 = standard feed + feed containing 500 ppm hordenine, G 50 = standard feed + feed containing 50 ppm gramine and G 500 = standard feed + feed containing 500 ppm gramine.)

The total amount of eac ı type of feed (standard and alkaloid-treated) consumed by the mice is presented in Figure 4.4. Mice tended to ingest more standard feed than alkaloid-treated feed for all treatment groups except the 50 ppm hordenine. Total feed consumption tended to be lowest in the 50 ppm hordenine group.

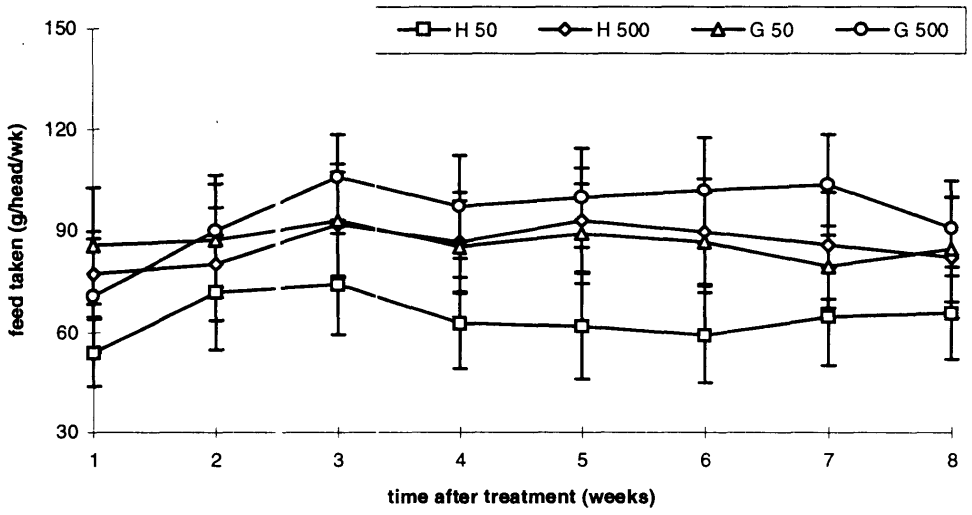

Figure 4.3. Mouse feed ingested for eight weeks of treatment

(Values are Means ± SEM, H 50 = standard feed + feed containing 50 ppm hordenine, H 500 = standard feed + feed containing 500 ppm hordenine, G 50 = standard feed + feed containing 50 ppm gramine and G 500 = standard feed + feed containing 500 ppm gramine.)

4.3.3. Liver Weight

The weights of mouse livers in this experiment, presented in Figure 4.5 and Appendix I, were not significantly different between treatment groups (P = 0.072), although liver weight tended to be lower at the higher levels of the alkaloids.

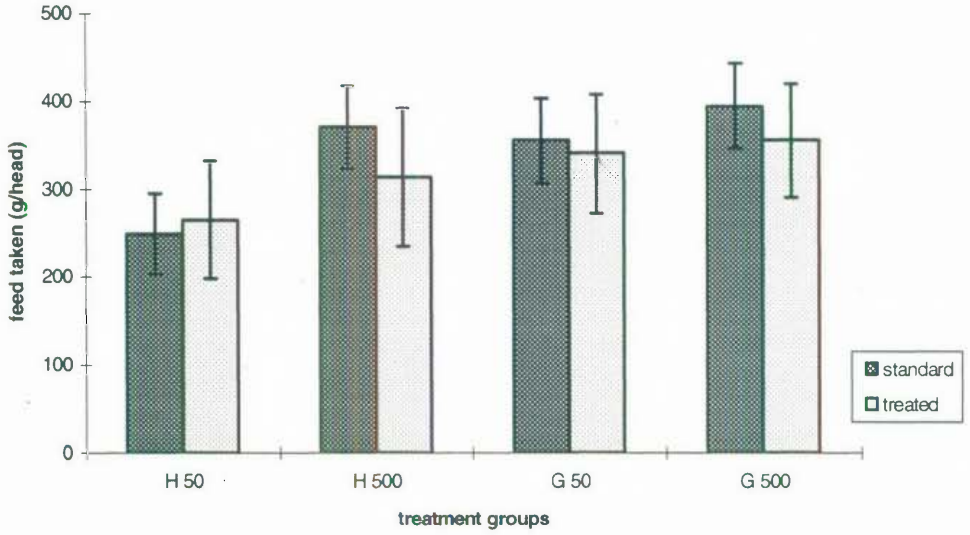

Figure 4.4. Total amount of feed taken by mice during the experiment

(Values are Means ± SEM, H 50 = standard feed + feed containing 50 ppm hordenine, H 500 = standard feed + feed containing 500 ppm hordenine, G 50 = standard feed + feed containing 50 ppm gramine and G 500 = standard feed + feed containing 500 ppm gramine.)

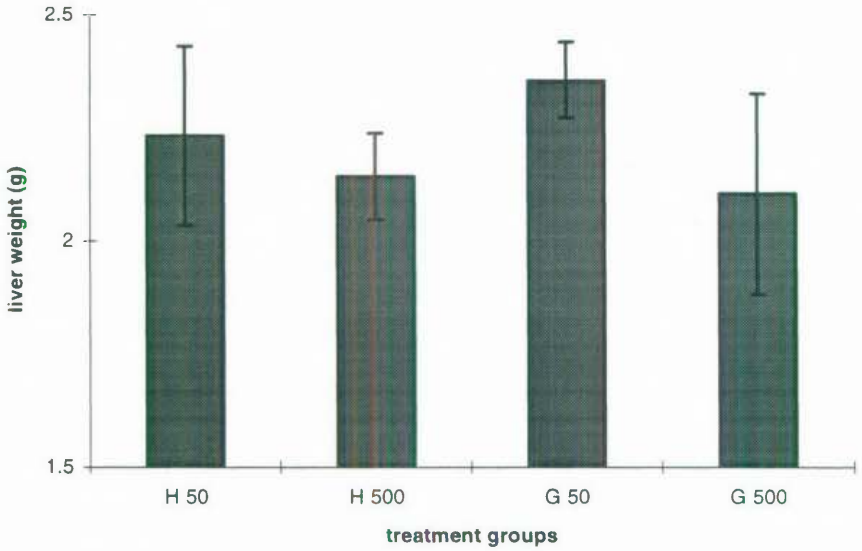

Figure 4.5. Liver weight of mice after eight weeks of treatment

(Values are Means ± SEM, H 50 = standard feed + feed containing 50 ppm hordenine, H 500 = standard feed + feed containing 500 ppm hordenine, G 50 = standard feed + feed containing 50 ppm gramine and G 500 = standard feed + feed containing 500 ppm gramine.)

4.3.4. HPLC Analysis of Liver

The means of gramine and hordenine content obtained by HPLC analysis of liver in this experiment are presented in Table 4.1. For each of the 500 ppm hordenine and 500 ppm gramine treatments groups, the alkaloids were detected in only 9 out of 15 livers tested. The values shown in Table 4.1 are the means ± SEM for the 9 livers.

Table 4.1. Gramine and Hordenine Content in Liver (μg/g fresh wt.)

Treatment Group	Mean (n) ± SEM
Feed containing hordenine 50 ppm + standard feed	Not Detectable
Feed containing hordenine 500 ppm + standard feed	10.72(n=9) ± 0.42
Feed containing gramine 50 ppm + standard feed	Not Detectable
Feed containing gramine 500 ppm + standard feed	0.38(n=9) ± 0.08

Consistent with liver weight trends, alkaloids were recovered only from the treatments containing the higher level of alkaloids. Levels of gramine and hordenine in the liver varied between treatment groups. No gramine and hordenine was detected in low alkaloid treatment (50 ppm) groups. However, the amount of hordenine detected in the liver of high alkaloid treatment groups was much higher (twenty eight-fold) than that of gramine.

4.4. Discussion

While the results observed from this experiment did not show any statistically significant effects of treatment, some interesting trends emerged. Body weight showed a consistent upward trend with all treatments but did not differ significantly between treatment groups. This finding contrasted with the work of Marten *et al.* (1976) who showed that total alkaloid concentration of reed canarygrass was highly negatively

associated with average daily gains by lambs and steers (r = -0.91). These workers found, however, that animals had less diarrhoea when grazing gramine-containing plants as compared to tryptamine-carboline-containing plants.

In addition, Marten, Jordan and Hovin (1981) demonstrated that animal performance improved when grazing low-alkaloid cultivars of reed canarygrass. An indole alkaloid concentration of 0.2% dry weight caused reduced weight gain in lambs. As the alkaloid concentration increased beyond this level the adverse effect on animal performance became progressively greater. Similarly, Glick and Joslyn (1970) reported that a 4% level of tannic acid in the diet caused weight loss and growth retardation in weanling rats and an 8% tannic acid level killed them.

Feed intake increased with age in all treatment groups. Although there were no significant differences between treatment groups, feed intake tended to be highest for the 500 ppm gramine plus standard feed group and lowest for 50 ppm hordenine plus standard feed. In contrast, the work of Marten *et al.* (1976) showed that, when a choice is given, total basic alkaloid concentration (particularly indole alkaloids, Simons and Marten 1971) of reed canarygrass is negatively correlated with palatability of the grass to ruminant animals.

The data presented in Figure 4.4 indicate that all treatment groups, except for 50 ppm hordenine plus standard feed, tended to consume more standard feed than alkaloid- containing feed. This observation is consistent with the common belief that animals have the ability to select food less deleterious to them when a choice is given. For example, when laboratory rats were presented with a series of foods, each containing a different concentration of selenium, they consistently ate the least toxic food (Franke and Potter 1936).

In terms of liver weight lower mean weights were associated with the higher alkaloid treatments and, consistent with this trend, alkaloids were recovered by HPLC analysis only from livers in the high alkaloid treatment groups.

There is no significant evidence that palatability was affected more by high alkaloid than low alkaloid treatments although the data indicate that liver weight was less affected by a lower alkaloid content in the diet. This finding is further addressed in Chapters 6 and 7.

The results of this experiment indicate that in a situation where all treatment groups had simultaneous access to standard feed, the levels of alkaloids presented to the mice had no adverse effects on growth rate.

Chapter 5

Effects of Gramine and Hordenine on Mice

5.1. Introduction

The effect of secondary metabolites has been demonstrated by many workers with a variety of animal species (broadly discussed in Chapter 2). The previous experiment (see Chapter 4), in which mice were given feeding choice between standard feed and feed containing different concentrations of gramine and hordenine, did not show any significant effect of treatment. The fifty ppm hordenine treatment groups, however, tended to consume the lowest total amount of standard feed. In the present experiment (described in this chapter), mice were given feed containing different levels of the alkaloid (gramine and hordenine) without feeding choice. The objective of this experiment was to examine the effect of gramine and hordenine on mice, without access to standard feed in all treatment groups.

5.2. Materials and Methods

The experiment was commenced when mice were 4 weeks of age and continued for 12 weeks (February to May 1994). Room temperature was maintained at 23 $\pm$ 1^{o}C and 12/12 hour light cycle, onset at 7 am. Relative humidity was maintained at 60%.

The Completely Randomised Design had 5 treatments and 5 replications, with 3 mice in every box to make a total of 75 mice. The treatments were different levels (w/w) of gramine and hordenine which were incorporated in the feed i.e.:

A = standard feed (control)

B = feed containing 50 ppm hordenine

C = feed containing 500 ppm hordenine

D = feed containing 50 ppm gramine

E = feed containing 500 ppm gramine

The process of preparing feed for this experiment was the same as that for the previous experiment (see Section 3.3.1). The collection of data followed the procedure as described in Section 4.2.

5.3. Results

5.3.1. Body Weight

Body weight increased significantly ($P < 0.0001$) over the twelve week experimental period in all treatment groups (Figures 5.1 and 5.2). However, there were no significant differences between treatment groups and no significant interaction between age and treatment (Appendix L).

The control group tended to achieve the highest mean body weight and 500 ppm hordenine treatment group had a tendency to be lowest.

5.3.2. Feed Intake

The average intakes of feed containing different levels of gramine and hordenine are presented in Figure 5.3. There was a statistically significant effect of both treatment ($P < 0.0001$) and age ($P < 0.0001$) and a significant interaction between treatment and age ($P < 0.0001$).

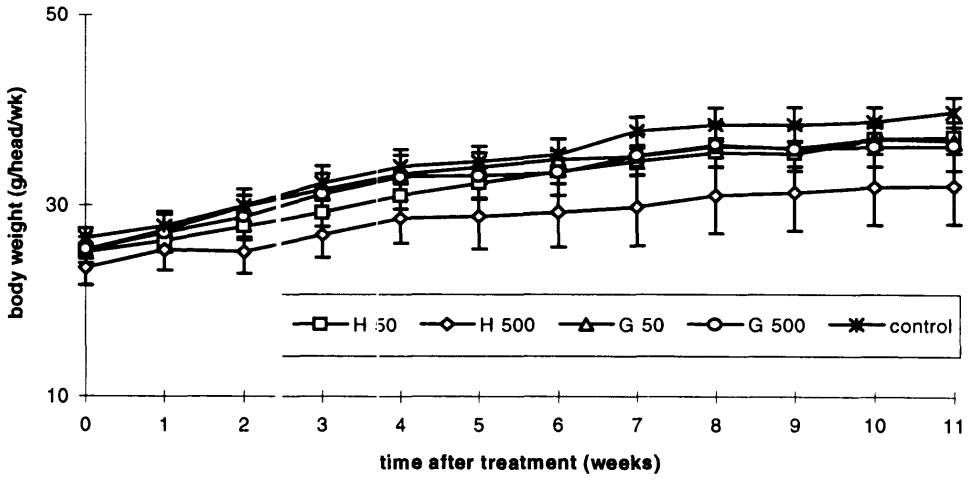

Figure 5.1. Body weight of mice over twelve weeks of experimental period

(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 and control = no gramine and hordenine)

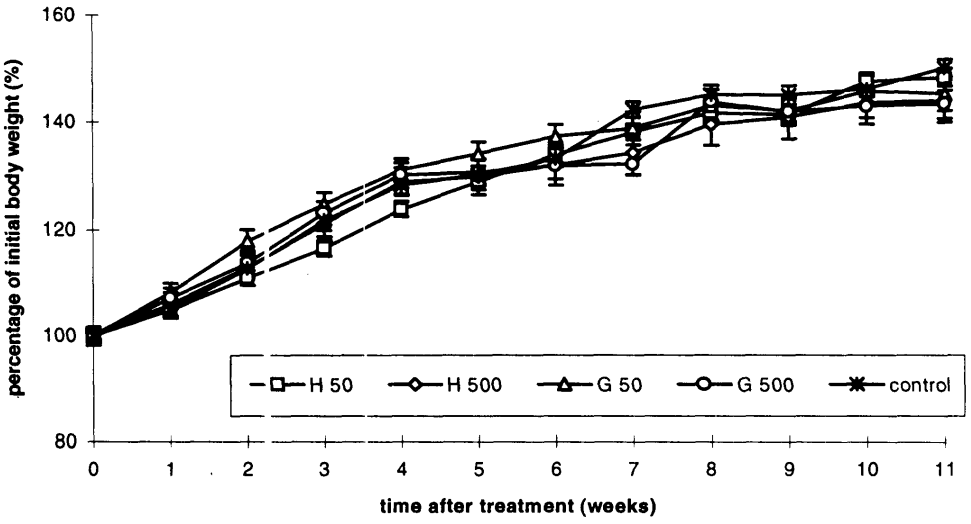

Figure 5.2. Body weight of mice as a percentage of initial body weight

(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 and control = no gramine and hordenine)

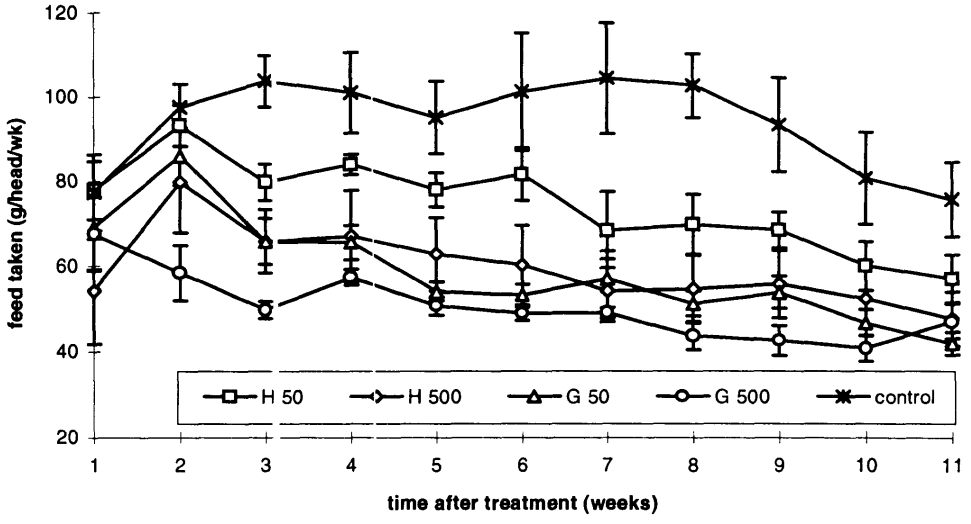

Figure 5.3. Mouse feed ingested for alkaloid treatments

(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 and control = no gramine and hordenine)

The feed presented to the control group (nil gramine and hordenine) was ingested to a greater extent than that of other treatment groups from week two of the treatment (Figure 5.3). In contrast, the treatment containing 500 ppm gramine was consumed least. There was a tendency for feed intake to decrease with increased concentration of the alkaloids. Data of weekly feed intake of each treatment group for this experiment are presented in Table 5.1.

5.3.3. Liver Weight

The average weights of mouse liver for all treatments are presented in Figure 5.4 and Appendix J. The liver weights were not significantly different between treatment groups. However, there was a tendency for the 500 ppm gramine treatment group to have the highest mean liver weight.

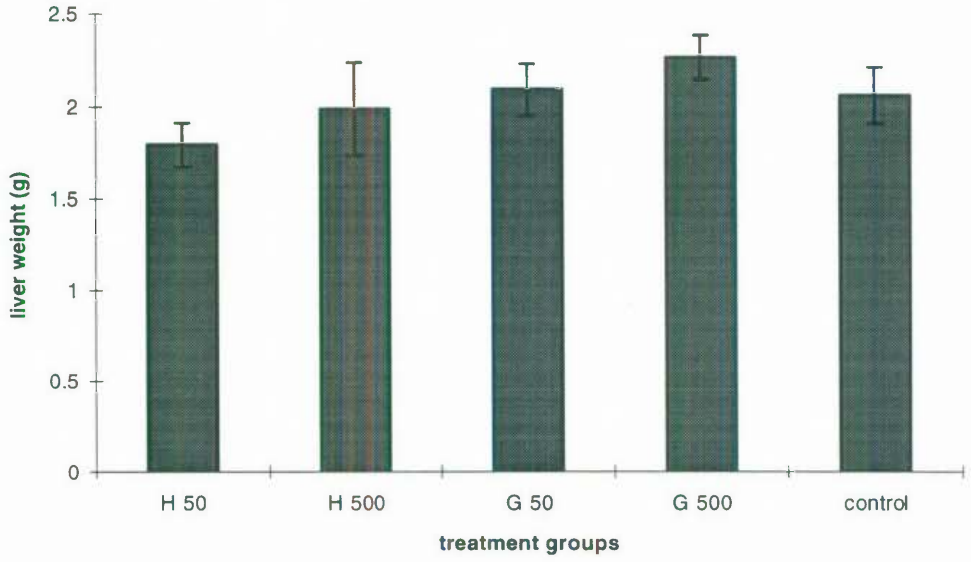

Figure 5.4. Liver weight of mice after twelve weeks of alkaloid treatment

(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 and control = no gramine and hordenine)

Table 5.1. Feed intakes (g) of mice receiving different levels of alkaloids gramine and hordenine in the feed

Treatment Group	Week after treatment										
	1	2	3	4	5	6	7	8	9	10	11
H 50	77.91^{a} ± 6.88	93.21^{a} ± 4.96	79.85^{b} ± 4.36	84.11^{ab} ± 2.51	78.03^{ab} ± 4.04	81.71^{ab} ± 6.20	68.49^{b} ± 8.98	69.91^{b} ± 7.06	68.55^{ab} ± 4.22	60.17^{ab} ± 5.70	56.99^{ab} ± 5.57
H 500	54.10^{a} ± 12.30	79.91^{ab} ± 12.00	65.84^{b} ± 7.59	67.24^{b} ± 10.70	62.87^{b} ± 8.65	60.28^{b} ± 9.50	54.32^{b} ± 7.30	54.63^{b} ± 7.84	55.80^{b} ± 7.87	52.47^{ab} ± 8.65	47.72^{b} ± 6.27
G 50	69.08^{a} ± 9.97	85.95^{ab} ± 6.93	65.86^{b} ± 5.48	65.64^{b} ± 4.09	54.01^{b} ± 2.16	53.26^{b} ± 2.40	57.03^{b} ± 6.63	51.26^{b} ± 2.85	53.91^{b} ± 3.64	46.75^{b} ± 2.99	41.98^{b} ± 2.61
G 500	67.53^{a} ± 8.74	58.50^{b} ± 6.44	49.75^{b} ± 2.06	57.37^{b} ± 1.87	50.75^{b} ± 2.18	48.90^{b} ± 1.67	49.13^{b} ± 1.20	43.77^{b} ± 3.24	42.75^{b} ± 3.48	40.90^{b} ± 3.04	47.10^{b} ± 4.03
Control	77.37^{a} ± 9.05	97.6^{a} ± 5.40	103.82^{a} ± 6.17	101.07^{a} ± 9.66	95.26^{a} ± 8.58	101.32^{a} ± 13.9	104.50^{a} ± 13.2	102.80^{a} ± 7.62	93.55^{a} ± 11.10	80.87^{a} ± 10.90	75.81^{a} ± 8.87

(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 ppm and 500 ppm hordenine respectively, G 50 and G 500 = feed containing 50 ppm and 500 ppm gramine respectively, control = no hordenine and gramine)

5.3.4. HPLC Analysis of Liver

The mean contents of gramine and hordenine recovered by HPLC analysis of liver in this experiment are presented in Table 5.2. For each treatment group, the number of livers in which alkaloid was detected out of 15 livers tested is indicated in brackets after the mean.

Table 5.2. Gramine and Hordenine Content of Liver (μg/g fresh wt.)

Treatment Group	Mean (n)* ± SEM
Feed containing hordenine 50 ppm	8.43 (n=3) ± 0.36
Feed containing hordenine 500 ppm	26.89 (n=7) ± 3.33
Feed containing gramine 50 ppm	0.39 (n=7) ± 0.39
Feed containing gramine 500 ppm	0.54 (n=10) ± 0.27
Standard feed	Not Detectable

* n = amount of liver showed HPLC result

Levels of gramine and hordenine in the liver varied between treatment groups. All treatment groups demonstrate recovery of the alkaloids (either gramine or hordenine) through the HPLC analysis in at least some livers. The 50 ppm hordenine group showed the least number of livers in which alkaloid was detected while the group of 500 ppm gramine had the highest. In addition, the highest level of alkaloid recovered was from the livers of the 500 ppm hordenine group.

5.4. Discussion

Body weight increased significantly with age in all treatment groups. The presence of the alkaloids in the feed had no significant effect on growth rate. The final body weight achieved was highest in the control group (39.8 g) and lowest for the 500

ppm hordenine group (32.0 g) which was consistently lowest from the second week of treatment.

Interesting trends emerged with feed intakes which show large treatment differences, particularly between gramine 500 ppm (lowest) and the control group (highest). The second highest feed intake was by the hordenine 50 ppm group. The effect of treatment on feed intake might be associated with an unpalatability effect of gramine in the diets. This suggestion is consistent with the finding of Kendall *et al.* (1979) who worked with meadow vole (*Microtus pennsylvanicus*) and found that gramine inhibited the feed intake of meadow voles more than did hordenine. The inhibition was maximum at about 1% of gramine in diets. Similarly, gramine affected the feeding behaviour of the aphids *Schizaphis graminum* and *Rhopalosiphum padi* (Zuniga *et al.* 1988). When incorporated into diets, gramine decreased survival and amount of diet ingested by *R. padi* (Zuniga and Corcuera 1986).

Gramine and other similar compounds in *Phalaris* pastures are toxic to sheep and cattle (Marten *et al.* 1976) and the grazing animals showed different levels of acceptance of *Phalaris arundinacea* L. (O'Donovan, Barnes, Plumlee, Mott and Packett 1967). An extreme case reported was that *P. arundinacea* L. was one of the pasture species that caused acute bovine pulmonary emphysema in cattle with a mortality was about 30% of infected animals (Carlson and Breeze 1984). All the workers mentioned, above, demonstrated that the defensive role of gramine may be a result of its toxic and feeding-deterrent properties.

The fact that the hordenine 50 ppm group had the second highest feed intake, after the control group, agreed with the findings of Williams *et al.* (1971). In separation of alkaloids using gas-liquid chromatography, these workers identified hordenine as the major component of the more palatable clones of reed canarygrass. This suggests that gramine is more likely to be associated with negative effects than hordenine.

Liver weight was not significantly different between treatment groups even though the 500 ppm gramine group tended to be the highest. This might be due to the effect of the alkaloid which caused liver enlargement. Cheeke (1991) reported that one of characteristic signs of Pyrrolizidine alkaloids toxicosis in animals was enlargement of hepatocytes (megalocytosis). However, for both alkaloids the higher the content of the feed, the higher the content of the livers. Consistent with the finding of previous experiment, discussed in Chapter 4, the highest amount of alkaloid recovered through the HPLC analysis was found from the 500 ppm hordenine group.

The main findings of the experiment are that mouse growth rate was not significantly affected by ingestion of the alkaloids gramine and hordenine at levels of 50 ppm and 500 ppm. It is assumed that the concentrations of alkaloid given were not high enough to depress the growth rate of mice because plant secondary metabolitess have dosage dependent toxic properties (Freeland 1991). However, data indicate that palatability of the feed was adversely affected by incorporation of 500 ppm gramine. The workers demonstrated that palatability of fodder in sheep and cattle is decreased by a high content of indole-alkaloid compounds (Marten *et al.* 1976). In the present study, total feed intake by the control group was almost double that of gramine 500 ppm (571.10 and 1015.10 g respectively).

At this stage of the experimentation, the use of laboratory mice as a model for the effects of allelochemicals on vertebrate species was evaluated. Mice are relatively slow-growing and it is not possible to expose them to alkaloid-containing feed until they have been weaned onto laboratory feed. Therefore, experiments could not begin until mice were 4 weeks of age and had already achieved approximately 89% of their adult body weight.

It was considered that broiler chickens might provide a more appropriate model because they are precocial birds, are able to seek food soon after hatched and have a very fast growth rate. Therefore, the experiments presented in Chapters 6 and 7 were conducted using broiler chickens.