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Incorporation of [^{3}H]-cholesterol in various tissues during ovarian development of *Locusta migratoria* L. (Orthoptera: Acrididdae)

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Abstract

Experiments were conducted to study the incorporation of [^{3}H]-cholesterol in different tissues of adult female locust *Locusta migratoria* (5 days, 12 days and 22 days old). The results showed that radioactivity from the haemolymph was taken up by all the tissues namely fat body, ovary and muscles. The concentration of tritium in the haemolymph was initially more in all the three stages of development studied but declined gradually with time and cholesterol was incorporated in other tissues of the locust body. The fat body of the female locust picked up radioactivity more in younger stages i.e. 5 days (5.01%) and 12 days (6.86%) after the adult moult as compared to the fat body in 22 (3.38%) days old adult 8 hours after injection. The uptake of radioactivity by the immature ovary in 5 days (1.26%) old locust was very little which, however, increased a little in 12 days (3.03%) old females but tremendously in 22 days (19.21%) old adult 16 hours after injection. This may perhaps be correlated with development of oocytes which occurs between 12 and 22 days. The uptake of cholesterol was comparatively rapid in muscles of 12 days (9.89%) old adults 8 hours after injection.

Keywords: *Locusta migratoria*; cholesterol; ovarian development; haemolymph; fat body; muscles

Introduction

In insects, sterols serve as components of membranes of cellular and subcellular structures, as precursors of moulting hormone, ecdysteroids and also function as a signalling molecule bound to the hedgehog group of protein that influence development (Ikekawa *et al.*,1993; Svoboda and Weirich, 1995; Behmer and Nes, 2003) [2, 15, 28]. Insects are, however, unable to synthesize cholesterol de novo since the essential enzymes required in the biosynthetic pathway are lacking (Clark and Bloch, 1959 & Li and Jing, 2020) [6, 22]. Thus insects must obtain these sterols from their diet. Insects can, however, convert the dietary phytosterols to cholesterol to fulfill their nutritional requirements (Behmer *et al.*, 1999) [3]. The normal growth studies in *Locusta migratoria* (Goel and Agarwal 1987) [12] have shown that while there appears to be not very much net movement of sterols in the body of male locusts, between 12 to 22 days of adult life, the sterol content and concentration in certain organs and tissues of the adult females change greatly indicating lot of mobilization and transport of sterols in the body. Thus, in corporation in different organs/tissue of [^{3}H]-cholesterol, injected into the body was investigated. Ovary of reproductively competent *Locusta migratoria* females have the capacity of synthesizing ecdysteroid from the precursor cholesterol and the ecdysteroid conjugates present in the newly laid eggs are of maternal origin (Laguex *et al.*, 1977) [21]. Hence, it would be worthwhile to study the variation if any, in the uptake of cholesterol by the ovary of locust at different period of ovarian maturity. Although, sterols have been considered as essential nutrients for growth and development in insects, their turnover and site of deposition has not been investigated previously in *Locusta*. A few studies have been undertaken on the distribution of cholesterol among individual organs of American cockroach (Casida *et al.*, 1957) [5], *Eurycotis floridana* (Clayton and Edwards, 1961) [7] and *Rhodnius prolixus* (Entringer *et al.*, 2021) [9]. There is, however, a dearth of investigation on the dynamic state of sterols in the female locust as affected by the reproductive state of insect. A study was thus undertaken to follow the incorporation of Δ^5-sterol, cholesterol (cholest-5-en-3β-ol) in different tissues in relation to the age of locusts using radioactive sterol. Since cholesterol was found to be the major sterol in *Locusta migratoria* (Goel and Agarwal, 1987) [12], [^{3}H]-cholesterol was used in the present investigation.

Material and Method

Insect rearing

Culture of *L. migratoria* was maintained in the laboratory in aluminium cages on fresh green leaves of the host plant, *Zea mays* (maize) and bran (Goel and Agarwal, 1987) [12]. Water was supplied once a day in the form of soaked cotton wool pads. The locusts were reared at 25-30^0 C, 60-80% RH and 12:12 LD regime. The cages were cleaned daily to remove the faeces and dead animals, and fresh food added. Adult female locusts of different age groups were used in the present study. The locusts used were obtained from the culture maintained in the laboratory. Newly emerged adult (within 24 hours of the last moult) were marked with colored paints for future recognition and transferred to new cages. Experimental animals were not isolated i.e. male and female locusts were kept together so that the insects used were always kept in crowded condition. The insects used in the present study were:

1. Five days old females representing the stage of adult before the start of oocyte development.

2. Twelve days old females in which yolk was being deposited in the terminal oocytes
3. Twenty two days old females with fully developed oocytes.

Groups of locusts of each stage were injected with [^{3}H]-cholesterol (Sp. act.8Ci/m mol, Radiochemical Centre, Amerashan, U.K.) dissolved in 5µl of ethanol with Hamilton micro syringe. The injections were given laterally at the intersegment membrane of the fifth and sixth abdominal sternite, with the needle directed anteriorly in the locust. Sterol was purified by column chromatography and purity checked before use by GLC. The insects were kept in normal rearing conditions in the cage illuminated with 25 w bulb until dissection. The locusts were sacrificed after a desired time period. A known amount of haemolymph (10µl) was withdrawn from the insect by puncturing the hind leg region of the metathorax and drawing the blood into a graduated micro-pipette. Haemolymph was transferred to the scintillation vials containing 100µl ethanol and radioactivity estimated after mixing with 10 ml of Scintillation Fluid [{2,5-diphenyloxazole(PPO)-5gm} and {1,4-bis-2(5-phenyl oxazolyl)-benzene (POPOP)-50 mg} in 1 liter of Toluene].

Preparation of the tissue homogenate for radioactivity assay

The insects were dissected after collecting the haemolymph. Fat body, ovary and muscles were then removed as completely as possible and rinsed in ice cold buffered saline (40 mM potassium chloride-8mM sodium phosphate buffer, pH 6.5). The perivisceral fat body, which contains fewer oenocytes than the peripheral fat body was removed from the insect and freed from the ovary (Coupland, 1957) [8]. The tissues separated were washed three times with cold buffered saline, blotted on a filter paper to remove excess saline, weighed and homogenized in chloroform-methanol (2:1, v/v) using Virtis homogenizer according to the method of Folch *et al*. (1957) [10].

Extraction of sterols

Sterols were purified by column chromatography using florisil (PR grade, 60-100 mesh, Sigma, USA) as per Carroll (1961) [4] and by TLC using solvent system, hexane-diethyl ether-acetic acid (83:16:1, v/v/v) (Goel and Agarwal 1987; Rath *et al*., 1993) [12, 25]. A 10µl aliquot of the extract of the sterol sample was used for the estimation of total radioactivity. Minimum of three replicates were used in each case.

Assay of radioactivity

The radioactivity incorporated into various tissues was measured by automatic Packard tricarb liquid scintillation Spectrometer model 3320. The sample was dissolved in a known volume of hexane and 10 µl aliquot of it was taken for counting of [^{3}H]. The efficiency of counting for the samples was determined by channel ratio method and [^{3}H] estimations were corrected for background and quenching effects.

Results

Experiments were conducted to study the distribution of cholesterol in different tissues of adult locusts during various stage of ovarian development whereby 5 days old females represented immature adults. The 12 days old females represented beginning of the development of oocytes which was more or less mature by the 22nd day. The distribution of cholesterol was examined at different intervals after its injection into the locust. The tissues examined were haemolymh, fat body, ovary and flight muscles. The incorporation of [^{3}H]-cholesterol is expressed in terms of the percentage of the total cholesterol injected into each insect. Cholesterol was incorporated by all the tissues including haemolymph, fat body, ovary and muscles but in different kinetics.

Hemolymph

The maximum level of [^{3}H]-cholesterol in the haemolymph of 5 and 12 days old adults was found 8 hours after injection and declined thereafter (Fig.1). In 10 µl haemolymph of 5 days old adults only 0.39 per cent of cholesterol injected was detected one hour after injection which gradually increased to a maximum of 1.38 per cent after 8 hours and this declined the reafter. In 12 and 22 days old adults the [^{3}H]-cholesterol detected in the haemolymph remained more or less constant up to 8 hours after injection and declined gradually thereafter.

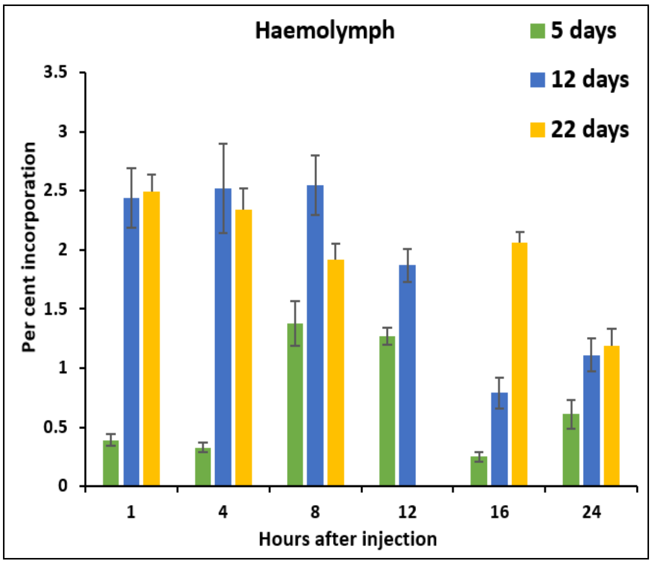

Fig 1: Per cent incorporation of [^{3}H]-cholesterol in the haemolymph (10µl) of female *L. migratoria* in relation to age. Each locust of the particular age group was injected with 0.22 mµ moles of ^{3}H cholesterol (Sp. Act. 8Ci/m mol). Insects were sacrificed at specific time intervals. Minimum of three replicates were used in each case.

Fat Body

The incorporation of [^{3}H]-cholesterol in the fat body of locust in shown in (Fig. 2). Maximum cholesterol incorporation was observed in the fat body of 5 days old females at 16 hours after injection, followed by12 days old females at 8 hours. In 22 days old females maximum incorporation took place at 4 hours after injection. In 5 days old locusts [^{3}H]-cholesterol incorporation by the fat body was 2.72 per cent one hour after injection. This value increased to 12.94 per cent at 16 hours after injection. In 12 days old locusts a maximum of 8.63 per cent cholesterol was detected in the fat body at 8 hours. A much lower incorporation of 5.85 per cent cholesterol was shown to be the maximum uptake by the fat body of 22 days old locusts at 4 hours after injection.

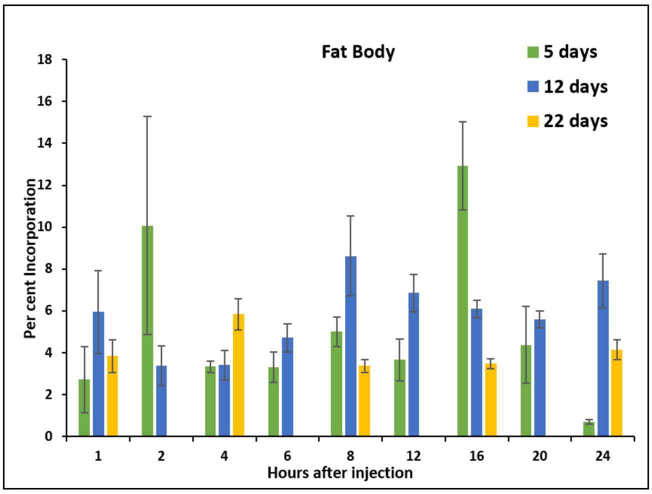

Fig 2: Per cent incorporation of $[^3H]$-cholesterol in the fat body of female *L. migratoria* in relation to age. Each locust of the particular age group was injected with 0.22 mμ moles of $[^3H]$-cholesterol (Sp. Act. 8Ci/m mol). Insects were sacrificed at specific time intervals. Minimum of three replicates were used in each case.

Ovary

Uptake of $[^3H]$-cholesterol by the ovaries from the hemolymph at different time intervals is shown in Fig. 3. Maximum incorporation in the ovaries was observed at about 12 to 16 hours after the cholesterol was injected in all the three age groups. In 5 days old locusts the uptake of $[^3H]$-cholesterol by the ovaries was only 0.37 per cent one hour after injection which increased gradually with time reaching a maximum cholesterol incorporation of 2.63 per cent 12 hours of injection, and declining gradually thereafter. In 12 days old locust, cholesterol uptake after 1 hour of injection was 0.85 per cent increasing to a maximum of 3.03 per cent 16 hours after injection. In 22 days old insects, however, a much higher uptake was observed. Even within an hour after injection as much as 9.12 per cent of cholesterol was detected which further increased to a maximum of 19.21 per cent at 16 hours after injection.

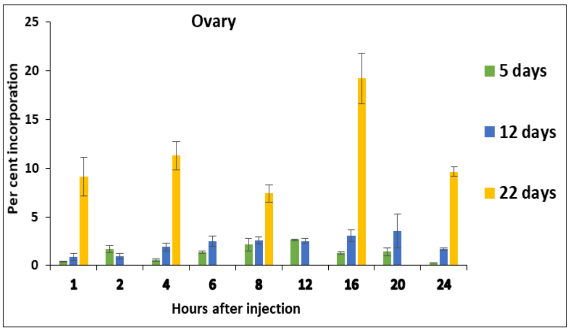

Fig 3: Per cent incorporation of $[^3H]$-cholesterol in the ovary of female *L. migratoria* in relation to age. Each locust of the particular age group was injected with 0.22 mμ moles of $[^3H]$-cholesterol (Sp. Act. 8Ci/m mol). Insects were sacrificed at specific time intervals. Minimum of three replicates were used in each case.

Flight muscles

The cholesterol incorporation by the flight muscles of locust showed a gradual increase with an increase in the time interval after injection inn 5 days old adults (Fig. 4). The maximum incorporation was about 3 per cent after16 hours. The uptake of cholesterol was comparatively rapid in muscles of 12 days old adults. The maximum incorporation of about 10 per cent was observed 8 hours after injection. Again as the locust aged to 22 days, cholesterol pick up was much less and per cent incorporation was almost similar to that observed in 5 days old adult female locusts.

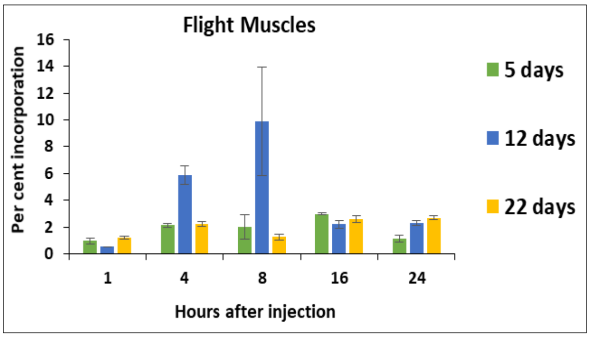

Fig 4: Per cent incorporation of $[^3H]$-cholesterol in the flight muscles of female *L. migratoria* in relation to age. Each locust of the particular age group was injected with 0.22 mμ moles of $[^3H]$-cholesterol (Sp. Act. 8Ci/m mol). Insects were sacrificed at specific time intervals. Minimum of three replicates were used in each case.

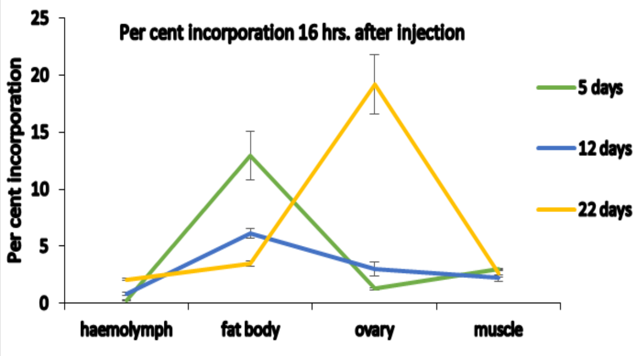

Fig 5: Per cent incorporation of $[^3H]$-cholesterol in various tissues of adult female *L. migratoria* 16 hours after injection. Each locust of the particular age group was injected with 0.22 mμ moles of $[^3H]$-cholesterol (Sp. Act. 8Ci/m mol). Minimum of three replicates were used in each case.

Discussion

Our earlier study on the sterol content in various tissues of adult female locust, *Locusta migratoria*, during development, when fed on fresh green leaves of the host plants, *Zea mays* and *Sorghum bicolor* has shown that certain tissues accumulate more sterol while in others the retention of sterols is comparatively less (Goel and Agarwal, 1987) [12]. It thus became important to study the sterol turnover in different tissues in relation to the age of female *Locusta migratoria* using labelled cholesterol.

The results presented in this study show that radioactivity was detected in all the tissues tested and at all the time interval taken but the distribution was not uniform indicating that *L. migratoria* is capable of directly incorporating cholesterol from the hemolymph into its tissues according to the needs at different stages of development process. Tissue incorporation of cholesterol has also been shown in *Periplaneta americana* (Joshi and Agarwal, 1976) [17], *Heliothis armigera* (Kuthiala and Ritter, 1988) [20] and *Rhodnius prolixus* (Entringer *et al*., 2021) [9]. It is reported that during the period of locust development from 12 to 22 days of adult life, cholesterol deposition increases in ovary and decreases in fat body (Goel and Agarwal, 1987) [12]. The cholesterol serves as the transport form of sterols between organs and tissues mediated by specific lipoprotein acceptors (Siakotos, 1960) [27]. Our data indicating that considerable amount of cholesterol was taken up by the tissues of locust may suggest that sterol is utilized as elements of cellular structure as has also been shown by Gilby and McKellar (1970) [11] in blowfly. In haemolymph, the radioactivity peaked at 8 hours after injection in 12 days old adult and declined thereafter. It indicated a dynamic state of cholesterol in the hemolymph during the transport depending upon the somatic and reproductive growth of the locusts. A gradual decrease with time, in the labelled cholesterol concentration in haemolymph, in all the three age groups tested, further confirms that the transport of cholesterol from haemolymph in the tissues is taking place. Hill *et al*., (1968) [13] reported that by about 12 days of adult life, somatic growth in locust is completed. Somatic growth is attributed mainly to growth of cuticle, flight muscles and an increase in haemolymph volume. During this period, materials accumulate in fat body and muscle. Later when ovarian growth starts, lipids, proteins and carbohydrates are transported from the fat body to ovary.

In the fat body the radioactivity level peaked in 5 days old adult locusts, 16 hours after injection and then returned to less than the initial level by 24 hours after injection. However, cholesterol incorporation was much less in all other stages tested. This profile indicates a high uptake and outflow of the cholesterol from the fat body. Similar studies on *Rhodnius prolixus* have shown a radioactivity peak of dietary cholesterol in fat body on the fifth day after feeding which returned to the original level on the seventh day (Entringer *et al*., 2021) [9].

Maximum incorporation in the ovaries was observed in 22 days old locusts at about 12 to 16 hours after the cholesterol was injected. It was observed that even one hour after injecting the cholesterol, the incorporation in ovary was much more in 22 days old females as compared to all other age groups at different time after injection. In 5 days old locusts in which the oocytes were not well developed, the uptake of [^{3}H]-cholesterol by the ovary from haemolymph is very little. As the development progresses, whereby in 12 days old females the oocyte development has started, the uptake of cholesterol by the ovary also increased. However, the incorporation by ovary increased sharply in 22 days old female in which oocytes are more or less fully developed so much so that almost 20 per cent of the injected cholesterol was detected at 16 hours after injection. In fat body on the other hand, maximum uptake of cholesterol was seen in case of 5 days old females. The fat body and ovary, hence behave opposite to each other as far as the cholesterol incorporation is concerned (Fig.5). Apparently, transfer of [^{3}H]-cholesterol to the developing ovary took place from the fat body which was initially serving as cholesterol depots. The studies on the bug *Pyrrhocoris* which is not able to ingest sufficient amount of lipid during vitellogenesis, temporary storage by the fat body is a physiological adaptation that makes the insect independent from a constant food supply during vitellogenesis (Martin, 1969) [24]. Ichimasa 1975[14] showed that in *Bombyx mori*, ovaries continue to take up lipids or their precursors from hemolymph throughout development, indicating that ovarian lipids were supplied mainly from fat body. Studies on *Manduca sexta* (Jouni *et al*., 2002a, 2002b) [18, 19] have also shown that cholesterol is transferred to the developing oocytes with the help of lipophorin via hemolymph. Maternal cholesterol is the major source of the sterols found in the embryo and thus there should be an appropriate absorption and transport of the cholesterol to the developing oocyte for the successful reproduction (Behmer and Grebenok, 1998; Jing and Behmer, 2020) [1, 16]. Studies on the sterol composition of *Locusta* reared on *Sorghum* have shown that 7 days old eggs contain 3.5 mg sterol/g dry wt. of eggs and cholesterol accounted for almost 95% of the total sterol (Goel and Agarwal, 1987) [12]. The higher rate of cholesterol incorporation in fully developed ovaries seen in the present investigation and presence of large amount of cholesterol in the eggs together suggest that cholesterol incorporated in the ovary of adult female locust is passed on to the eggs, to be utilized later for embryogenesis and synthesis of moulting hormone, ecdysone. Similar studies were performed in Manduca sexta by Thompson *et al*., (1986) [29] and showed that [^{14} C] cholesterol injected in the female pupae was metabolized and converted to ecdysteroid conjugates (mainly 26-hydroxyecdysone 26 phosphate) in ovaries and eggs. Sall *et al*., (1983) [26] have also shown that in the adult *L. migratoria* females, the injected cholesterol was transferred to the eggs and could be used as a precursor for ecdysone biosynthesis in the embryonic prothoracic gland. Lagueux and coworkers (1977) [21] showed that the injected [^{3}H]-cholesterol is used as a precursor for ecdysteroid biosynthesis by the ovaries in *L. migratoria*. The egg laying corresponds to the disappearances of ecdysteroid from the female insects and the hormonal molecules could be recovered from the eggs. In the present study, presence of tritium in haemolymph during entire experimental period indicate that exchange and transport of cholesterol occurs between organs and tissues of locusts via haemolymph which is acting as transport medium. Function of haemolymph as a transport medium has been demonstrated in *Heliothis armigera* (Kuthiala and Ritter, 1988) [20] *Manduca sexta* (Yun *et al*., 2002) [30] and *Rhodnius prolixus* (Majerowicz *et al*., 2013) [23].

Maximum incorporation of cholesterol by the flight muscles was observed after 12 days wherein the radioactive cholesterol increased in muscles and decreased in fat body. It appears that there is probably a transfer of [^{3}H]-cholesterol from fat body to flight muscles which must be utilizing the sterol for their growth. At the end of ovarian development, the cholesterol uptake by the muscles decrease while that of ovary increases, corresponding to a continued decrease in the uptake by the fat body. The correlation of ovarian development with the reduction of fat body has also been shown earlier in *Pyrrhocoris* (Martin, 1969) [24].

Hence, our findings suggest that ovaries grow and incorporate cholesterol from the fat body via haemolymph during growth and maturation in adult female locusts. It will be interesting to investigate a mechanism whereby the cholesterol incorporation in the ovary is impeded, in turn inhibiting egg laying or moulting process due to lack of ecdysteroids production.

Further, *in vitro* studies using some inhibitors that can prevent cholesterol from being incorporated in ovarian tissues of *Locusta migrtoria* might serve as an effective strategy to control the locust population to a certain extent.

Conclusion

1. Cholesterol is taken up by various tissues from the haemolymph.
2. Fat body of the young female picks up cholesterol more

as compared to the fat body of 22 days old female to serve as a store depot
3. Cholesterol uptake by ovary of 22 days old female is much greater than that of young female for the developing ovary/oocytes.
4. Maximum incorporation by the flight muscles was observed when the somatic growth is completed and muscles are almost fully developed.
5. There is a lot of mobilization and transport of sterols in the body of locust

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Conflict of interest

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

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