CHAPTER

FOUR

EFFECTS OF GRAMINE AND HORDENINE ON INSECTS

4.1 INTRODUCTION

According to the results of Experiment 2 in Chapter 3, a barley cultivar containing a high gramine level (cv.Lara) showed significant negative effects on the growth and development of *M. convecta* when compared to a low-gramine cultivar (cv.Schooner). On the other hand, the study on effects of gramine when incorporated in artificial diet (Experiment 4 in Chapter 3) showed no significant negative effects on the insect. Therefore, the question now arises whether or not other allelochemicals in barley leaves may also have biological effects on the insect, and whether they have synergistic effects with gramine.

Hordenine, normally found in roots of barley, is a potential allelochemical that may enhance the effect of gramine on *M. convecta*. Liu (1991) demonstrated synergistic effects of the two allelochemicals in reducing radicle length of white mustard. Although some researchers have isolated hordenine only in the barley roots (Leete *et al.* 1952, Leete and Marion 1953, Frank and Marion 1956), this study found it in barley leaves (cv.Lara) at the concentration of about 60 ppm fresh weight (see Section 4.3.1). The presence of both substances in the leaves means that they could both be affecting foliage feeders such as *M. convecta*.

The objective of the work described in this chapter was to study the effects of gramine and hordenine when incorporated into the artificial diets, and their synergistic effects on insects. The insects used were *M. convecta*, *H. punctigera* and *A. ipsilon*. Although *H. punctigera* is not a pest of barley, it was used to provide a comparison with *M. convecta* and *A. ipsilon*. *H. punctigera* feeds almost exclusively on broad leaf plants (Zalucki *et al.* 1986, 1994). It therefore may not have evolved with gramine and hordenine, and perhaps similar alkaloids in other grasses, as have *M. convecta* and *A. ipsilon*. The concentrations of gramine and hordenine used were based on the concentrations found in leaves of cv.Lara (gramine 500 ppm and hordenine 60 ppm). The combination of gramine 500 ppm and hordenine 500 ppm was used because there was evidence that the synergistic effects of these allelochemicals on white mustard were higher when they were combined in equal concentrations (Liu 1991).

4.2 MATERIALS AND METHODS

4.2.1 Hordenine in barley leaves

Barley plants, cv.Lara, were sown in 15 cm diameter pots on 15th May 1992 in the glasshouse maintained at 20-30°C (barley from Experiment 2 in Chapter 3). The leaves were analysed for hordenine by using High Performance Liquid Chromatography techniques. The method for extraction and quantification was that used by Hoult and Lovett (1993) (see Section 4.2.5). Barley leaves used for analysis were 8 days old and were cut from different pots.

4.2.2 Experiment 5 - Effect of gramine and hordenine on armyworms reared on artificial diets

This experiment was conducted in the insectary at 25°C and day length 16 hours per day. The experiment commenced on 23rd July 1993 and finished on 5th October 1993.

There were 5 treatments in this experiment:

Treatment 1 gramine 500 ppm
Treatment 2 hordenine 60 ppm
Treatment 3 gramine 500 ppm+hordenine 60 ppm
Treatment 4 gramine 500 ppm+hordenine 500 ppm
and Treatment 5 control (no gramine and hordenine)

Gramine and hordenine for each treatment were dissolved in 10 ml of ethanol and mixed into the armyworm artificial diet (Appendix A). The ethanol concentration in the final diet was approximately 1%. The same volume of ethanol, without gramine and hordenine, was also added to control diet. In order to avoid the degradation of gramine from high temperature, the solvent of each treatment was mixed into the artificial diet when the diet was still liquid, but after the temperature fell below 50°C.

Larvae used in this experiment were neonates from egg masses laid by moths of *M. convecta*, fed on a 5% sucrose solution via soaked dental wicks. The moths were the adults of larvae reared on armyworm artificial diet (Appendix A) using the same method as in Chapter 3 - Experiment 1. Both larvae and moths were reared in the insectary at 25°C, 16:8 L:D. Thirty neonate larvae per treatment were put individually in clear 35-

ml plastic cups with holes in the lids, and placed in the insectary in a completely randomised design.

Diets were changed every week and both new and old diets of each batch of each treatment were kept for gramine and hordenine analysis until the larvae pupated. Pupae were placed individually into clear 35-ml plastic cups and covered with a 20 mm layer of moistened vermiculite to maintain moisture. Data on survival, date of pupation, weight of pupae, sex of pupae and date of adult emergence were collected.

4.2.3 Experiment 6 - Effect of gramine and hordenine on *H. punctigera* reared on artificial diets

This experiment was conducted in the insectary at 25°C, 16:8 L:D. The experiment started on 7th February 1994 and was completed on 6th April 1994.

There were 6 treatments in this experiment. Treatments 1-5 were the same as in Experiment 5. Treatment 6 was control without ethanol. This treatment was added to the experiment in order to observe the effect of ethanol, which was mixed in artificial diets as a solvent for the allelochemicals, on survival, growth and development of the insect. 10 ml of ethanol was used for each treatment and was added to treatment 5 (control) without gramine and hordenine. These solutions were mixed into *Helicoverpa* diet (Appendix F) when the diet was still liquid but after the temperature fell below 50°C for the same reason as in Experiment 5

The larvae used for this experiment were neonates from egg masses laid by moths of *H. punctigera*, fed on 5% sucrose solution via soaked dental wicks. The moths were the adults of larvae reared on *Helicoverpa* artificial diet (Appendix F) in the insectary. The method used for rearing the insect was as described in Chapter 3-Experiment 1, except the pupae were left in the diet until adult emergence. This was done because the mortality was higher when they were removed and then covered with a layer of moistened vermiculite, as in the earlier experiment.

Forty neonate larvae per treatment were put individually in clear 35-ml plastic cups with holes in the lids and were placed in the insectary in a completely randomised design. Diets were changed every week and both new and old diets of each batch of each treatment were kept for gramine and hordenine analysis until the larvae pupated. Pupae were left in the diets until adult emergence.

Data on survival and dead larvae were collected. The dead larvae were classified into four categories using the following scale (Anon 1994):

VS = very small (<0.3 cm in body length)
S = small (0.3-0.7 cm in body length)
M = medium (0.7-2.0 cm in body length)
and L = large (>2.0 cm in body length)

This scale of classification was used because there are no reliable data relating head widths to instar number, in contrast to the situation with *M. convecta* (Sections 3.3.1.2 and 3.3.2.2).

Larvae which reached the pupal stage were then classified as follows:

A = the larva died during pupation
B = the larva developed into a malformed pupa
C = the larva successfully pupated but died before adult emergence
D = the larva underwent successful pupation but developed into severely malformed adult or the adult did not eclose completely from the pupal case
E = the larva underwent successful development through to the adult stage

Data on days to pupation, weight of pupae, sex of pupae and days to adult emergence were recorded for each pupa.

4.2.4 Experiment 7 - Effect of gramine and hordenine on *A. ipsilon* reared on artificial diets

This experiment was conducted in the same insectary as Experiments 5 and 6 at 25°C and 16:8 L:D. It commenced on 13th May 1994 and finished on 12th July 1994.

The treatments were the same as in Experiment 6. Gramine for each treatment was dissolved in 10 ml of ethanol while hordenine for each treatment was dissolved in water, because it dissolved better in water than in ethanol. Treatment 5 (control without gramine and hordenine) had 10 ml of ethanol, whereas no ethanol was added to the control treatment 6. The solution for each treatment was mixed into the armyworm artificial diet (Appendix A) using the method described in Section 4.2.2.

The larvae used for this experiment were neonates from egg masses laid by moths of *A. ipsilon*, fed on 5% sucrose solution via soaked dental wicks. The moths

were the adults of larvae reared on armyworm artificial diet (Appendix A) in the insectary. The method used for rearing was as described in Chapter 3-Experiment 1, except the pupae were left in the diet until adult emergence. These moths were the first laboratory generation from moths collected in the field from a light trap at University of New England in April 1994.

There were forty neonate larvae per treatment, put individually in clear 35-ml plastic cups with holes in the lids. The larvae were put on the diet on 13th May 1994 and placed in the insectary in a completely randomised design.

Diets were changed every week and both new and old diets of each batch of each treatment were kept for gramine and hordenine analysis until pupation. Pupae were left in the diets until adult emergence.

Data on survival and dead larvae were collected. The dead larvae were classified into four categories using a scale that was modified from the *Helicoverpa* scale by comparing the body length of final instar larvae of *Helicoverpa* and *Agrotis*. The body length of *Agrotis* larvae was found average 0.94 of the body length of *Helicoverpa* larvae (the mean body length of *Helicoverpa* and *Agrotis* larvae were 3.456 and 3.244 cm, respectively and the number of larvae measured was 9 larvae for each species). Therefore, the scale for *Agrotis* larvae was as follows:

VS = very small (<0.28 cm in body length)
S = small (0.28-0.66 cm in body length)
M = medium (0.66-1.88 cm in body length)
and L = large (>1.88 cm in body length)

Larvae surviving to the pupal stage were classified into A-E categories as described in Experiment 6. In addition, data on days to pupation, weight of pupae, sex of pupae and days to adult emergence were recorded for each pupa.

4.2.5 Gramine and hordenine analysis

Gramine and hordenine concentrations in diets of the experiments in this chapter were measured by HPLC using the method of Hoult and Lovett (1993). The extraction process of gramine and hordenine was the same as in Section 3.2.5 of Chapter 3 and the purification procedure was as follows;

acetonitrile (ACN) 2 ml
|
0.001 M KH_2PO_4 pH 7 2 ml
|
aliquot applied
|
0.05 M KH_2PO_4 pH 9.5 / isopropanol (85:15) 2 ml
|
0.05 M KH_2PO_4 pH 2.3 / isopropanol (70:30) 1.5 ml

As with Section 3.2.5, acetonitrile and 0.001 M KH_2PO_4 were used for preparing Sep-pak cartridges before using the sample. For barley leaves and Experiment 5, the final eluates were evaporated to dryness under nitrogen at 40°C. For Experiments 6 and 7, the final eluates were evaporated to dryness using an Evaporator concentrator. Then these dried samples were taken up in 1 or 10 ml mobile phase (0.025 M KH_2PO_4 + 0.1%TEA pH 7.15/ ACN 2/1) depending on the concentrations of gramine and hordenine in the samples (1 ml for control diets, 10 ml for the others, and 10 ml for barley leaves). Ten µl samples were injected into the Waters HPLC system comprising M40 pump, flowrate 2 ml/min, U6K injector, Water's µBondapak Phenyl column 10 µ (3.9 mm x 300 mm) and UV vis spectrophotometer, wavelength 219 nm. Elution was isocratic (the mobile phase). Gramine and hordenine in samples were identified by retention time relative to the authentic compounds and quantification of both compounds was by peak area using a Waters 745 data module.

4.2.6 Analysis of data

In each experiment, data on the survival of the insects and the sex ratio were analysed by χ^2 analysis. Data on time to pupation, weight of pupae, time to adult emergence and duration of pupal stage of insects were analysed by analysis of variance and the general linear model (GLM) using the MINITAB statistical package (as in Section 3.2.6). The general linear model was used when the data were unbalanced, which often happened because mortality varied between treatments. The differences in means between treatments were compared by using Bonferroni Multiple Comparisons Procedure (Devore and Peck 1986) whenever appropriate. This procedure allows differences in means from varying sample sizes to be tested, unlike other methods such as Duncans multiple range tests.

4.3 RESULTS

4.3.1 Hordenine in barley leaves

Hordenine concentration found in barley leaves varied from 31.80 to 101.24 ppm fresh weight and mean hordenine concentration was 61.75 ppm (Table 4.1). Although barley leaves used were the same age, hordenine concentrations varied between replications. This may be due to the effect of shading on the production of allelochemicals (Rice 1984) as barley leaves were cut from different pots and these pots were placed one behind the other in the glasshouse.

Table 4.1. Hordenine concentration (ppm) in barley leaves (cv.Lara)

Rep	Hordenine content (ppm fresh wt.)
1	60.72
2	31.80
3	53.25
4	101.24
Mean±se	61.75±14.52

4.3.2 Experiment 5 - Effect of gramine and hordenine on armyworms reared on artificial diets

4.3.2.1 Survival to the pupal stage

There were highly significant differences ($p<0.005$) in survival to the pupal stage of *M. convecta* reared on artificial diets containing different concentrations of gramine and hordenine. The lowest and the highest survival were those of pupae from larvae reared on diets containing gramine 500 ppm+hordenine 60 ppm and control, respectively (Table 4.2).

Table 4.2. Survival to the pupal stage of *Mythimna convecta* reared on artificial diets containing gramine and hordenine.

Treatments	Survived	Died	Total
1. 500 ppm gramine	16 (10)	14 (20)	30
2. 60 ppm hordenine	11 (10)	19 (20)	30
3. 500 ppm gramine + 60 ppm hordenine	0 (10)	30 (20)	30
4. 500 ppm gramine + 500 ppm hordenine	4 (10)	26 (20)	30
5. control	19 (10)	11 (20)	30
Total	50	100	150

The numbers in parenthesis are expected counts based on the null hypothesis. The treatments had highly significant differences (χ^2 = 38.1, df = 4, p<0.005). Initial N was 30.

Further χ^2 tests using 2x2 contingency tables gave significant differences from the control for hordenine 60 ppm (χ^2 = 4.27, p<0.05), gramine 500 ppm+hordenine 60 ppm (χ^2 = 27.80, p<0.001) and gramine 500 ppm+hordenine 500 ppm (χ^2 = 15.86, p< 0.001), all on 1 df. There was no significant difference for gramine 500 ppm.

4.3.2.2 Survival to the adult stage

There were also highly significant differences (p<0.005) in survival to the adult stage of *M. convecta* reared on artificial diets containing different concentrations of gramine and hordenine. The lowest and the highest survival were those of adults from pupae of larvae reared on diets containing gramine 500 ppm+hordenine 60 ppm and control, respectively (Table 4.3).

Table 4.3. Survival to the adult stage of *Mythimna convecta* reared on artificial diets containing gramine and hordenine.

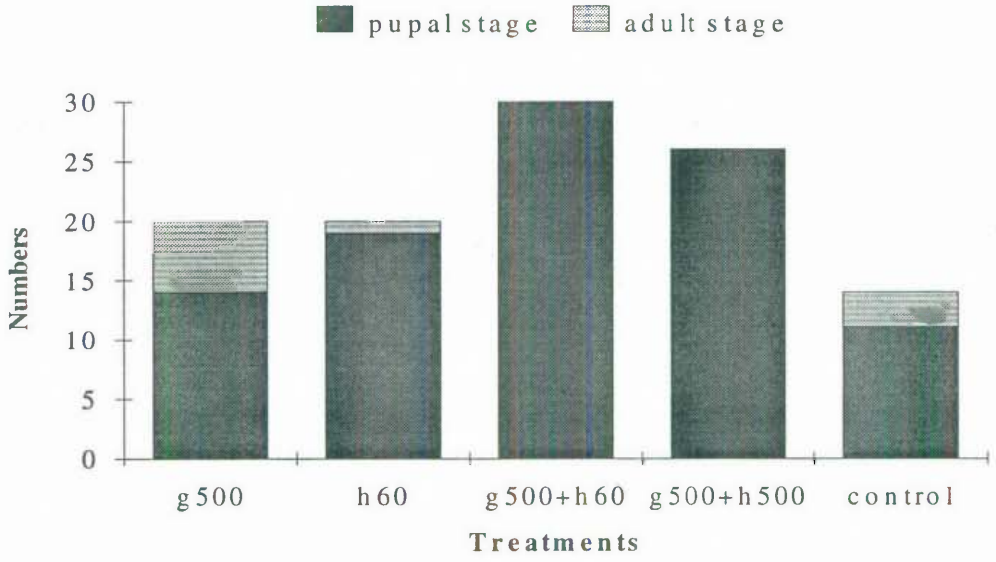
Treatments	Survived	Died	Total
1. 500 ppm gramine	10 (8)	20 (22)	30
2. 60 ppm hordenine	10 (8)	20 (22)	30
3. 500 ppm gramine + 60 ppm hordenine	0 (8)	30 (22)	30
4. 500 ppm gramine + 500 ppm hordenine	4(8)	26 (22)	30
5. control	16(8)	14 (22)	30
Total	40	110	150

The numbers in parenthesis are expected counts based on the null hypothesis. The treatments had highly significant differences (χ^2 = 25.91, df = 4, p<0.005). Initial N was 30.

Further χ^2 tests using 2x2 contingency tables gave significant differences from the control for gramine 500 ppm+hordenine 60 ppm ($\chi^2 = 21.82$, $p<0.001$) and gramine 500 ppm+hordenine 500 ppm ($\chi^2 = 10.80$, $p<0.005$), both on 1 df, but not the other treatments.

Figure 4.1 illustrates the results presented in Tables 30 and 31 showing that most insects died before the pupal stage and the highest mortality was found in diets containing both gramine and hordenine.

Figure 4.1. Numbers of dead pupae and adults of *Mythimna convecta* reared on artificial diets containing gramine and hordenine (g500 = gramine 500 ppm, h60 = hordenine 60 ppm, g500+h60 = gramine 500 ppm+hordenine 60 ppm, g500+h500 = gramine 500 ppm+hordenine 500 ppm).



4.3.2.3 Time to pupation

There were highly significant differences in time to pupation of *M. convecta* reared on artificial diets containing different gramine and hordenine concentrations ($p = 0.001$). Using the Bonferroni Multiple Comparisons Procedure, it was found that there was a significant difference between gramine 500 ppm and control but not between any other two treatments (Table 4.4).

Table 4.4. Time to pupation (days) of *Mythimna convecta* reared on artificial diets containing gramine and hordenine.

Treatments	n	Time to pupation (Mean±se)
1. 500 ppm gramine	16	40.75±1.48 ^{b}
2. 60 ppm hordenine	11	36.91±1.93 ^{ab}
3. 500 ppm gramine + 60 ppm hordenine	-	-
4. 500 ppm gramine + 500 ppm hordenine	4	41.25±2.56 ^{ab}
5. control	19	33.74±0.58 ^{a}

The treatments had highly significant differences ($p = 0.001$) by one-way analysis of variance and means bearing the same superscript were not significantly different by the Bonferroni Multiple Comparisons Procedure (Devore and Peck 1986).

There was no significant difference (by one-way analysis of variance) in time to pupation of male and female *M. convecta* from all treatments combined ($p = 0.829$). Mean time to pupation of male and female *M. convecta* was 37.09 and 37.44 days, respectively (Table 4.5).

Table 4.5. Time to pupation (days) of male and female *Mythimna convecta* reared on artificial diets (all treatments combined).

Sexes	n	Time to pupation (Mean±se)
Males	23	37.09±1.12
Females	27	37.44±1.19

non significant ($p = 0.829$) by one-way analysis of variance

The general linear model showed that there were highly significant differences ($p = 0.001$) between treatments in time to pupation. But, as in the one-way analysis of variance, time to pupation was not significantly different between the sexes ($p = 0.627$). There was no significant interaction between treatment and the sex ($p = 0.510$) (Table 4.6).

Table 4.6. GLM analysis of time to pupation of *Mythimna convecta* reared on artificial diets containing gramine and hordenine.

SOURCE	DF	Seq SS	Adj SS	Adj MS	F	P
TREAT	3	495.74	476.47	158.82	6.24	0.001
SEX	1	0.00	6.10	6.10	0.24	0.627
TREAT*SEX	3	59 81	59.81	19.94	0.78	0.510
ERROR	42	1068.52	1068.52	25.44		
TOTAL	49	1624.08				

4.3.2.4 Weight of pupae

There were no significant differences (p = 0.792) in weight of pupae of *M. convecta* reared on artificial diets with gramine 500 ppm, hordenine 60 ppm, gramine 500 ppm+hordenine 500 ppm and control (Table 4.7).

Table 4.7. Weight of pupae of *Mythimna convecta* (mg) reared on artificial diets containing gramine and hordenine.

Treatments	n	Weight of pupae (Mean±se)
1. 500 ppm gramine	16	371.37±10.68
2. 60 ppm hordenine	11	355.09±19.35
3. 500 ppm gramine + 60 ppm hordenine	-	-
4. 500 ppm gramine + 500 ppm hordenine	4	364.00±21.05
5. control	19	355.79±11.31

non significant (p = 0.792) by one-way analysis of variance

There was no significant difference (p = 0.216) in weight of pupae of male and female *M. convecta* from all treatments combined (Table 4.8). Mean weight of pupae of male and female insects were 351.83 and 369.33 milligrams, respectively.

Table 4.8. Weight of pupae (mg) of male and female *Mythimna convecta* reared on artificial diets (all treatments combined).

Sexes	n	Weight of pupae (Mean±se)
Males	23	351.83±9.46
Females	27	369.33±10.07

non significant ($p = 0.216$) by one-way analysis of variance

The general linear model showed that there were no effects on weight of pupae of treatment (p = 0.862), sex (p = 0.412) or the interaction between treatment and the sex (p = 0.813) (Table 4.9).

Table 4.9. GLM analysis of weight of pupae of *Mythimna convecta* reared on artificial diets containing gramine and hordenine.

SOURCE	DF	Seq SS	Adj SS	Adj MS	F	P
TREAT	3	2654	1980	660	0.25	0.862
SEX	1	3744	1817	1817	0.69	0.412
TREAT*SEX	3	2520	2520	840	0.32	0.813
ERROR	42	111348	111348	2651		
TOTAL	49	120266				

4.3.2.5 Sex ratio

There were no significant differences between treatments in the sex ratio of pupae (p>0.75) and of adults (p>0.95) (Table 4.10).

Table 4.10. Sex ratio of *Mythimna convecta* pupae and adults from larvae reared on artificial diets containing gramine and hordenine.

Treatments	Sex ratio (pupae)		Sex ratio (adults)	
	Males	Females	Males	Females
1. 500 ppm gramine	7	9	5	5
2. 60 ppm hordenine	4	7	4	6
3. 500 ppm gramine + 60 ppm hordenine	-	-	-	-
4. 500 ppm gramine + 500 ppm hordenine	2	2	2	2
5.control	10	9	8	8

The treatments were not significantly different (χ^2 = 0.81, df = 3, p>0.75 for sex ratio of pupae, χ^2 = 0.30, df = 3, p>0.95 for sex ratio of adults)

4.3.2.6 Time to adult emergence

There were highly significant differences in time to adult emergence of *M. convecta* reared on artificial diets containing different gramine and hordenine concentrations (p = 0.003). The Bonferroni Multiple Comparisons Procedure showed significant differences between gramine 500 ppm and control, and also gramine 500 ppm+hordenine 500 ppm and control (Table 4.11).

Table 4.11. Time to adult emergence (days) of *Mythimna convecta* reared on artificial diets containing gramine and hordenine.

Treatments	n	Time of adult emergence (Mean±se)
1. 500 ppm gramine	10	53.60±1.82 ^{b}
2. 60 ppm hordenine	10	50.80±2.19 ^{ab}
3. 500 ppm gramine + 60 ppm hordenine	-	-
4. 500 ppm gramine + 500 ppm hordenine	4	54.50±2.63 ^{b}
5. control	16	46.44±0.65 ^{a}

The treatments had highly significant differences (p = 0.003) by one-way analysis of variance and means bearing the same superscript were not significantly different by the Bonferroni Multiple Comparisons Procedure (Devore and Peck 1986).

Time to adult emergence of males and females from all treatments combined was not significantly different (p = 0.436) (Table 4.12).

Table 4.12. Time to adult emergence (days) of male and female *Mythimna convecta* reared on artificial diets (all treatments combined).

Sexes	n	Time of adult emergence (Mean±se)
Males	19	50.90±1.36
Females	21	49.43±1.27

non significant (p = 0.436) by one-way analysis of variance

The general linear model showed that there were highly significant differences (p = 0.005) between treatments in time to adult emergence of *M. convecta*. There were no significant effects of sex (p = 0.309) or the interaction between treatment and the sex (p = 0.813) (Table 4.13).

Table 4.13. GLM analysis of time to adult emergence of *Mythimna convecta* reared on artificial diets containing gramine and hordenine.

SOURCE	DF	Seq SS	Adj SS	Adj MS	F	P
TREAT	3	419.44	420.28	140.09	5.18	0.005
SEX	1	23.65	28.88	28.88	1.07	0.309
TREAT*SEX	3	25.67	25.67	8.56	0.32	0.813
ERROR	32	865.62	865.62	27.05		
TOTAL	39	1334.37				

The pattern of the results with time to adult emergence was similar to that with time to pupation. This result might be expected since time to pupation is an important component of time to emergence.

4.3.2.7 Duration of pupal stage

There were no significant differences in duration of pupal stage of *M. convecta* reared on artificial diets containing different gramine and hordenine concentrations (Table 4.14).

Table 4.14. Duration of pupal stage (days) of *Mythimna convecta* reared on artificial diets containing gramine and hordenine.

Treatments	n	Duration of pupal stage (Mean±se)
1. 500 ppm gramine	10	13.40±0.22
2. 60 ppm hordenine	10	13.40±0.27
3. 500 ppm gramine + 60 ppm hordenine	-	-
4. 500 ppm gramine + 500 ppm hordenine	4	13.25±0.48
5. control	16	13.00±0.22

The treatments had no significant differences ($p = 0.576$) by one-way analysis of variance.

There was significant difference ($p = 0.027$) in duration of pupal stage of male and female *M. convecta* reared on artificial diets when data from all treatments were combined. Males had a slightly longer duration of pupal stage than females (Tables 4.15 and 4.16).

Table 4.15. Duration of pupal stage (days) of male and female *Mythimna convecta* reared on artificial diets (all treatments combined).

Sexes	n	Duration of pupal stage (Mean±se)
Males	19	13.53±0.14
Females	21	12.95±0.20

Table 4.16. Analysis of variance on duration of pupal stage of male and female *Mythimna convecta* reared on artificial diets (all treatments combined).

SOURCE	DF	SS	MS	F	P
SEXES	1	3.286	3.286	5.27	0.027
ERROR	38	23.689	0.623		
TOTAL	39	26.975			

The general linear model showed that there were no significant effects on duration of pupal stage of treatment ($p = 0.432$), sex ($p = 0.060$) or the interaction between treatment and the sex ($p = 0.550$) (Table 4.17). However, the effect of sex was almost significant. The fact that the significance of sex found by one-way analysis of

variance disappeared in the GLM analysis indicates some degree of confounding between treatment and sex.

Table 4.17. GLM analysis of duration of pupal stage of *Mythimna convecta* reared on artificial diets containing gramine and hordenine.

SOURCE	DF	Seq SS	Adj SS	Adj MS	F	P
TREAT	3	1.83	1.83	0.61	0.94	0.432
SEX	1	3.52	2.45	2.45	3.80	0.060
TREAT*SEX	3	1.38	1.38	0.46	0.71	0.550
ERROR	32	20.65	20.65	0.65		
TOTAL	39	26.98				

4.3.2.8 Gramine and hordenine concentration in artificial diets

For treatment 1 (gramine 500 ppm), gramine concentration in new diets (the diets before feeding by larvae) varied from 512.4 to 537.4 ppm fresh weight (2069.4 to 2155.6 ppm dry weight) and hordenine concentration in new diets varied from 0.0 to 2.0 ppm fresh weight (0.0 to 8.0 ppm dry weight). The old diets (the diets left from larvae feeding) of treatment 1 had gramine concentrations from 561.5 to 607.2 ppm fresh weight (1911.9 to 2172.6 ppm dry weight) and hordenine concentrations from 3.4 to 6.8 ppm fresh weight (11.6 to 24.5 ppm dry weight).

The new diets of treatment 2 (hordenine 60 ppm) had gramine concentrations from 0.4 to 1.4 ppm fresh weight (2.0 to 5.8 ppm dry weight) and hordenine concentrations from 12.1 to 18.3 ppm fresh weight (54.4 to 80.8 ppm dry weight). The old diets of treatment 2 had gramine concentrations from 1.3 to 1.6 ppm fresh weight (5.4 to 6.5 ppm dry weight) and hordenine concentrations from 9.5 to 16.2 ppm fresh weight (33.9 to 65.2 ppm dry weight).

Treatment 3 (gramine 500 ppm+hordenine 60 ppm) had gramine concentrations in new diets from 506.0 to 534.5 ppm fresh weight (2191.5 to 2192.0 ppm dry weight) and hordenine concentrations in new diets from 16.9 to 32.0 ppm fresh weight (72.9 to 131.1 ppm dry weight). For the old diets, gramine concentration varied from 548.2 to 589.1 ppm fresh weight (2044.6 to 2165.2 ppm dry weight) and hordenine concentration varied from 30.2 to 77.3 ppm fresh weight (119.1 to 268.1 ppm dry weight). There was no third batch of diets for this treatment because of the high mortality of larvae.

Treatment 4 (gramine 500 ppm+hordenine 500 ppm) had gramine concentrations in new diets from 476.1 to 537.4 ppm fresh weight (2036.2 to 2188.3 ppm dry weight) and hordenine concentrations in new diets from 69.0 to 135.0 ppm fresh weight (295.0 to 547.6 ppm dry weight). Gramine concentration in the old diets varied from 546.9 to 559.4 ppm fresh weight (1963.3 to 2115.7 ppm dry weight) and hordenine concentration varied from 45.8 to 59.1 ppm fresh weight (177.1 to 206.8 ppm dry weight). As with Treatment 3, there was no third batch of diets for this treatment because of the high mortality of larvae.

Gramine and hordenine concentration in the new diets of treatment 5 (control) varied from 0.4 to 1.2 ppm fresh weight (1.8 to 4.8 ppm dry weight) and 1.7 to 3.1 ppm fresh weight (7.5 to 13.3 ppm dry weight), respectively. For the old diets, gramine and hordenine concentration varied from 0.4 to 1.9 ppm fresh weight (1.4 to 7.4 ppm dry weight) and 2.2 to 3.0 ppm fresh weight (8.1 to 11.7 ppm dry weight), respectively (Figure 4.2 and Appendix K).

Figure 4.2. Gramine and hordenine concentration (ppm) in artificial diet used for feeding *Mythimna convecta* (The concentrations of gramine and hordenine at the 1st, 12th, 13th, 26th, 27th and 33rd days were the concentrations of the first batch/new diet, the first batch/old diet, the second batch/new diet, the second batch/old diet, the third batch/new diet and the third batch/old diet, respectively). ———— fresh weight ---------- dry weight

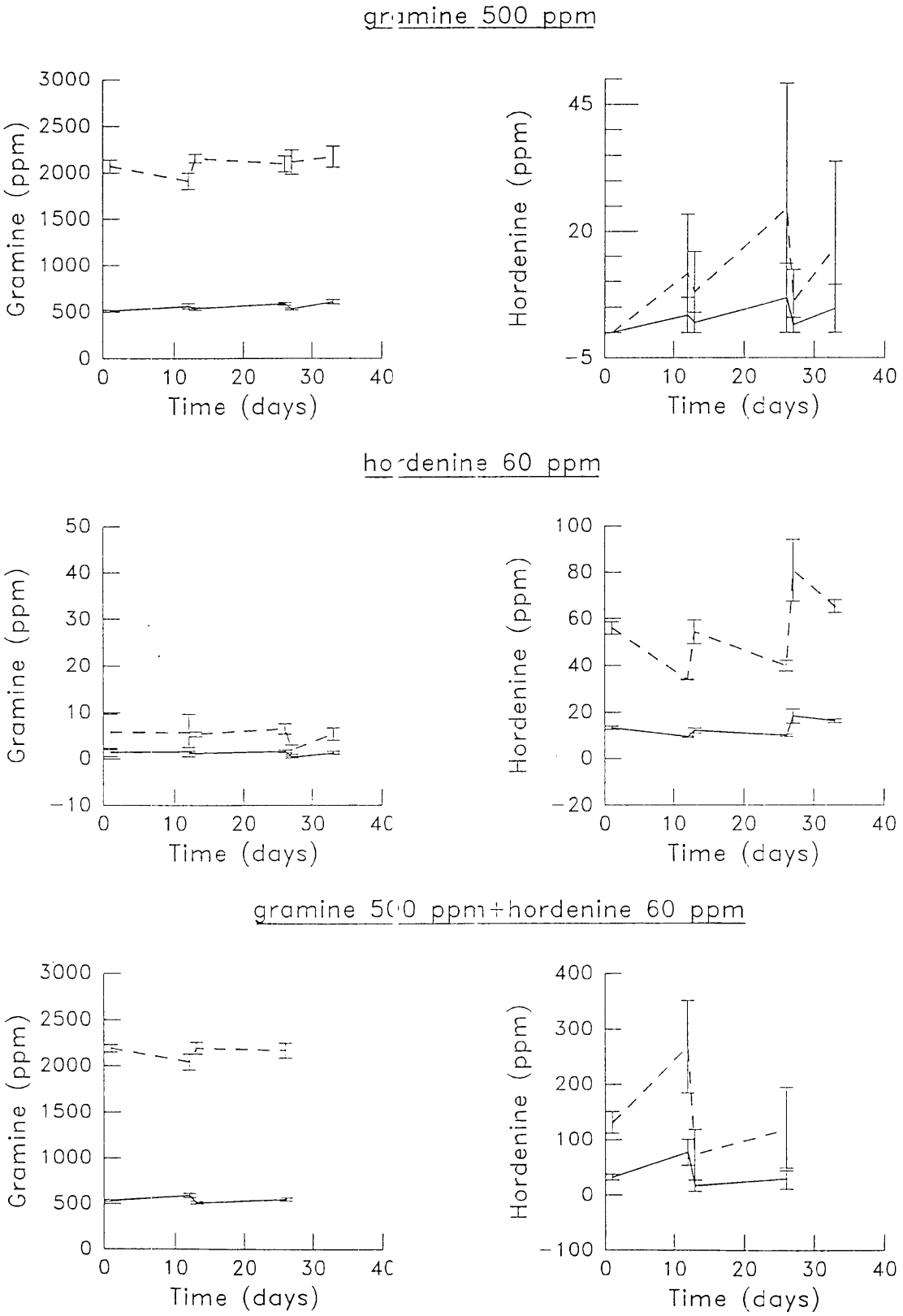
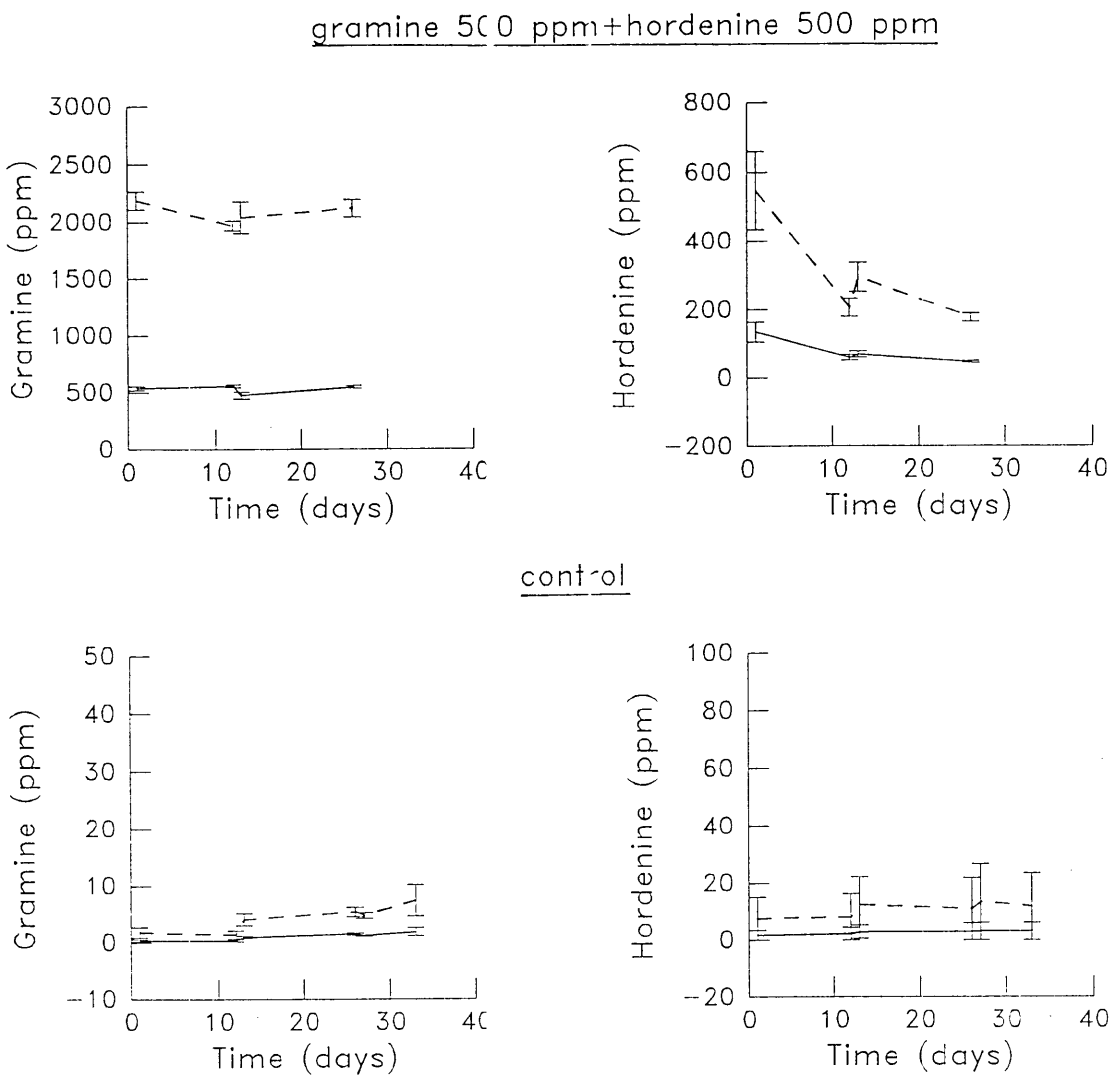


Figure 4.2. (continued)



4.3.3 Experiment 6 - Effect of gramine and hordenine on *H. punctigera* reared on artificial diets

4.3.3.1 Survival

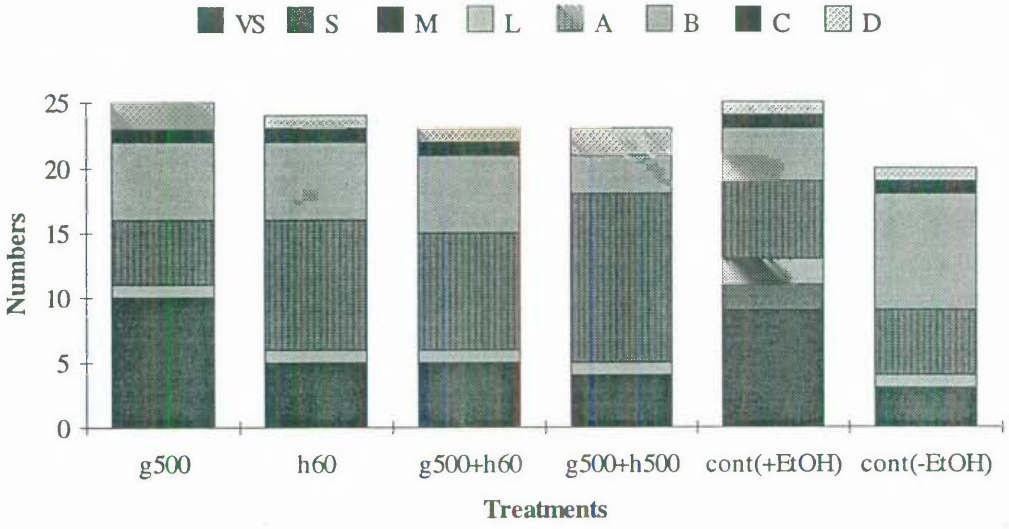
Numbers of *H. punctigera* surviving to each category are shown in Table 4.18.

Table 4.18. Survival numbers of *Helicoverpa punctigera* reared on artificial diets containing gramine and hordenine (Numbers surviving to stages: VS = very small larva, S = small larva, M = medium larva, L= large larva, A = larva died during pupation, B = malformed pupa, C = died before adult emergence and D = severely malformed adult). Initial N was 40.

Treatments	VS	S	M	L	A	B	C	D
1. 500 ppm gramine	30	30	30	29	24	18	17	15
2. 60 ppm hordenine	35	35	35	34	24	18	17	16
3. 500 ppm gramine + 60 ppm hordenine	35	35	35	34	25	19	18	17
4. 500 ppm gramine + 500 ppm hordenine	36	36	36	35	22	19	19	17
5. control (with ethanol)	31	29	29	27	21	17	16	15
6. control (without ethanol)	37	37	37	36	31	22	21	20

Figure 4.3 shows that most insects died when they were very small and during pupation. The number of dead insects was lowest in control diet without ethanol.

Figure 4.3. Numbers of dead larvae, dead pupae, malformed pupae, dead adults and malformed adults of *Helicoverpa punctigera* reared on artificial diets containing gramine and hordenine (VS = very small larva; S = small larva; M = medium larva; L= large larva; A = larva died during pupation; B = malformed pupa; C = died before adult emergence; and D = severely malformed adult, g500 = gramine 500 ppm; h60 = hordenine 60 ppm; g500+h60 = gramine 500 ppm+hordenine 60 ppm; g500+h500 = gramine 500 ppm+hordenine 500 ppm; cont(+EtOH) = control with ethanol; and cont(-EtOH) = control without ethanol).



A χ^2 analysis of the numbers surviving to the large larvae stage is shown in Table 4.19.

Table 4.19. Survival of *Helicoverpa punctigera* (large larvae) reared on artificial diets containing gramine and hordenine.

Treatments	Survived	Died	Total
1. 500 ppm gramine	29 (32.5)	11 (7.5)	40
2. 60 ppm hordenine	34 (32.5)	6 (7.5)	40
3. 500 ppm gramine + 60 ppm hordenine	34 (32.5)	6 (7.5)	40
4. 500 ppm gramine + 500 ppm hordenine	35 (32.5)	5 (7.5)	40
5. control (with ethanol)	27 (32.5)	13 (7.5)	40
6. control (without ethanol)	36 (32.5)	4 (7.5)	40
Total	195	45	240

The numbers in parenthesis are expected counts based on the null hypothesis, the numbers that died are cumulative to the large larval stage. The treatments were not significantly different (χ^2 = 10.75, df = 5, p> 0.05). Initial N was 40.

Further χ^2 tests using 2x2 contingency tables showed significant differences from the control (without ethanol) treatment for gramine 500 ppm (χ^2 = 4.02, $p<0.05$) and the control with ethanol (χ^2 = 6 05, $p<0.025$), both on 1 df.

Similar χ^2 analyses were carried out on cumulative mortality to earlier developmental stages, and all gave no significant differences between treatments (χ^2 = 7.84 ($p>0.10$) for VS larvae, 10.38 ($p>0.05$) for S and 10.38 ($p>0.05$) for M, all on 5 df).

An analysis of the number of larvae which survived the pupal period is shown in Table 4.20.

Table 4.20. Survival of *Helicoverpa punctigera* (larva died during pupation) reared on artificial diets containing gramine and hordenine.

Treatments	Survived	Died	Total
1. 500 ppm gramine	24 (21.9)	5 (7.1)	29
2. 60 ppm hordenine	24 (25.6)	10 (8.4)	34
3. 500 ppm gramine + 60 ppm hordenine	25 (25.6)	9 (8.4)	34
4. 500 ppm gramine + 500 ppm hordenine	22 (26.4)	13 (8.6)	35
5. control (with ethanol)	21 (20.4)	6 (6.7)	27
6. control (without ethanol)	31 (27.1)	5 (8.9)	36
Total	147	48	195

The numbers in parenthesis are expected counts based on the null hypothesis. The treatments were not significantly different (χ^2 = 6.61, df = 5, p> 0.25).

A χ^2 analysis of the numbers surviving as complete pupae is shown in Table 4.21.

Table 4.21. Survival of *Helicoverpa punctigera* (malformed pupa) reared on artificial diets containing gramine and hordenine.

Treatments	Survived	Died	Total
1. 500 ppm gramine	18 (18.5)	6 (5.6)	24
2. 60 ppm hordenine	18 (18.5)	6 (5.6)	24
3. 500 ppm gramine + 60 ppm hordenine	19 (19.2)	6 (5.8)	25
4. 500 ppm gramine + 500 ppm hordenine	19 (16.9)	3 (5.1)	22
5. control (with ethanol)	17 (16.1)	4 (4.9)	21
6. control (without ethanol)	22 (23.8)	9 (7.2)	31
Total	113	34	147

The numbers in parenthesis are expected counts based on the null hypothesis. The treatments were not significantly different (χ^2 = 2.03, df = 5, p >0.75).

An analysis of the number of larvae which survived to the adult stage is shown in Table 4.22.

Table 4.22. Survival of *Helicoverpa punctigera* (died before adult emergence) reared on artificial diets containing gramine and hordenine.

Treatments	Survived	Died	Total
1. 500 ppm gramine	17 (17.2)	1 (0.8)	18
2. 60 ppm hordenine	17 (17.2)	1 (0.8)	18
3. 500 ppm gramine + 60 ppm hordenine	18 (18.2)	1 (0.8)	19
4. 500 ppm gramine + 500 ppm hordenine	19 (18.2)	0 (0.8)	19
5. control (with ethanol)	16 (16.3)	1 (0.8)	17
6. control (without ethanol)	21 (21.0)	1 (1.0)	22
Total	108	5	113

The numbers in parenthesis are expected counts based on the null hypothesis. The treatments were not significantly different ($\chi^2 = 1.11$, df = 5, $p > 0.95$).

A χ^2 analysis of the numbers surviving as complete adults is shown in Table 4.23.

Table 4.23. Survival of *Helicoverpa punctigera* (severely malformed adult) reared on artificial diets containing gramine and hordenine.

Treatments	Survived	Died	Total
1. 500 ppm gramine	15 (15.7)	2 (1.3)	17
2. 60 ppm hordenine	16 (15.7)	1 (1.3)	17
3. 500 ppm gramine + 60 ppm hordenine	17 (16.7)	1 (1.3)	18
4. 500 ppm gramine + 500 ppm hordenine	17 (17.6)	2 (1.4)	19
5. control (with ethanol)	15 (14.8)	1 (1.2)	16
6. control (without ethanol)	20 (19.4)	1 (1.6)	21
Total	100	8	108

The numbers in parenthesis are expected counts based on the null hypothesis. The treatments were not significantly different ($\chi^2 = 1.13$, df = 5, $p > 0.95$).

There were no significant differences between treatments in overall survival of insects to the adult stage (Table 4.24).

Table 4.24. Overall survival of *Helicoverpa punctigera* reared on artificial diets containing gramine and hordenine.

Treatments	Viable adults	Died or non-viable	Total
1. 500 ppm gramine	15 (16.7)	25 (23.3)	40
2. 60 ppm hordenine	16 (16.7)	24 (23.3)	40
3. 500 ppm gramine + 60 ppm hordenine	17 (16.7)	23 (23.3)	40
4. 500 ppm gramine + 500 ppm hordenine	17 (16.7)	23 (23.3)	40
5. control (with ethanol)	15 (16.7)	25 (23.3)	40
6. control (without ethanol)	20 (16.7)	20 (23.3)	40
Total	100	140	240

The numbers in parenthesis are expected counts based on the null hypothesis. The treatments were not significantly different ($\chi^2 = 1.78$, df = 5, $p \geq 0.75$). Initial N was 40.

Further χ^2 tests using 2x2 contingency tables gave no significant differences between the control (without ethanol) and any other treatment.

4.3.3.2 Time to pupation

There were highly significant differences ($p<0.001$) between treatments in time to pupation of *H. punctigera*. Time to pupation of insects reared on artificial diet containing gramine 500 ppm was significantly longer than those reared on artificial diet containing gramine 500 ppm+hordenine 500 ppm and control without ethanol. There were no other significant differences, and there were also no significant differences between time to pupation of insects reared on control diets with and without ethanol (Table 4.25).

Table 4.25. Time to pupation (days) of *Helicoverpa punctigera* reared on artificial diets containing gramine and hordenine.

Treatments	n	Time to pupation (Mean±se)
1. 500 ppm gramine	24	23.79±0.61 ^{a}
2. 60 ppm hordenine	24	23.08±0.86 ^{ab}
3. 500 ppm gramine + 60 ppm hordenine	25	22.40±0.59 ^{ab}
4. 500 ppm gramine + 500 ppm hordenine	22	20.41±0.41 ^{b}
5.control (with ethanol)	21	21.76±0.39 ^{abc}
6.control (without ethanol)	31	19.16±0.75 ^{bc}

The treatments had highly significant differences ($p<0.001$) by one-way analysis of variance and means bearing the same superscript were not significantly different by the Bonferroni Multiple Comparisons Procedure (Devore and Peck 1986).

Time to pupation of male and female *H. punctigera* reared on artificial diets were not significantly different ($p = 0.819$) when data from all treatments were combined (Table 4.26).

Table 4.26. Time to pupation (days) of male and female *Helicoverpa punctigera* reared on artificial diets (all treatments combined).

Sexes	n	Time to pupation (Mean±se)
Males	66	21.74±0.38
Females	81	21.61±0.44

non significant ($p = 0.819$) by one-way analysis of variance

The general linear model showed that there were highly significant differences ($p<0.001$) between treatments in time to pupation of *H. punctigera*. There was no significant effect of sex ($p = 0.853$) or the interaction between treatment and the sex ($p = 0.646$) (Table 4.27).

Table 4.27. GLM analysis of time to pupation of *Helicoverpa punctigera* reared on artificial diets containing gramine ar d hordenine.

SOURCE	DF	Seq SS	Adj SS	Adj MS	F	P
TREAT	5	399 55	392.84	78.57	7.30	<0.001
SEX	1	0.74	0.37	0.37	0.03	0.853
TREAT*SEX	5	36.13	36.13	7.23	0.67	0.646
ERROR	135	1452.24	1452.24	10.76		
TOTAL	146	1888.67				

4.3.3.3 Weight of pupae

There were highly significant differences ($p<0.001$) between treatments in weight of pupae of *H. punctigera*. Pupal weights of insects reared on control diet without ethanol were significantly heavier than those in the other treatments. There were no other significant differences (Table 4.28).

Table 4.28. Weight of pupae of *Helicoverpa punctigera* (mg) reared on artificial diets containing gramine and hordenine.

Treatments	n	Weight of pupae (Mean±se)
1. 500 ppm gramine	24	284.17±9.23 [a]
2. 60 ppm hordenine	24	291.38±7.54 [a]
3. 500 ppm gramine + 60 ppm hordenine	25	284.04±8.50 [a]
4. 500 ppm gramine + 500 ppm hordenine	22	289.05±6.64 [a]
5.control (with ethanol)	21	287.05±9.66 [a]
6.control (without ethanol)	31	331.84±5.20 [b]

The treatments had highly significant differences ($p< 0.001$) by one-way analysis of variance and means bearing the same superscript were not significantly different by the Bonferroni Multiple Comparisons Procedure (Devore and Peck 1986).

There was a highly significant difference ($p = 0.009$) in weight of pupae of male and female of *H. punctigera* reared on artificial diets when data from all treatments were combined. Male pupae were significantly heavier than females (Tables 4.29 and 4.30).

Table 4.29. Weight of pupae (mg) of male and female *Helicoverpa punctigera* reared on artificial diets (all treatments combined).

Sexes	n	Weight of pupae (Mean±se)
Males	66	306.41±4.33
Females	81	288.46±5.03

Table 4.30. Analysis of variance on weight of pupae of male and female *Helicoverpa punctigera* reared on artificial diets (all treatments combined).

SOURCE	DF	SS	MS	F	P
SEXES	1	11721	11721	6.94	0.009
ERROR	145	244788	1688		
TOTAL	146	256509			

The general linear model showed that there were highly significant differences ($p<0.001$) between treatments in pupal weights. There were also highly significant differences ($p = 0.001$) between the sexes but there was no interaction effect between treatment and the sex ($p = 0.567$) (Table 4.31).

Table 4.31. GLM analysis of weight of pupae of *Helicoverpa punctigera* reared on artificial diets containing gramine and hordenine.

SOURCE	DF	Seq SS	Adj SS	Adj MS	F	P
TREAT	5	49975	51087	10217	7.41	<0.001
SEX	1	15005	14798	14798	10.73	0.001
TREAT*SEX	5	5358	5368	1074	0.78	0.567
ERROR	135	186162	186162	1379		
TOTAL	146	256509				

4.3.3.4 Sex ratio

There were no significant differences between treatments in the sex ratio of pupae ($p>0.50$) and of adults ($p>0.10$) (Table 4.32).

Table 4.32. Sex ratio of *Helicoverpa punctigera* pupae and adults from larvae reared on artificial diets containing gramine and hordenine.

Treatments	Sex ratio (pupae)		Sex ratio (adults)	
	Males	Females	Males	Females
1. 500 ppm gramine	11	13	7	10
2. 60 ppm hordenine	13	11	12	5
3. 500 ppm gramine + 60 ppm hordenine	10	15	8	10
4. 500 ppm gramine + 500 ppm hordenine	8	14	6	11
5.control (with ethanol)	12	9	10	5
6.control (without ethanol)	12	19	11	10

The treatments were not significantly different ($\chi^2 = 3.49$, df = 5, p>0.50 for sex ratio of pupae, $\chi^2 = 6.74$, df = 5, p>0.10 for sex ratio of adults).

Further χ^2 analyses using 2x2 contingency tables gave no significant difference between the control (without ethanol) and any other treatment.

4.3.3.5 Time to adult emergence

There were highly significant differences (p<0.001) between treatments in time to adult emergence. Time to emergence of insects was shortest in the control without ethanol diet. This treatment was significantly different from all others except the 500 ppm gramine+500 ppm hordenine. There were no other significant differences (Table 4.33).

Table 4.33. Time to adult emergence (days) of *Helicoverpa punctigera* reared on artificial diets containing gramine and hordenine.

Treatments	n	Time of adult emergence (Mean±se)
1. 500 ppm gramine	17	37.47±0.58 ^{a}
2. 60 ppm hordenine	17	36.88±0.84 ^{a}
3. 500 ppm gramine + 60 ppm hordenine	18	36.44±0.77 ^{a}
4. 500 ppm gramine + 500 ppm hordenine	17	35.47±0.61 ^{ab}
5.control (with ethanol)	15	36.60±0.62 ^{a}
6.control (without ethanol)	21	33.19±0.75 ^{b}

The treatments had highly significant differences (p< 0.001) by one-way analysis of variance and means bearing the same superscript were not significantly different by the Bonferroni Multiple Comparisons Procedure (Devore and Peck 1986).

There was a highly significant difference (p = 0.001) in time to adult emergence of male and female *H. punctigera* reared on artificial diets when data from all treatments were combined. Males took longer time to emerge than females (Tables 4.34 and 4.35).

Table 4.34. Time to adult emergence (days) of male and female *Helicoverpa punctigera* reared on artificial diets (all treatments combined).

Sexes	n	Time of adult emergence (Mean±se)
Males	54	36.89±0.44
Females	51	34.84±0.42

Table 4.35. Analysis of variance on time to adult emergence of male and female *Helicoverpa punctigera* reared on artificial diets (all treatments combined).

SOURCE	DF	SS	MS	F	P
SEXES	1	109.77	109.77	11.28	0.001
ERROR	103	1002.08	9.73		
TOTAL	104	1111.85			

The general linear model showed that there were highly significant differences (p<0.001) between treatments in time to adult emergence. Similarly, there was a highly significant difference (p = 0.001) between the sexes. However, there was no interaction effect between treatment and sex (p = 0.122) (Table 4.36).

Table 4.36. GLM analysis of time to adult emergence of *Helicoverpa punctigera* reared on artificial diets containing gramine and hordenine.

SOURCE	DF	Seq SS	Adj SS	Adj MS	F	P
TREAT	5	228.33	234.79	46.96	6.18	<0.001
SEX	1	108.66	90.52	90.52	11.91	0.001
TREAT*SEX	5	68.14	68.14	13.63	1.79	0.122
ERROR	93	706.72	706.72	7.60		
TOTAL	104	1111.85				

4.3.3.6 Duration of pupal stage

There were no significant differences between treatments in duration of pupal stage of *H. punctigera* (Table 4.37)