Barley Allelochemicals of Gramine and Hordenine: Their Effects on Broiler Chickens

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Abstract. An experiment aimed at examining the effects of gramine and hordenine, incorporated into diets, on the growth and histological structures of the chicken liver has been carried out at the animal house complex, Analytical Laboratory, and Histology laboratory of the University of New England, Armidale, Australia. Five treatment groups (50 and 500 ppm hordenine or gramine, and standard feed as control) were administered to one-week old broiler chickens. Each treatment group had six heads of chickens and were replicated 6 times. Data collected including body weight and feed intake (recorded weekly), liver tissue collection for histological examination, and determination of gramine and hordenine in the liver. Data were analysed using Analysis of Variance (ANOVA) at 5% level of confident. Results demonstrated that gramine had more effects than hordenine on the animal tested. A high concentration (500 ppm) of either gramine or hoedenine was sufficient to induce changes in liver structure of the chickens as indicated by cell vacuolation even though they were not necessarily associated with reduced feed intake or growth rate of the animals. Scab-like lesions on their feet during weeks 4 and 5 of the experiment were observed from the group of 500-ppm gramine.

Key Words : gramine, hordenine, chicken

Introduction

Plant allelochemicals are secondary metabolites (Swain, 1977) and are identified as non-nutritional compounds, synthesized by a plant species that are able to affect growth, health, and behavior or population biology of another species either as a stimulator or an inhibitor (Whittaker, 1971). Zoologist defines allelochemicals as a non-nutritional chemicals produced by one organism that affects the growth, health, behavior or population biology of other species (Reese, 1979). This definition is very close to the one used for plant allelochemicals. However, whether a substance should be identified as a nutrient or an allelochemical is actually dependent upon the context rather than on its biosynthetic origin (Berenbaum, 1995).

The allelochemicals present maintain the overall integrity of the plants against competitors, predators and pathogens. In general, allelochemicals (except at high concentrations) cannot penetrate the plasmalemma of the recipient cells (Roshchina, 1999), indicating that there may be a special recognition system in the plasma membrane. The physical structure of the plasma membrane itself may also limit allelochemical penetration into the target cells. Carr et al. (1989) noted that in some cases, the surfaces of the plasmalemma and the cell walls are covered by a liquid excretion or hydrophilic excretions. This occurs in insect and mammal olfaction where slime mainly serves as a primary sensory element. In other cases, many surface enzymes such as peroxidases, aminoxidases, and proteases recognise their enzyme-substrates and change the enzymatic activity without penetrating into the target cell (Roshchina, 1999), emphasising that allelochemicals may face some barriers before entering the target cell. If enzymatic reactions occur on the cell surface, the cells are protected against allelopathic invasion.

Respiration is one physiological aspect of plants that has been affected by allalochemicals. For instance, α-pinene at concentrations lower than 250 µM, α-pinene stimulated respiration in maize in the absence of adenine dinucleotide phosphate (ADP), but inhibited respiration in the presence of ADP. In contrast, at concentrations higher than 250 µM, α-pinene inhibited respiration regardless of the presence of ADP (Abrahim, 2003a). Similarly, a variety of flavonoids also inhibited ATP production in mitochondria by lowering the ADP/O ratio in yeast mitochondria (Einhellig, 1995) and papers therein}. However, it is unlikely that the allelopathic interference of these compounds have an effect on mitochondrial energy transduction. Abrahim et al., (2003b) found that ferulic, coumaric and vanillic acids at 0.1 to 10 mM did not affect mitochondrial respiration and related observations such as the ADP/O ratio in soybean hypocotyl axes. In addition, the monoterpenes they used (camphor, limonene, and α–pinene) at 0.1 to 10 mM strongly inhibited the respiratory activity of the soybean hypocotyl mitochondria, suggesting that this group of allelochemicals may cause phytotoxicity to mitochondria which in turn affects respiration (Abrahim, 2003a). Different group of allelochemicals clearly have different effects on respiration and their activity is species specific and concentration dependent. Chaniago et al. (2007) reported the effects of aqueous extracts of nutgrass on the respiration of soybean seedlings.

Compounds such as allelochemicals that are secondary metabolites and produced by various plant species could play an extremely important role against pests. This is due to plant inability to run away from the predators. Different mechanism has been found in plants as ways to protect themselves against pathogens and herbivores, such as anatomical structures and the accumulation of secondary metabolites (Ryan and Jagendorf, 1995). These defensive chemicals affect different animals in different ways.

One common form of self-defence mechanisms is that the plant renders itself unpalatable to and/or indigestible by the herbivores when the leaves are consumed (Ryan and Jagendorf, 1995) and may cause poisoning in animals. For instance, Pyrrolizidine alkaloilds, mainly found in plants of the families Boraginaceae, Compositae and Leguminacae, have proven to experience intoxication leading to pulmonary damage in livestocks (Mosher, 1982) hepatic damages in livestock (Cheeke, 1988) and liver megalocytosis in yaks (*Bos grunnies*) (Winter et al., 1990). Another forage grasses, *Phalaris* sp., produce tryptamine and carboline alkaloids that may cause neurological damage in livestock (Cheeke, 1995). The alkaloid riddelliine, a member of a class of pyrrollizidine alkaloids, reduced bodyweight gains in both rats and mice and causing developmental toxicity in rodents (Chan et al., 1994).

Terminal liver disease developed in calves fed tansy ragwort (*Senecio jacobaea*)-contaminated pellets and vacuolar changes occurred in the liver due to the pyrrolizidine alkaloids (Craig et al., 1991). Poisoning in ruminants from ingestion of the plant Lantana camara, which contains the triterpene acids lantadene A and lantadene B has been reviewed (McKenzie, 1991). Jaundice (liver injury), photosensitisation, and ruminal stasis have been recorded as a consequence of lantana poisoning (Pass, 1986).

Two alkaloids, gramine (N,N-dimethylindolemethylamine) and hordenine (N,N-dimethylthramine), have been isolated from leaves and roots of germinating barley (Bowden and Marion, 1951; Leete et al., 1952; Liu and Lovett, 1990). Many works have been conducted to examine the role of gramine and hordenine on other organisms such as bacterium *Pseudomonas syringae* (Sepulveda and Corcuera, 1988) and inhibitory effect on a

fungal pathogen (*Drechslera teres*) and armyworm (*Mythimna convecta*) (Lovett and Hoult, 1993). These lead to the possibility of exploring gramine and hordenine for self-defence agents by barley against other organisms. The work reported here was aimed at examining the effects of gramine and hordenine, incorporated into diets, on the growth and histological structures of the chicken liver.

Materials and Methods

Five experimental groups and six replications in a Completely Randomised Design were carried out at the Animal House complex of the University of New England, Armidale, Australia. 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 concentration (w/w) of gramine and hordenine which were incorporated into feed as follows: 50 ppm hordenine, 500 ppm hordenine, 50 ppm gramine, 500 ppm gramine, and standard feed as a control group.

Broiler strain chickens (*Gallus domesticus*) of both sexes were obtained from a commercial hatchery (BAIADA) at Kootingal, NSW, Australia. The chickens were obtained at one-day-old and kept in an electrically-heated brooder (Multiple Electric Brooder, Multiplo Incubator and Brooder Pty. Ltd. Sydney, Australia, 100 chickens for each level) at 35°C for one week and provided with chick starter feed and water ad libitum. Light was provided continuously. The chickens were then weighed and fitted with wing tags. Only those chickens which were within an average body weight range (107.33±6.21 g) were used for the experiment. At one week of age, chickens were group according to treatment and began receiving chemically treated feed as per treatment. Sub-group of 6 chickens were placed into individual compartments (72 cm long, 42 cm wide, and 26 cm high) of a bigger brooder (Superior Quality Equipment, F. Rynan Pty. Ltd., Austral, NSW, Australia) until they were three weeks of age. At this stage, sub-groups were transferred to metabolism cages (75 cm long, 75 cm wide and 35 cm high) which were placed in a different room. Chickens were still given starter feed for another week (until they were four weeks of age) after which the chicks starter feed was replaced by broiler finisher feed. The chickens were kept until they were 7 weeks of age. Room and cages were maintained at maximum cleanliness. Litter was removed regularly for sanitary reasons and the floor was cleaned every day.

Chick starter feed and Broiler finisher feed (both of Fielders Agricultural Products, Tamworth, NSW, Australia) were used as the chicken diets. Ten kg of feed for each treatment was placed into a cement mixer and the prepared solution of either gramine or hordenine (50 or 500 ppm) was sprayed onto the feed while the mixer was continuously turning. Having finished the solution, the mixer was kept turning for a further 10 minutes to maximise the mixing process. The feed was then air-dried overnight and put into labelled plastic bag. Due to a very large amount of feed required by the chickens, the mixing process was conducted every week during the experimental period.

Data collected including body weight and feed intake (recorded weekly), liver tissue collection for histological examination, and determination of gramine and hordenine in the liver. At the end of the experiment (seven weeks of age), animals were euthanased with an overdose of CO_2 gas. Small portion of liver (right lobe) were fixed in 10% neutral-buffered formalin solution for histological examination. The remainder of the livers were frozen at -20°C for later HPLC analysis of gramine and hordenine. The carcases were disposed of through the UNE Animal House disposal unit. Data were analysed using Analysis of Variance (ANOVA) at 5% level of confident.

Histological examination of chicken livers was carried out at the Histology Laboratory, department of Physiology, the University of New England. Five mm section of fixed liver was placed into labelled histological cassettes and placed into a jar containing 10% buffered formalin solution. The tissues were then ready for dehydration (Humason, 1972). Then the cassettes were transferred into a cup filled with molten wax for 30 minutes. Once the wax had solidified, the block of embedded tissue were trimmed to fit properly onto the microtome (Einz leitz, Germany). Three sections of µ5 m tissue were placed onto each slide glass prior to air-dry overnight and staining process according to Ehrlich's Haemotoxylin and Eosin (Humason, 1972). After staining, the samples were mounted by using Eukitt (O. Kindler, West Germany) and were ready to be examined under a microscope.

Gramine and hordenine 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. Ten µL aliquots of liver samples were injected into a Waters HPLC system consisting of a M40 pump (with flow rate 2 mL/min); U6K injector; UV/VIS Spectrophotometer (wavelength 221 nm). A Water's µBondapak Phenyl 10 µ (3'9 mm x 30 cm) was used as the column.

Results and Discussion

Body weight of broiler chickens increased significantly over the experimental period (P<0.001) in all treatment groups (Table 1). Although there were no significant differences between treatment groups, there was a significant interaction between treatment and age (P=0.001).

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.

Feed intake of chickens 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 consume the least (Figure 1).

Table 1. Body weight of chickens (g) six weeks after receiving different levels of alkaloids incorporated into diets

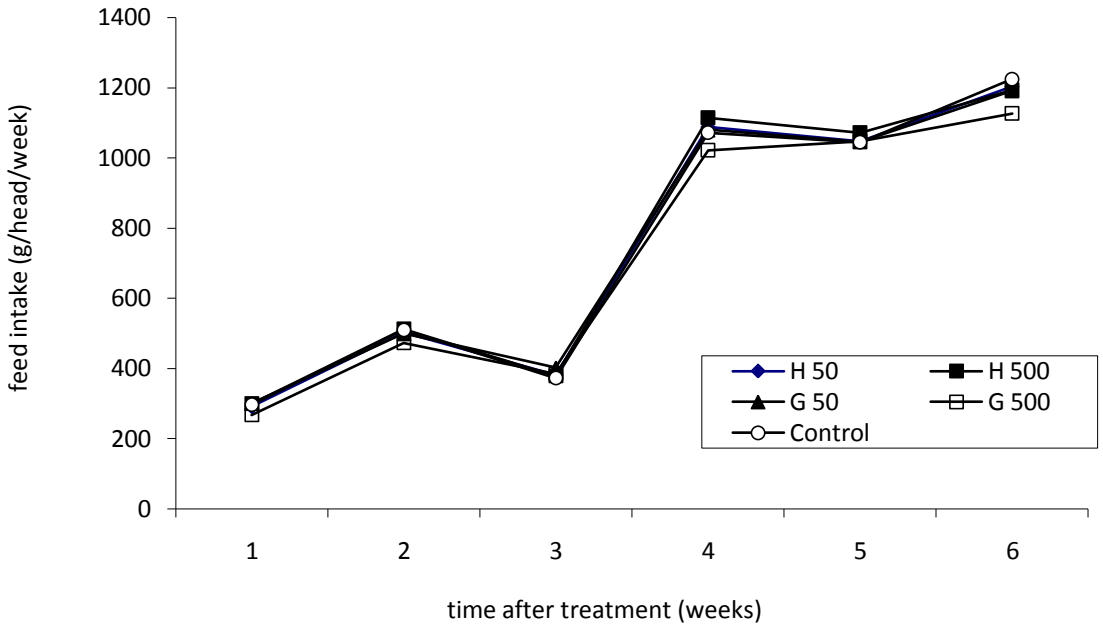
Group	Weeks after treatment						
	0	1	2	3	4	5	6
H 50	125.9^{a}±5.42	293.6^{a}±14.9	568.2^{a}±22.3	838.0^{a}±35.0	1340.1^{a}±34.4	1832.1^{a}±39.3	2356.8 ^{ab}±56.0
H 500	129.5^{a}±5.48	306.1^{a}±13.1	589.3^{a}±18.7	863.2^{a}±20.8	1332.5^{a}±16.4	1786.6^{a}±17.4	2276.9^{ab}±36.3
G 50	127.8^{a}±4.91	305.8^{a}±13.4	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}±34.5	2260.0^{b}±42.4
control	126.2^{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 bearing different superscript at the same column differ significantly (P<0.05)
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 or gramine in the feed)

Table 2. Gramine and hordenine content in chicken livers at seven weeks of age (µg/g fresh weight) receiving various concentrations of both alkaloids incorporated into diets

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 of alkaloids



(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 or gramine in the feed)

Figure 1. Amount of feed ingested by broiler chickens over seven weeks of age (Values are Mean ± SEM.

The mean content of gramine and hordenine in the chicken livers, recovered by HPLC analysis, are presented in Table 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. 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.

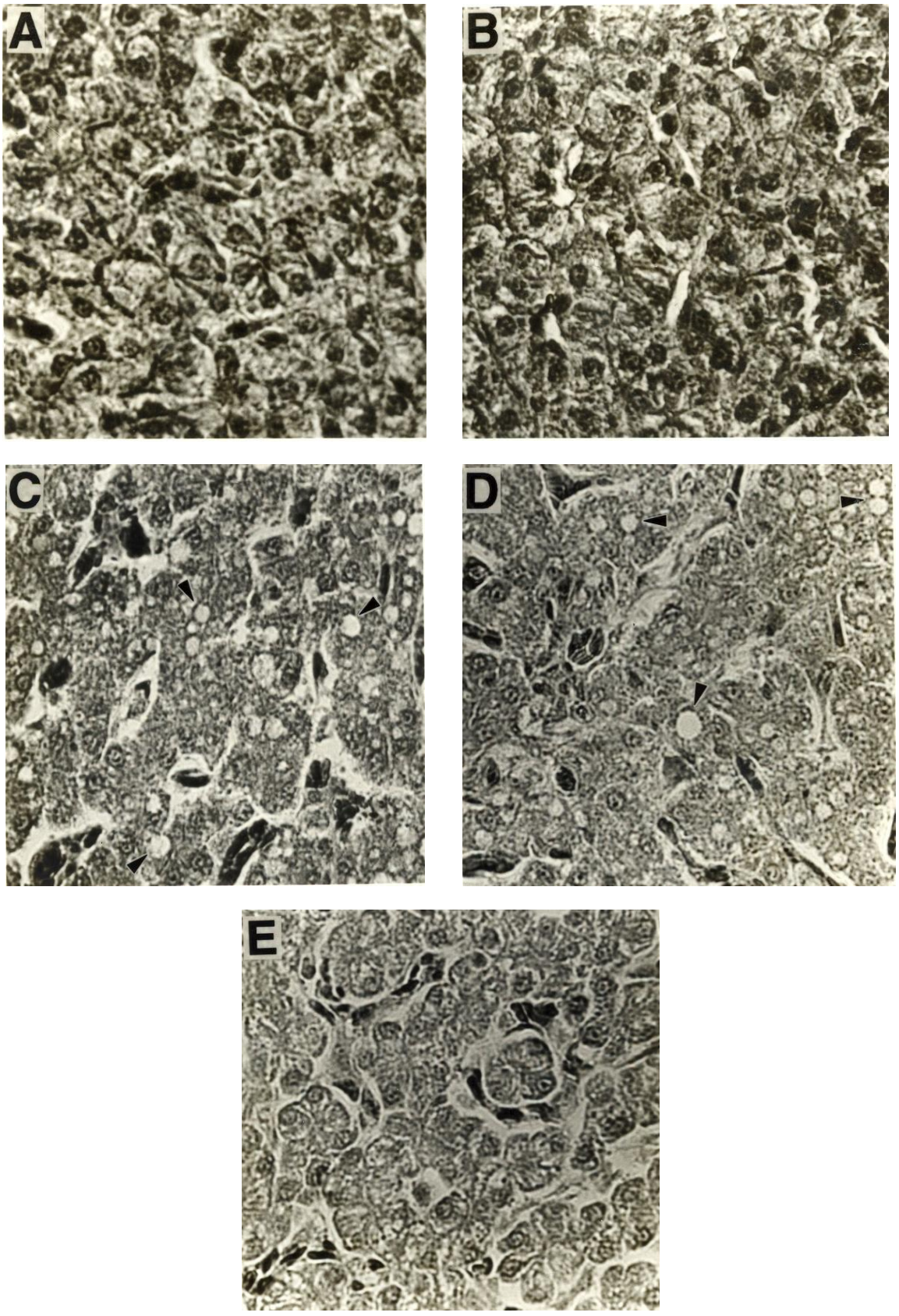
The livers of chickens exposed to low concentration (50 ppm) of gramine and hordenine did not show any abnormalities in the liver tissues. However, there was a difference between normal tissue from the group given standard feed and tissue from groups receiving high concentrations (500 ppm) of gramine 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 Figure 2. Interestingly, “scab-like” lesions were observed on the feed of chickens during approximately weeks four and five of the experiment.

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 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 chickens. The non-significant effects of gramine and hordenine on the growth of chickens might be due to a very low concentration of both alkaloids incorporated into diets. Therefore, it is assumed that the levels given were not enough to depress chicken growth rate. In addition, the effect of both alkaloids is dependent upon the age and state of health of animals and the length of time in which animal exposed to the alkaloid. Frank and Reed (1990) reported that alkaloid coniine administered at 50 mg/kg body weight to chickens resulted in 90% of morbidity in chickens. 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 feed. Factors such as the abundance and distribution of plants, their nutrient content, the digestive capability and capacity of the herbivore, the presence of the predators and learned behaviours can significantly influence feeding strategy of the animals (Mc Arthur et al., 1991)

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. One study showed that feed consumption of chickens decreased as the level of tannic acid increased in their diets. A level of 5% tannic acid caused chick mortality of about 70% between 7 and 11 days of the experiment (Vohra et al., 1966). As shown in Figure 1, 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 the chickens were removed from the large cage 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 the feed at the end of week 3 from chick starter feed to broiler finisher feed. However, over the last two weeks of the experiment, feed intake increased only slightly.

The amount of alkaloids recovered was associated with the treatment concentrations. The HPLC analysis indicates that the higher the concentration of alkaloids in feed, the higher their content in the livers. Histological examination showed that high concentrations of alkaloids caused liver damages as indicated by many vacuoles in the liver tissue. This finding demonstrates that both gramine and hordenine at high concentration (500 ppm) affected broiler chickens as indicated by liver abnormalities.

Microscopic examination of liver tissue clearly showed an abundance of vacuoles distributed evenly. This might be due to neutral fat 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. The ingestion of 500 ppm of gramine or 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 structures. However, the changes were not necessarily associated with reduced feed intake or growth rate of the animals. This finding agreed with the work of Chaniago (2004, unpublished thesis) that similar phenomenon of vacuolation in leaf tissue of soybean in response to aqueous



(A = 50 ppm gramine; B = 50 ppm hordenine; C = 500 ppm gramine; D = 500 ppm hordenine; E = standard feed as control. Arrows heads over white empty circles indicate some vacuoles)

Figure 2. Micrographs of livers from chickens receiving different levels of gramine and hordenine incorporated into feed (400 X magnification).

extract of nutgrass (*Cyperus rotundus* L.). Hepatic damages due to pyrrolizidine alkaloid were demonstrated by Peterson and Jago (1984) who found that 0.06% of the alkaloid caused 70% mortality in young rats. They concluded that this death was due to acute haemorrhagic necrosis and chronic liver damage.

The visible effects of 500 ppm gramine on chickens, Figure 3, were scab-like lesions on their feet during weeks 4 and 5 of the experiment. Similarly, a toxic phenol, hypericin, induced swelling, extreme itching oedema and cracking on the skin of livestock (Levin, 1976). Winter et al. (1990) reported unusual skin lesions in yaks (*Bos grunnies*) which were consistently associated with pyrrolozidine 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 keratinised epithelial cells. Therefore, it is possible that gramine had a similar effect on the feet of broiler chickens.

Conclusions

The main finding of this experiment was that gramine had more effects than hordenine on the animal tested. A high concentration (500 ppm) of either gramine or hoedenine was sufficient to induce changes in liver structure of the chickens as indicated by cell vacuolation even though they were not necessarily associated with reduced feed intake or growth rate of the animals. The present study and previous investigation on a number of different species indicate that barley has the potential for self-defence against other organisms through its biologically active secondary metabolites, gramina and hordenine.

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