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In vivo and *in vitro* studies on the uptake of [^{3}H]-Cholesterol by different tissues of the gram pod borer, *Helicoverpa armigera* (Hubner) in the presence of sterol metabolism inhibitors-The azasteroids

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Abstract
Certain compounds such as azasteroids are known to disrupt growth and development in insects. 25-azacholestane and 25-azacoprostane, the two azasteroids tested in the present study, caused a decrease in cholesterol incorporation in the sixth instar larvae of *Helicoverpa armig*era which were fed [^{3}H]-cholesterol 24 hours before sacrificing indicating that cholesterol formation as well as cholesterol incorporation is responsible for their inhibitive effect on the growth in *H. armigera*. In the adult females, these azasteroids caused a decrease in the cholesterol incorporation in fat body (7.2% in controls to 0.8% at 50 ppm azacholestane treatment) and ovaries (9.6% in controls to 6.9% at 50 ppm azacholestane treatment) as well as reduction in their weights. The azasteroids might in some way be affecting the ecdysteroid biosynthesis by the ovaries which in turn affects vitellogenin synthesis by the fat body and their subsequent release and uptake by the ovaries. Due to azacholestane treatment, in the *in vitro* studies, decrease in cholesterol incorporation by ovaries was observed but no such effect was seen in the fat body. The studies on the binding of cholesterol to the lipoproteins of hemolymph showed that maximum cholesterol binding occurred in LP-I out of six bands obtained. In the presence of azasteroid an increase in binding of cholesterol to LP-I was seen, which was reversed when additional cholesterol was given along with the azasteroid. In adult males, cholesterol incorporation decreased in fat body (4.8% in controls to 1.4% at 50 ppm azacholestane) with concomitant increase in the weight when treated with 25- azacholestane though not with 25-azacoprostane. Testes, however showed an increase in cholesterol incorporation in presence of the azasteroids both *in vivo* and *in vitro.*

Keywords: *Helicoverpa armigera*, hemolymph, fat body, ovary, testes, cholesterol, 25-azacholestane, 25-azacoprostane, sterol incorporation, lipoprotein

Introduction
A need for exogenous sterol for normal growth, development, metamorphosis and reproduction is characteristic of insects, differentiating them from other animals (Li and Jing, 2020; Entringer *et al*., 2021; Goel *et al*., 2021 [18, 6, 9]. Most phytophagous insects fulfill their sterol requirements by dealkylating C_{28} and C_{29} phytosterols to cholesterol with the help of enzymes like Δ^{24}- sterol reductase (Kuthiala *et al*., 1987; Agarwal *et al*., 1990; Jing and Behmer, 2020) [14, 1, 12]. These pathways of sterol metabolism and utilization are known to be inhibited by certain azasteroids in many insects (Svoboda *et al*., 1972; Agarwal *et al*., 1990) [27, 1] and nematodes (Cheong *et al*., 2011) [3].Therefore, this ability of the azasteroids to affect the sterol metabolism pathways in different tissues could be a potential target for exploiting the knowledge of their inhibitory effects in developing alternative pest management strategies. Such studies have been done only in few insects and very limited information is available of their effect on a very economically important pest, the gram pod borer, *Helicoverpa armigera*. *H. armigera* is a polyphagous pest causing extensive damage to many agricultural crops worldwide (Haile *et a*l., 2021) [10]. Therefore, studies were undertaken to understand the inhibitory mechanisms of two azasteroids namely, 25-azacholestane and 25-azacoprostane on the uptake of cholesterol by various tissues such as hemolymph, fat body, ovaries and testes in the larva and adults of *H. armigera*. Both, *in vivo* and *in vitro* studies were conducted to ascertain the fate of cholesterol by giving ^{3}H-cholesterol in the diet of the sixth instar larvae which were reared with azasteroids since first instar.

Materials and Methods
Maintenance of stock culture of *H. armigera*
The larvae of *H. armigera* used in these studies were reared on an artificial diet as described by Rath and Agarwal, 1988 [22]. They were reared individually in glass vials plugged with cotton (7.5 cm height x 2.5 cm diameter) containing 7-9 ml diet and maintained in the laboratory under the controlled conditions of temperature, 26.1 ± 2 ºC, Relative Humidity of 70 ± 5% and a photoperiod regime of 16L:8D (Rath and Agarwal, 1988) [22].

After pupation, they were sexed and kept in glass jars (20 cm h x15 cm diameter) for adult emergence and egg laying. Eggs were sterilized using 0.02% solution of sodium hypochlorite, then rinsed with distilled water and kept for hatching in a Petri dish.

Experimental diet

The freshly hatched first instar larvae from the stock culture were fed on the stock artificial diet fortified with varying concentrations of the two azasteroids. The rearing conditions being same as the stock culture. The two azasteroids tested individually were 25-azacholestane and 25-azacoprostane (gift from Dr J. A. Svoboda, Insect and Nematode hormone laboratory, USDA, Beltsville, Maryland, USA) The concentration tested ranged from 5 to 50 ppm for 25- azacholestane (wet weight of the diet) and 5 to 25 ppm in case of 25-azacoprostane. Control insects were reared on stock diet without the azasteroids. For each concentration of the azasteroids, minimum of 25 larvae were tested.

1. *In vivo* studies

The sixth instar larvae which were fed on artificial diet containing azasteroids were starved for 12 hours. They were then given 20 mg of diet containing [^{3}H]-cholesterol (Sp. Act. 8 Ci/ m mol, Radiochemical Centre, Amerashan, U.K.) which was eaten within few minutes (sixth instar were given 113 mμ moles (0.9 μCi) to 124 mμ moles (0.99 μCi) of [^{3}H]-cholesterol in case of the 25- azacholestane fed insects and were given 120 mμ moles(0.96 μCi) to 131 mμ moles(1.05 μCi) of [^{3}H]-cholesterol in case of 25-azacoprostane).The larvae were then transferred back to the original diet with the azasteroids. After the lapse of 24 hours, 5 individuals from each concentration of the azasteroid tested were sacrificed and their sterols extracted by chloroform: methanol (2:1, v/v) (Goel and Agarwal, 1987; Goel *et al*., 2021) [7, 9]. Rest of the larvae were allowed to grow till adult emergence. Sterols were also extracted from the collected faeces. An aliquot of 5 μl of the extract (dissolved in 250 μl hexane) was used for radioactivity estimation (Goel *et al*., 2021) [9]. On the second day after emergence, the adults were sacrificed (5 individual male and female adults from each concentration of the azasteroid). Their fat body, ovaries and testes were removed, sterols extracted and assayed for radioactivity.

2. *In vitro* studies

a. **Effect of 25-azacoprostane on uptake of ^{3}H- cholesterol by hemolymph**

Freshly hatched larvae were reared on an artificial diet fortified with (i) 10 ppm azacoprostane (ii) 10 ppm azacoprostane plus 0.1 % cholesterol. Insects reared on stock diet served as control. Hemolymph was collected from the sixth instar larvae and incubated with [^{3}H]-cholesterol (Sp. Act. 5.7Ci/ mmol) for 90 minutes at 30ºC with constant shaking. The labelled hemolymph was then subjected to PAGE (Polyacrylamide gel electrophoresis) (Davis, 1964)[4] and stained for lipoproteins using Sudan Black B. The radioactivity in the gel was estimated according to the method of Tishler and Epstein, 1968[30]. The stained bands in the gels were cut and transferred to scintillation vials containing 0.2 ml of 30% hydrogen peroxide. The vials were kept at 50ºC for 24 hours to allow complete solubilization of the gel slices. Two drops of ammonia liquor were then added to each vial to neutralize the contents of the vials. 5 ml of naphthalene containing scintillation fluid was added and counted in the liquid Scintillation Spectrometer with appropriate deductions for background.

b. **Effect of 25-azacholestane on [^{3}H]-cholesterol uptake by sixth instar larval fat body**

The fat body and hemolymph were collected from the sixth instar H. armigera larvae reared on an artificial diet containing 25-azacholestane (5 ppm or 25 ppm). The insects reared without azacholestane served as controls. Fat body was incubated for 2 hours at 30ºC with constant shaking in 100μl media containing 25 μl larval hemolymph, 75 μl buffered saline and 7.3 mμ moles (0.06 μCi) to 9.8 mμ moles (0.08 μCi) of [^{3}H]-cholesterol (Sp. Act. 8 Ci/m mol). Fat body was incubated in the labelled hemolymph in the combinations as in table 1.

Table 1: Incubation combinations of sixth instar larval fat body in larval hemolymph

S. No	Hemolymph (larvae)	Fat body (larvae)
1.	Control	Control
2.	5 ppm	Control
3.	25 ppm	Control
4.	Control	5 ppm
5.	5 ppm	5 ppm
6.	5 ppm	25 ppm
7.	25 ppm	25 ppm

All the incubations were carried out simultaneously. Minimum of three replicates were used in each case. After the incubation the fat body was removed sterols extracted and processed for radioactivity estimation.

c. **Effect of 25- azacholestane on [^{3}H]-cholesterol uptake by the ovaries of 2-day old adults**

Ovary was collected from 2-day old adults and hemolymph from female pupae of insects reared on an artificial diet containing 5 ppm or 25 ppm of 25-azacholestane. Insects reared in the absence of the azasteroid served as control. Ovary was incubated for 2 hours at 30ºC with constant shaking in 100μl media containing 10 μl female

pupal hemolymph,90 µl buffered saline and 7.6 mµ moles (0.06 µCi) to 10.8 mµ moles (0.09 µCi) of [^{3}H]-cholesterol (Sp. Act. 8 Ci/m mol). The incubation of ovary was carried out in the combinations as in table 2.

Table 2: Incubation combinations of 2-day old adult ovary in female pupal hemolymph

S. No	Hemolymph (female pupae)	Ovary (adults)
1.	Control	Control
2.	5 ppm	Control
3.	25 ppm	Control
4.	Control	5 ppm
5.	5 ppm	5 ppm
6.	Control	25 ppm
7.	25 ppm	25 ppm

Single ovary was used for each incubation. Minimum of three replicates were used in each case. After incubation the ovary was processed for radioactivity estimation

d. **Effect of 25- azacholestane on [^{3}H]-cholesterol uptake by the testes of 2-day old adults**

Testes were collected from 2-day old adults and hemolymph from male pupae of *H. armigera* reared on an artificial diet containing 5 ppm or 25 ppm of 25-azacholestane. Insects reared without azasteroids served as controls. Testes were incubated for 2 hours at 30ºC with constant shaking in 100µl media containing 10 µl male pupal hemolymph,90 µl buffered saline and 7.6 mµ moles (0.06 µCi) to 10.8 mµ moles (0.09 µCi) of [^{3}H]-cholesterol (Sp. Act. 8 Ci/m mol). The incubation of testes was carried out in the combinations as in table 3.

Table 3: Incubation combinations of 2-day old adult testis in male pupal hemolymph

S. No	Hemolymph (male pupae)	Testis (adults)
1.	Control	Control
2.	5 ppm	Control
3.	25 ppm	Control
4.	Control	5 ppm
5.	5 ppm	5 ppm
6.	Control	25 ppm
7.	25 ppm	25 ppm

Single testis was used for each incubation. Minimum of three replicates were used in each case. After incubation the testis was processed for radioactivity estimation.

All the statistical analysis for the data was done using Sigma Plot 14.0 software.

Results

In vivo studies

a. Effect of 25-azacholestane and 25-azacoprostane on [^{3}H] incorporation in sixth instar *H. amigera* larvae and their faeces 24 hours after [^{3}H]-cholesterol intake

The result showed that there was a significant decrease in [^{3}H] incorporation in larvae with increasing concentrations of 25-azacholestane in the diet as compared to the control group ($p < 0.001$) (Fig. 1). A similar trend was observed in the larval faeces. However, when the diet was treated with varying concentrations of 25-azacoprostane, significant decrease in [^{3}H] incorporation for both larvae ($p < 0.05$) and faeces ($p < 0.001$) was observed only at 5 ppm as compared to their respective control groups (Fig.1).

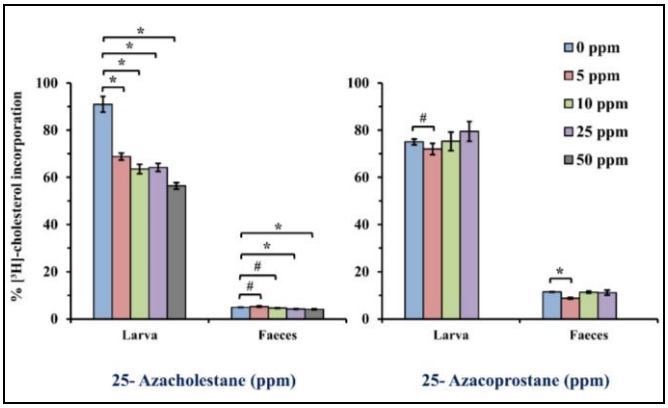

Fig 1: Effect of 25-azacholestane and 25-azacoprostane on [^{3}H] incorporation in sixth instar *H. amigera* larvae and their faeces 24 hours after [^{3}H]-cholesterol intake.

The data is presented in the bar graphs as mean ± S.D (n = 5). Statistical analysis was done by Student t-test (two-tailed) between the control and other groups for larvae and their faeces. * $p < 0.001$, ## $p < 0.01$, # $p < 0.05$.

b. Effect of dietary 25-azacholestasne and 25-azacoprostane on the weight in the tissues of *H. armigera* adults *in vivo*.

Adult females: The weight of the fat body in female declined from 21.8 mg in control to 6.9 mg at 50 ppm 25-azacholestane. The ovary also showed a decrease in weight from 43.1 mg in untreated insects to 11.5 mg at 25 ppm treatment (Table 4). With 25-azacoprostane, the weight of the fat body of female adult decreased from 17.2 mg in control to 10.6 mg at 25 ppm. The weight of the ovary, however, did not show much variation with the increase in azacoprostane concentration.

Adult males: The fat body showed a slight increase in weight from 7.9 mg in controls to 10.6 mg at 50 ppm treatment. The weight of the testes was, however, not much affected by the presence of 25-azacholestane. In case of azacoprostane treatment, the fat body showed an increase in weight till 10 ppm treatment though the weight of the testes did not vary much (Table 4).

Table 4: Effect of 25-azacholestane and 25-azacoprostane on the weight of the adult tissues *in vivo*

Additive Conc. (ppm)	25-azacholestane				25-azacoprostane			
	Fat body (mg)		Adult tissue (mg)		Fat body (mg)		Adult tissue (mg)	
	Female	Male	Female (ovary)	Male (Testis)	Female	Male	Female (ovary)	Male (Testis)
0	21.8 ± 2.9	7.9 ± 0.9	43.1 ± 0.4	2.5 ± 0.2	17.2 ± 5.4	9.2 ± 1.2	18.5 ± 4.6	2.6 ± 0.1
5	22.4 ± 6.5	7.2 ± 0.7	21.1 ± 6.3	4.2 ± 0.1	15.5 ± 5.9	14.4 ± 1.6	16.3 ± 4.5	5.2 ± 0.4
10	12.0 ± 2.5	9.5 ± 0.6	17.1 ± 3.4	2.8 ± 0.3	17.8 ± 3.4	17.6 ± 0.9	16.2 ± 3.3	7.9 ± 1.2
25	2.1 ± 0.1	3.5 ± 0.2	11.5 ± 2.1	2.4 ± 0.1	10.6 ± 1.3	9.6 ± 0.4	18.8 ± 3.5	9.8 ± 1.4
50	6.9 ± 0.7	10.6 ± 1.1	33.2 ± 7.3	3.7 ± 0.1	—	—	—	—

c. Effect of 25-azacholestane and 25-azacoprostane on [^{3}H] incorporation in the tissues of *H. amigera* adults *in vivo*.

Adult females: In both fat body and ovary, significant decrease in the incorporation of [^{3}H] was observed in all the analyzed concentrations of 25-azacholestane in the diet as compared to their respective controls ($p < 0.001$) (Fig. 2). A similar trend was also observed in these tissues when the diet was fortified with 25-azacoprostane (Fig. 2)

Adult males: The [^{3}H]-incorporation in fat body was significantly low in the male adults that were fed on 25-azacholestane as compared to the controls ($p < 0.001$) (Fig.2). However, in testes, surprisingly the [^{3}H]-incorporation was significantly higher at all the concentrations of 25-azacholestane in the diet as compared to the control ($p < 0.001$). In case of 25-azacoprostane, significant decrease in the percent incorporation of [^{3}H] in the fat body was observed at 10 ppm ($p = 0.001$) and 25 ppm ($p = 0.002$) as compared to the control. In testes, the [^{3}H] incorporation was significantly higher at 10 ppm ($p = 0.031$) and 25 ppm ($p < 0.001$) as compared to the control group (Fig.2).

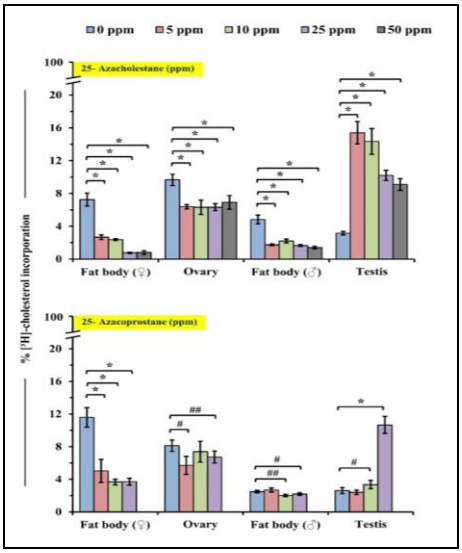

Fig 2: Effect of dietary 25-azacholestane and 25-azacoprostane on [^{3}H] incorporation in the tissues of *H. armigera* adults *in vivo*.

The data is presented in the bar graphs as mean ± S.D (n = 5). Statistical analysis was done by Student t-test (two-tailed) between the control and other groups for adult tissues. * $p < 0.001$, ## $p < 0.01$, # $p < 0.05$.

***In vitro* studies**

a. Effect of 25-azacoprostane on the uptake of [^{3}H]- cholesterol by the larval hemolymph lipoproteins of *H. armigera*

Electrophoretic analysis of the hemolymph from *H. armigera* larvae showed the presence of six lipoprotein bands (designated as LP-I, II, III, IV, V and IV) according to their migration towards the anode. The electropherogram of hemolymph of sixth instar larvae of *H. armigera* reared on artificial diet with or without azacoprostane and cholesterol showed that [^{3}H] was associated with all the six lipoprotein bands. The distribution of the radioactivity in all bands when the hemolymph was labelled *in vitro* is shown in table 5. Out of the six bands, maximum binding of [^{3}H] occurred in LP-I in all the cases. In control, 53.5% binding occurred to LP-I which increased to 67.7% in case of azacoprostane fed insects. It decreased to 28.2% binding to LP-I in insects fed on azacholestane plus 0.1% cholesterol though appreciable amounts of [^{3}H] was also associated with LP-II (25.4%) and LP-III (27.3%) in this case (Table 5)

Table 5: Percent distribution of [^{3}H] in the lipoproteins of larval hemolymph of *H.armigera* reared on an artificial diet containing various additives

Additives	Lipoprotein bands					
	I	II	III	IV	V	VI
	Percent [^{3}H]					
Control	53.5	12.6	10.1	7.6	10.0	6.2
10 ppm of 25-azacoprostane	67.7	15.3	9.4	1.4	2.3	3.9
10 ppm 25-azacoprostane+0.1%cholesterol	28.2	25.4	27.3	5.5	5.9	7.7

b. Effect of 25-azacholestane on [^{3}H]-cholesterol uptake by sixth instar larval fat body

Fat body of control larvae (no azacholestane in the diet) incubated in its own hemolymph was used as control group. Significantly higher uptake of [^{3}H]-cholesterol was observed when the fat body of control larvae was incubated in the hemolymph obtained from 25 ppm azacholestane reared larvae as compared to the control group ($p = 0.009$) (Fig. 3). Similarly, uptake of [^{3}H]-cholesterol was significantly higher than the control group in the fat body of larvae that were fed on 5 ppm and 25 ppm azacholestane and then incubated *in vitro* in the hemolymph obtained from 5 ppm ($p = 0.01$) and 25 ppm ($p < 0.001$) azacholestane reared larvae respectively. Interestingly, no difference was observed when the fat body of larvae fed on 5 ppm and 25 ppm azacholestane were incubated in the hemolymph of control larvae. However, no significant difference was observed between the control and other six groups when the uptake of [^{3}H]-cholesterol was calculated for per 10 mg of fat body (Fig. 3).

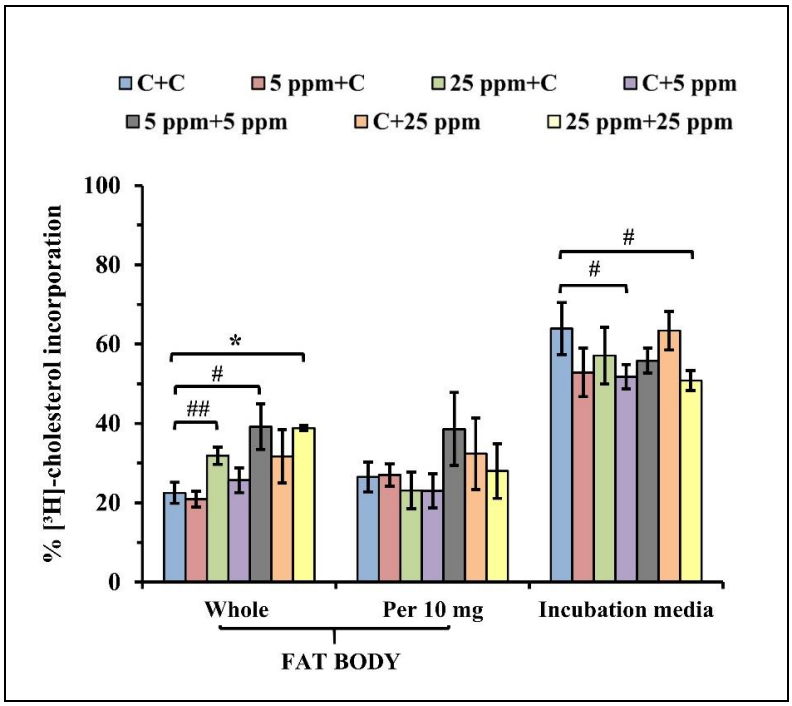

Fig 3: Percent [^{3}H]-cholesterol uptake *in vitro* by fat body of sixth instar larvae of *H. armigera* reared on a diet containing 25-azacholestane.

The data is presented in the bar graphs as mean ± S.D (n = 3). Statistical analysis was done by Student t-test (two-tailed) between the control and other groups. * $p < 0.001$, ## $p < 0.01$, # $p < 0.05$.

Incubation combinations: *Hemolymph (H) + Fat body (FB)* — C+C: Control larvae (H) + Control larvae (FB); 5 ppm + C: 5 ppm fed larvae (H) + Control larvae (FB); 25 ppm + C: 25 ppm fed larvae (H) + Control larvae (FB); C+5 ppm: Control larvae (H) + 5 ppm fed larvae (FB); 5 ppm+5 ppm: 5 ppm fed larvae (H) + 5 ppm fed larvae (FB); C+25 ppm: Control larvae (H) + 25 ppm fed larvae (FB); 25 ppm+25 ppm: 25 ppm fed larvae (H) + 25 ppm fed larvae (FB).

c. Effect of 25-azacholestane on [^{3}H]-cholesterol uptake by ovaries

Ovary of control adults (no azacholestane in the diet) incubated in the female pupal hemolymph (no azacholestane in the diet) was used as a control group. The uptake of [^{3}H]-cholesterol significantly declined in all the other groups (mostly $p < 0.001$) as compared to control group (Fig 4). No difference was observed when the ovary of control adults was incubated in the hemolymph obtained female pupae reared in 5 ppm azacholestane as compared to the control group.

When the uptake of [^{3}H]-cholesterol was calculated for per 10 mg of ovaries, the decline still remained significant in two groups as compared to control group. The first group being when the ovary of control adults was incubated in hemolymph of 25 ppm azacholestane reared female pupae ($p = 0.016$). The second group being the ovary of the adults reared on 5 ppm azacholestane when incubated *in vitro* in the hemolymph obtained from 5 ppm azacholestane reared female pupae ($p = 0.012$) (Fig. 4).

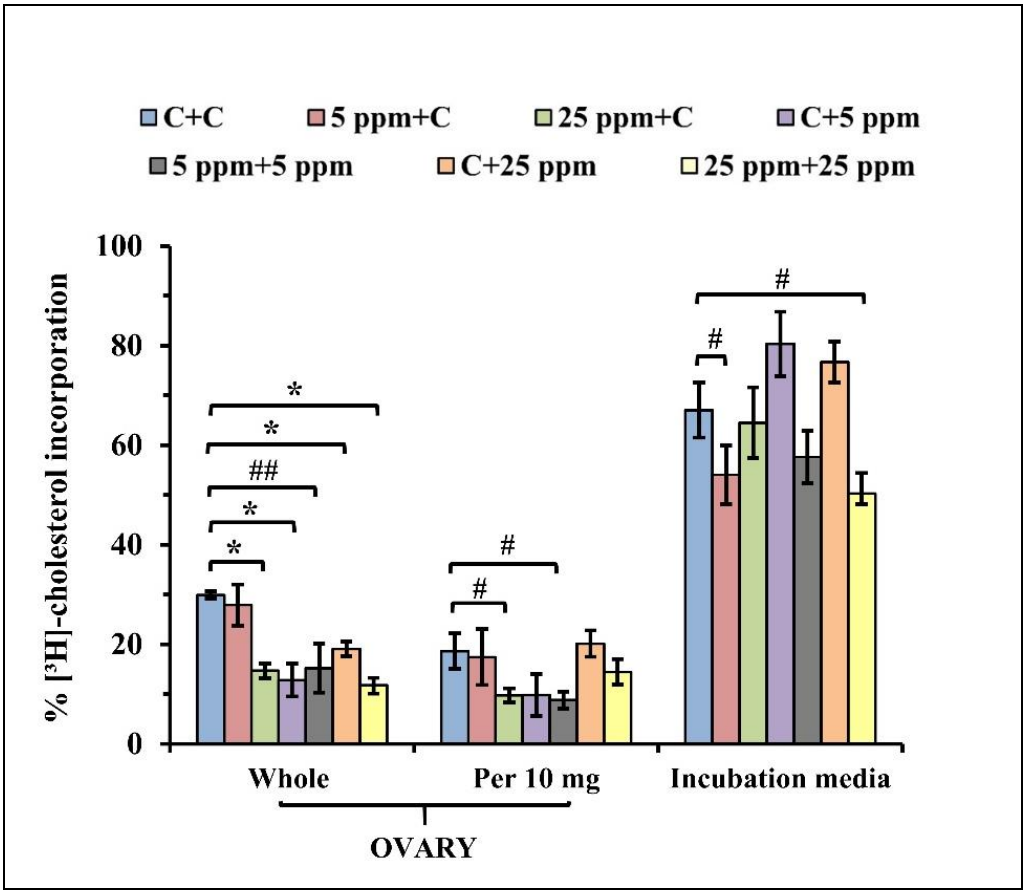

Fig 4: Percent [^{3}H]-cholesterol uptake *in vitro* by ovaries of 2-day old adults of *H. armigera* reared on a diet containing 25-azacholestane.

The data is presented in the bar graphs as mean ± S.D (n = 3). Statistical analysis was done by Student t-test (two-tailed) between the control and other groups. * $p < 0.001$, ## $p < 0.01$, # $p < 0.05$.

Incubation combinations: *Hemolymph (H) + Adult Ovary* — C+C: Control female pupa (H) + Control ovary; 5 ppm+C: 5 ppm fed female pupa (H) + Control ovary; 25 ppm+C: 25 ppm female pupa (H) + Control ovary; C+5 ppm: Control female pupa (H) + Ovary from 5 ppm fed female pupa; 5 ppm+5 ppm: 5 ppm fed female pupa (H) + Ovary from 5 ppm fed female pupa; C+25 ppm: Control female pupa (H) + Ovary from 25 ppm fed female pupa; 25 ppm+25 ppm: 25 ppm fed female pupa (H) + Ovary from 25 ppm fed female pupa

d. Effect of 25-azacholestane on [³H]-cholesterol uptake by testes

Testis of control adults (no azacholestane in the diet) incubated in the male pupal hemolymph (no azacholestane in the diet) was used as a control group in this. Contrary to the pattern observed in ovaries, in case of testes the uptake of [³H]-cholesterol significantly increased in three groups as compared to control group. The first group was when the testis of control adults was incubated in male pupal hemolymph obtained from pupae reared in 25 ppm azacholestane ($p = 0.003$) (Fig. 5). The second group was when the testis of the adults that were reared on 25 ppm azacholestane were incubated in hemolymph of control pupae ($p = 0.007$) and the third one was when the testis of the adults that were reared on 25 ppm azacholestane were incubated *in vitro* in the hemolymph obtained from 25 ppm azacholestane reared male pupae ($p = 0.046$). The increase in uptake of [³H]-cholesterol when calculated for per 10 mg of testes results was consistent with the results when whole tissue was taken into account ($p < 0.001$ for above mentioned first and second groups, and $p = 0.006$ for the third group). In addition, significant increase in uptake of [³H]-cholesterol was also observed in the group in which testis of the adults that were reared on 5 ppm azacholestane were incubated in hemolymph of control pupae ($p = 0.003$) (Fig. 5).

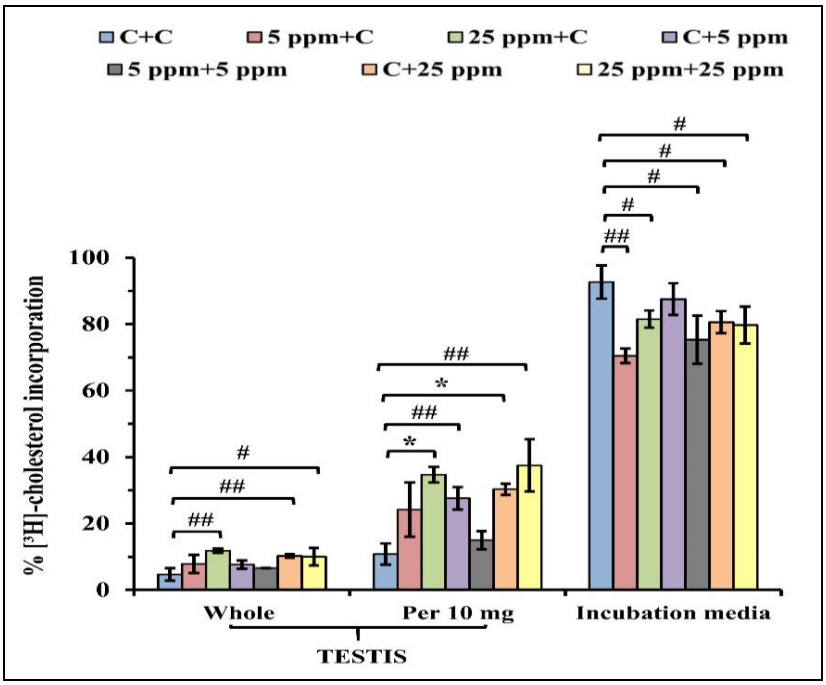

Fig 5: Percent [³H]-cholesterol uptake *in vitro* by testes of 2-day old adults of *H. armigera* reared on a diet containing 25-azacholestane.

The data is presented in the bar graphs as mean ± S.D (n = 3). Statistical analysis was done by Student t-test (two-tailed) between the control and other groups. * $p < 0.001$, ## $p < 0.01$, # $p < 0.05$.

Incubation combinations: *Hemolymph (H) + Adult Testis* — C+C: Control male pupa (H) + Control testis; 5 ppm+C: 5 ppm fed male pupa (H) + Control testis; 25 ppm+C: 25 ppm male pupa (H) + Control testis; C+5 ppm: Control male pupa (H) + Testis from 5 ppm fed male pupa; 5 ppm+5 ppm: 5 ppm fed male pupa (H) + Testis from 5 ppm fed male pupa; C+25 ppm: Control male pupa (H) + Testis from 25 ppm fed male pupa; 25 ppm+25 ppm: 25 ppm fed male pupa (H) + Testis from 25 ppm fed male pupa.

Discussion

Our earlier studies on the effect of azasteroids in insects like *Spodoptera* (Kuthiala et al., 1987) [14], *Chilo* (Agarwal *et al*., 1990) [1] and *Locusta* (Goel and Agarwal, 1987) [8] have shown these compounds to inhibit their growth and development. In view of the above findings, present studies were conducted to understand the effect of 25-azacholestane and 25- azacoprostane on cholesterol incorporation in the larvae of *H.armigera* 24-hours after [³H]-cholesterol intake in diet. It was observed that the 25-azacholestane treatment caused a decrease in the cholesterol incorporation in the larvae. This indicates that reduction in cholesterol formation as well as its incorporation is responsible for the retarded growth in *H. armigera*. However, azacoprostane treatment did not showed lesser variation in the incorporation of cholesterol suggesting that this could be a lesser potent inhibitor compared to 25-azacholestane. The low recovery of [³H] in the faeces could be due to their non-extractability. It has been reported in *Manduca sexta* (Yun *et al*., 2002) [31] that the transfer of cholesterol from midgut to the larval fat body is via the lipophorin (hemolymph lipoprotein) and is stored in the fat body both in the free and esterified form (Jouni *et al*., 2002) [13]. From the fat body it is transported to different tissues and organs to be used for other metabolic processes. Our results suggest that the azasteroids tested in the present studies might be inhibiting some steps in the transport and incorporation of cholesterol.

In the female adults, the cholesterol incorporation was inhibited in the fat body and ovaries due to the azasteroid treatment along with decrease in the weight of these tissues. However, the weight of the ovary was not much affected by the treatment of 25-azcoprostane indicating this azasteroid is less inhibitory. The decrease in the weight of the ovary could be correlated with a reduced oocyte growth and vitellogenesis. A suitable amount of cholesterol availability through absorption, incorporation and transport to the developing oocytes is essential for its proper development in insects, as maternal cholesterol is the major source of embryonic sterol (Behmer and Grebenok, 1998; Jing and Behmer, 2020; Entringer *et al.*, 2021; Goel *et a*l., 2021) [2, 12, 6, 9]. Vitellogenin synthesis has been shown to be controlled by juvenile hormone and the ecdysteroid, 20-hydroxyecdysone in hymenopterans, lepidopterans and dipterans (Wu *et al*., 2021) [32]. Vitellogenins are mostly synthesized in fat body (Roy *et al*., 2018; Lenaerts *et al*., 2019; Swevers, 2019) [24, 17, 28] and deposited in the developing oocytes. With the help of hemolymph lipoproteins, it is transported into the ovary and absorbed by maturing oocytes (Majerowicz *et al*., 2013; Goel *et al.*, 2021) [20, 9]. Ovaries are also known to be a source of these ecdysteroids in several insects (Lageux *et al*., 1977; Masler *et al*.,1980) [15, 21]. Ovaries incubated with [^{3}H]-cholesterol have been reported to secrete [^{3}H]-ecdysone in insects (Lageux *et a*l., 1977; Rees *et al*.,1980) [15, 23]. In lepidopterans, such as *Manduca sexta* and *H. armigera* vitellogenin synthesis is initiated in adults (Swevers and latrou, 2003)[29]. Our observations in *H. armigera* also suggest that the azasteroids might in some way be affecting the ecdysteroid biosynthesis by the ovaries, either due to a reduced cholesterol incorporation or by blocking the pathway of ecdysone synthesis from cholesterol. This, in turn could be affecting the vitellogenin synthesis in the fat body and their subsequent release and uptake by the ovaries. Hence, the observed decrease in the weight of the fat body and ovaries in adult females of *H. armigera*. This reduction in weight could therefore account for the reduction in the cholesterol incorporation in these tissues. However, the studies on incorporation of cholesterol in the fat body of azasteroid treated insects *in vitro* showed that the presence of azasteroid did not affect the uptake. This may mean that (1) the azasteroid has no effect on the cholesterol uptake by the fat body or (2) the doses tested were lower than the ones required to elicit any effect or (3) the tissue could not condition itself in the time given to show any effect on the cholesterol uptake. The *in vitro* studies on the uptake of [^{3}H]-cholesterol by the lipoproteins of the hemolymph in the presence or absence of azacoprostance and additional cholesterol indicates that the maximum binding occurs to LP-I ranging from 53.5% in controls to 67.7% in the presence of 10 ppm azacoprostane. This may mean that there was already sufficient cholesterol bound to LP-I in control and hence only 53.5 % was taken up from the incubation medium. Since the azasteroids are known cause reduction in cholesterol formation and due to less availibility of cholesterol, the lipoprotein had a higher capacity to bind and hence the observed increase in binding. This is further substantiated by the fact that when excess of cholesterol was added to the diet, the % of [^{3}H] bound to LP-I was reduced to 28.2%. Singhal, (1985) [26] has reported high affinity and low capacity of hemolymph lipoproteins for cholesterol.

Similar, *in vitro* studies conducted with ovaries showed that the presence of azacholestane either in the ovaries or hemolymph resulted in a decrease of cholesterol uptake. These results are in agreement with the *in vivo* studies in which the [^{3}H]-cholesterol incorporation decreased in ovaries of the azasteroid treated insects. In adult males of *H. armigera* there was a reduction in cholesterol incorporation *in vivo* in the fat body of azacholestane treated insects with a concomittant increase in the weight of the fat body. However, the testes showed an increase in cholesterol incorporation due to azasteroid treatment without any effect on its weight. Testes when incubated *in vitro* also showed an increase in cholesterol incorporation when either hemolymph or testes were taken from azacholestane fed insects. The reasons for this increase is not clear. In many insects spermatogenesis occurs late in larval life or during pupal stage and the role of 20-hydroxy ecdysone in promoting spermatogenesis is well documented (Dumser, 1980; Schubert, 2017) [5, 25]. The testes of adult males have been shown to be a potential source of ecdysteroids in some insects (Hoffmann and Behrens, 1982; Loeb *et al*., 1984) [11, 19]. However, the sperm and reproductive organs are already developed in adult male lepidopterans (Leclerq-Smekens, 1974) [16] and therefore the role of ecdysteroids produced by the adult testes is possibly to mediate bursts of sperm release and sexual behaviour (Loeb *et a*l., 1984) [19].

Conclusion

In our present studies, it was seen that due to the azasteroid treatment, the cholesterol incorporation is affected in the larval and adult tissues both *in vivo* and *in vitro*. There was a decrease in the cholesterol incorporation in the larval tissues and the adult tissues (fat body of male and female; ovaries of female) but increase of cholesterol incorporation in adult testes. The inhibition in cholesterol incorporation in the tissues in addition to the non-availability of sufficient cholesterol as a result of reduction in cholesterol formation could be responsible for the retardation of growth and development *in H. armigera.* This opens up a potential pathway of sterol metabolism and incorporation, to be targeted and exploited as a possible strategy in development of novel and safe pest control strategies. The inhibition of dietary sterol metabolism, uptake, incorporation or utilization may be an important area of research for planning effective control mechanisms for insect pests.

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Conflict of interest

RR and VG hereby declare that there is no conflict of interest.

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