**Precursor Supply for Insect Juvenile Hormone III Biosynthesis in a Cockroach**

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The biosynthesis of the sesquiterpenoid juvenile hormone III (JH III) was studied using corpora allata of the cockroach *Diploptera punctata* incubated *in vitro* and a radiochemical assay for the hormone produced. The influence of several exogenous precursors such as glucose, trehalose, acetate, amino acids, and mevalonate on JH synthetic rates was studied. Glucose or trehalose were needed for an optimal rate of JH synthesis. Highest rates were achieved at trehalose concentrations below the normal hemolymph levels (35-40 mM). About one-third of the glucose utilized for the biosynthesis of JH III was metabolized through a pentose pathway, but acetyl-CoA derived from glucose was significantly diluted by acetyl-CoA from other sources. Amino acids provided both a source of carbon for JH III synthesis and a source of energy that allowed JH III synthesis from acetate and stimulated JH III synthesis from glucose. Acetate was a poor substrate, because it could not support JH III synthesis in long term incubations. The incorporation of exogenous mevalonate into JH III was dependent on the physiological state of the glands, but there was a significant dilution with endogenous mevalonate. This dilution reflected in part the poor penetration of mevalonate into the corpora allata cells, because JH synthesis in mevalonol-treated cells was not fully rescued by mevalonate.

Insect juvenile hormones are a group of sesquiterpenoids biosynthesized in small endocrine organs, the corpora allata, under the control of the insect brain (1, 2). These hormones are best known for the role they play in preventing metamorphosis of larval forms. In adult females of many insect species, JH controls vitellogenin synthesis and oocyte growth. Evidence accumulated so far indicates that the major insect juvenile hormone is the simple sesquiterpenoid JH III (methyl-10,11-epoxy-3,7,11-trimethyl-2E,6E-dodecaadienolate) and that the higher homologs of JH III are produced only in the order Lepidoptera (1). For instance, the corpora allata of the viviparous cockroach *Diploptera punctata* produce only JH III *in vitro*, and analysis of the JH circulating in this insect’s hemolymph by gas chromatography coupled to mass spectrometry also showed the presence of only JH III (3, 4). Schooley et al. (5) first demonstrated that radioactivity from [2-14C]acetate and [2-14C]mevalonate was incorporated into JH III biosynthesized by corpora allata of *Manduca sexta* incubated *in vitro*, and further studies confirmed that JH III was synthesized according to a “classical” terpenoid pathway (1, 6, 7). In addition to JH III, the corpora allata from lepidopteran species produce higher homologs, such as JH II and JH I, in which one or two isoprene units have been replaced by a homoisoprene unit derived from 3′-homomevalonate (1, 6, 7). However, despite such structural information, virtually nothing is known about the mechanisms controlling both the nature of the JH produced and its rate of production. A better understanding of the demands of the corpora allata cells for the necessary precursors and their utilization for JH synthesis is needed. In this paper, we have studied some aspects of precursor supply for JH III synthesis in female adults of *D. punctata*. This insect is particularly suitable for this type of study, because its corpora allata synthesize large amounts of JH III *in vitro* (3, 4, 8). Throughout our experiments, we have taken advantage of an *in vitro* radiochemical assay for JH synthesis, which is based on the stoichiometric incorporation of label from [methyl-14C] or [methyl-3H]methionine into the methyl ester moiety of the biosynthesized juvenile hormone (2, 7, 9). To complement this known requirement of exogenous methionine for synthesis of the methyl ester group of JH, our aim in this paper was to clarify the metabolic source of carbon utilized for *de novo* synthesis of the sesquiterpenoid carbon chain of JH III.

**Experimental Procedures**

*Insects*—The viviparous cockroach *D. punctata* was reared as described previously (10). The activity of the corpora allata in *in vitro* is strictly dependent on the physiological state of the adult insect (3, 9), and thus day-5 mated female adults were chosen to provide high activity corpora allata, and day-9 mated females as well as day-5 virgin females provided low activity glands.

*Materials*—L-[methyl-3H]Methionine (200 mCi/mmol), L-[methyl-14C]methionine (49.6 mCi/mmol), RS-[5-3H]mevalonolactone (24 and 30 Ci/mmol), and L-[4,5-3H]Uracil (58.4 Ci/mmol) were obtained from New England Nuclear. D-1-[1-14C]Glucose (58 mCi/mmol) and D-6-[14C]glucose (59 mCi/mmol) were obtained from Amersham Corp. Labeled and unlabeled mevalonolactones (from Sigma) were converted to mevalonate by titration with 1 N NaOH (11). Mevalonolactone was a gift from A. W. Alberts (Merck Institute for Therapeutic Research, Rahway, NJ) and was used as its sodium salt.

*Incubation Medium*—In some experiments, the incubation medium was used medium 199 (Gibco) with Hank’s salts, L-glutamine, 25 mM HEPES at pH 7.2, and 2% Ficoll (Sigma). This medium contained 0.61 mM sodium acetate and 5.5 mM glucose and has been designated as “medium 199” throughout this paper. In other experiments, the medium was prepared from Gibco’s MEM Select-amine kit. Minimal medium consisted of Hank’s salts, phenol red, and 25 mM HEPES at pH 7.2, MEM vitamin mixture, and 2% Ficoll. The amino acids were Arg, Cys, Glu, His, Ile, Leu, Lys, Phe, Thr, Trp, Tyr, and Val at concentrations recommended in Gibco’s MEM. When so indicated,
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In short-term incubations were compared to control rates in complete MEM. Fig. 1 shows that optimal rates of JH synthesis were obtained at 5.5 mM glucose. No stimulation of JH synthesis was observed with higher concentrations, even at 100 mM glucose. With low activity glands (from day-5 virgin females), no significant difference was found between 5.5 and 100 mM.

Because trehalose, not glucose, is the major carbohydrate circulating in insect hemolymph, it was necessary to test the influence of trehalose on JH synthesis in vitro in a similar way. Fig. 2 shows that essentially identical results were obtained with trehalose in the absence of glucose and acetate. These results imply that the cells of the corpora allata contain sufficient trehalase activity to generate the levels of glucose needed for JH synthesis.

In addition, the hemolymph trehalose levels in *D. punctata* were found to be well within the range of trehalose concentrations that were optimal for JH synthesis in vitro. Table I shows that hemolymph trehalose levels were remarkably constant between 35 and 40 mM. These results indicate that glucose levels are not limiting in standard incubations of corpora allata and that the difference in the rate of JH synthesis between high and low activity glands is not caused by a limited supply of trehalose. The results also justify the use of glucose instead of trehalose in in vitro assays of JH synthesis.

Pathways of Glucose Utilization—Corpora allata from day-5 mated females were incubated with either [1-14C]glucose or [methyl-3H]methionine or as [methyl-3H]methionine at a final concentration of 0.05 mM. Preliminary experiments showed that the rate of juvenile hormone synthesis was equivalent in medium 199 and complete MEM. Yeager's saline. Following incubation of the corpora allata in medium containing the appropriate substrates and methyl-labeled methionine, JH III synthesis was measured by an isooctane partition assay (12). Because the glands are removed from the incubation medium before extraction, the assay measured JH released into the medium rather than total JH synthesized. However, there is a linear correlation between release and synthesis over the whole range of activity of the corpora allata (3, 9) and we have therefore designated the results as "synthesis" for the sake of clarity. In all double-label experiments the isooctane extract of the incubation medium was analyzed by thin-layer chromatography on plastic-backed silica plates in the solvent system hexane/ethyl acetate (6:1, v/v) and the JH III zone assayed by liquid scintillation spectrometry as described previously (12).

High Performance Liquid Chromatography—Analysis of the secretion products of the corpora allata by HPLC was done using a Perkin-Elmer model LC95 variable wavelength UV detector (4.5-μl flow cell), and LCI 100 integrator/plotter. Reversed-phase HPLC used a Supelcosil LC-18 column (250 × 4.6 mm, 5-μm particle size) and the solvent system hexane/ethyl acetate (70:30) at 1.5 ml/min. Normal phase HPLC used a Supelcosil LC-Si column (150 × 4.6 mm, 5-μm particle size) with a solvent system hexane (propanediol saturated)/ether (96.4) at 1 ml/min. Reference JH I, II, and III were obtained from Behring Diagnostics.

Molar Incorporation Ratio and Dilution Factor Calculations—For the incorporation of 14C label from glucose, the following formula was used for the molar incorporation ratio (MIR), taking advantage of the stoichiometric incorporation of label from [methyl-3H]methionine:

\[
\frac{\text{dpm}[14\text{C}]/\text{Glc specific activity}}{\text{dpm} [\text{H}]/\text{Met specific activity}} = \text{MIR}_{\text{ACOG}}
\]

Because [1-14C]glucose can generate a labeled acetyl-CoA only when metabolized through a glycolytic pathway, and assuming that the 2 triose phosphates generated by glycolysis are equivalent and that all acetyl-CoA derived from glucose are generated through either glycolysis or a pentose pathway, then the fractions of glucose utilization through glycolysis (G) and pentose pathway (P) are given, according to Katz and Wood (13) by the following relations:

\[
\frac{\text{MIR}_{\text{ACOG}}}{\text{MIR}_{\text{ACOG}}} = \frac{1 - P}{1 + 2P} \quad \text{and} \quad P = 1 - G
\]

The dilution factor of acetyl-CoA derived from exogenous glucose by acetyl-CoA generated from other sources can then be calculated knowing that 9 acetyl-CoA are needed for 1 JH III produced and that there are (2G + P) acetyl-CoA generated for each molecule of exogenous glucose utilized. Thus, the dilution factor is:

\[
9 = (2G + P) \text{MIR}_{\text{ACOG}} - (2G + P) \text{MIR}_{\text{ACOG}}
\]

For the incorporation of mevalonate, the molar incorporation ratio (MIR), taking advantage of the stoichiometric incorporation of label from [methyl-3H]methionine or as [methyl-3H]methionine at a final concentration of 0.05 mM. Preliminary experiments showed that the maximal incorporation of farnesoate would remove the 3H label from 1 of the 3 isoprene units.

RESULTS

Influence of Glucose and Trehalose Concentration on JH Biosynthesis—Corpora allata from day-5 mated females (high activity glands) were incubated in complete MEM containing trehalose or glucose but no acetate. The rates of JH synthesis were found to be well within the range of trehalose concentrations that were optimal for JH synthesis in vitro. Table I shows that hemolymph trehalose levels were remarkably constant between 35 and 40 mM. These results indicate that glucose levels are not limiting in standard incubations of corpora allata and that the difference in the rate of JH synthesis between high and low activity glands is not caused by a limited supply of trehalose. The results also justify the use of glucose instead of trehalose in in vitro assays of JH synthesis.

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For the incorporation of mevalonate, the molar incorporation ratio (MIR), taking advantage of the stoichiometric incorporation of label from [methyl-3H]methionine or as [methyl-3H]methionine at a final concentration of 0.05 mM. Preliminary experiments showed that the maximal incorporation of farnesoate would remove the 3H label from 1 of the 3 isoprene units.

Thus, the dilution factor is: (2 - MIR[mevalonate]/MIR[Met-labeled])

RESULTS

Influence of Glucose and Trehalose Concentration on JH Biosynthesis—Corpora allata from day-5 mated females (high activity glands) were incubated in complete MEM containing trehalose or glucose but no acetate. The rates of JH synthesis
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I. Incorporation of radioactivity from [1-14C]- and [6-14C]glucose into JH III

In each experiment, two pairs of corpora allata from day-5 mated females were incubated in MEM medium with amino acids and 0.6 mM glucose provided either as [1-14C]- or [6-14C]glucose for the times indicated. [methyl-3H]Methionine provided the mass marker for the JH synthesized. Molar incorporation ratios of 14C/3H are means ± S.E. obtained from 3-5 μl of hemolymph samples from number of insects.

| Age                          | Trehalose (mM) | n  |
|------------------------------|----------------|----|
| Last larval instar, day 8    | 41.4 ± 1.2     | 4  |
| Adult mated female, day 1    | 39.8 ± 2.9     | 9  |
| Adult mated female, day 5    | 35.7 ± 1.3     | 9  |
| Adult mated female, day 21   | 34.8 ± 2.6     | 8  |
| Adult virgin female, day 5   | 35.8 ± 1.4     | 10 |
| Adult male, day 5            | 34.9 ± 1.3     | 10 |

II. Incorporation of radioactivity from [1-14C]- and [6-14C]glucose into JH III

Incorporation of radioactivity from [1-14C]- and [6-14C]glucose into JH III was monitored at regular intervals (Fig. 3). In medium lacking carbon sources (other than methionine), JH synthesis drastically declined during the first hours and virtually stopped after 12 h. With acetate as sole carbon source, JH synthesis also stopped, although the glands initially utilized some acetate for JH synthesis. The addition of acetate to complete MEM did not enhance the rate of JH synthesis over the long term, although an initial and short term stimulation (25% for 3 h) was observed. These experiments show that the corpora allata do not normally utilize acetate as a precursor for JH synthesis.

Amino Acids and JH Biosynthesis—The utilization of amino acids for JH synthesis was studied in long term incubations as described above for acetate (Figs. 3 and 4). With amino acids as the only carbon source, JH synthesis reached a steady state which was about one-tenth the control rate of synthesis. When both amino acids and acetate were supplied, JH synthesis stabilized at about one-third the rate of controls, thus indicating that the metabolism of amino acids generated sufficient energy for the utilization of acetate. Glucose as the only source of carbon was slightly less effective than in combination with amino acids and could maintain a rate of only about 80% of the controls. These results suggested that the corpora allata utilized amino acids both as a source of carbon (presumably HMG-CoA, acetoacetyl-CoA, and acetil-CoA) and as a source of energy.

| incubation time | [1-14C]Glc | [6-14C]Glc | through a pentose pathway | Dilution of AcetylCoA |
|-----------------|------------|------------|--------------------------|---------------------|
| 0-3 h           | 0.62 ± 0.02| 1.41 ± 0.19| 29.8%                    | 2.75                |
| 3-6 h           | 0.73 ± 0.09| 1.64 ± 0.16| 29.3%                    | 2.21                |
| 6-11 h          | 0.99 ± 0.12| 1.77 ± 0.05| 20.8%                    | 1.84                |
| 0-3 h (+ 0.61 mM acetate) | 0.53 ± 0.07| 1.16 ± 0.10| 28.4%                    | 3.52                |

* Calculated ratio of endogenous acetyl-CoA to acetyl-CoA derived from exogenous glucose.
In order to provide direct evidence of the metabolism of amino acids to utilizable JH III precursor(s), corpora allata were incubated in medium 199 in the presence of [4,5-3H]leucine. The JH III synthesized was analyzed by silica TLC and reversed-phase HPLC (Fig. 5) and both methods revealed that radioactivity from the amino acid had been incorporated into JH III, although no determination of the incorporation rate was attempted.

Corpora allata incubated in complete MEM and 0.8 mM cycloheximide continued to produce JH III at a normal rate for at least 12 h (Fig. 6), and control experiments showed that this level of cycloheximide stopped incorporation of [4,5-3H]leucine into trichloroacetic acid-precipitable proteins in short term experiments. Fig. 6 also shows that corpora allata that stopped synthesizing JH because of a lack of adequate precursor supply almost immediately produced JH when transferred back to complete MEM. In fact a slight overshoot of JH synthesis was observed for the first 2 h following return to a normal medium, but the rate of JH synthesis then leveled off to the original control rate. These experiments indicated that the difference in synthetic rates between media containing glucose and glucose + amino acids (Fig. 4) was not due to a possible decrease of an essential protein with a rapid turnover caused by the lack of amino acid precursors. Rather, this difference was due to the function of amino acids as energy and/or carbon sources.

Mevalonate Incorporation into JH III—Fig. 7 shows that, when present in the incubation medium at 10 mM, mevalonate stimulated JH III biosynthesis in the corpora allata from day-9 mated females, but not from day-5 mated or virgin females. Because little stimulation was observed at lower concentrations, it was felt that poor penetration of mevalonate might prevent a possible stimulation of JH III biosynthesis. To test this possibility, the incorporation of mevalonate was studied directly, using [5-3H]mevalonate and [methyl-14C]methionine in a double labeling experiment. The molar incorporation
incorporation caused by mevinolin was similar for the three groups of corpora allata and virtually all JH synthesized in the presence of mevinolin were lower, but the molar incorporation ratio was even lower) rate of mevalonate penetration. Thus we concluded that exogenous mevalonate, even in the presence of mevinolin was derived from exogenous mevalonate. However, the level of inhibition was still sub-stantial (about 50% for the three groups of glands) when compared to inhibition rates observed in the absence of exogenous mevalonate (typically 88% over a 3-h incubation period). Thus we concluded that exogenous mevalonate, even at 10 mM, could not restore an adequate level of mevalonate within the cells of mevinolin-treated glands.

In the absence of mevinolin, the rates of mevalonate incorporation were lower, but the molar incorporation ratio was significantly higher for day-9 mated females when compared to day-5 mated or virgin females. Taken together, those results suggested that the lack of JH stimulation by mevalonate in corpora allata from day-5 mated females was not caused by a different (i.e. even lower) rate of mevalonate penetration. This would have been reflected in mevinolin-treated glands by a higher rate of inhibition of JH synthesis and/or a lower incorporation ratio.

**DISCUSSION**

The structural aspects of JH biosynthesis are relatively well understood (1). JH III appears to be synthesized from acetyl-CoA through the classical isoprenoid pathway to farnesy1 pyrophosphate. These early steps are shared with other pathways, such as the biosynthesis of cholesterol, ubiquinone, dolichols, etc. The later steps, oxidation of farnesy1 pyrophosphate to farnesoate, methylation, and epoxidation, are specific to the JH III pathway. However, the origin of acetyl-CoA has not been addressed experimentally, nor have the quantitative aspects of JH synthesis been pursued in detail. There are theoretically three sources of readily available hemolymph precursors capable of generating acetyl-CoA for JH III biosynthesis: carbohydrates, i.e. mainly trehalose, free amino acids present in high concentrations in insect hemolymph, and lipids carried by the lipoprotein lipophorin.

Matthews et al. (15) have determined that the glucose concentration in the hemolymph of the cockroach Periplaneta americana is about 0.15 mM and this has to be compared to trehalose levels of 45–53 mM in that cockroach (14), and 35–40 mM determined in this study. Obviously trehalose is the major carbohydrate available to the corpora allata. Because the dose-response for glucose and trehalose were comparable and because there was no significant difference in the response of corpora allata of low and high activity, we conclude that the levels of carbohydrate available and the trehalase activity of the gland cells are not limiting factors for JH III synthesis. Radioactivity from [5-3H]mevalonate is incorporated into methyl farnesoate and JH III (10) and glycolytic degradation of glucose to acetyl-CoA was thus presumed to occur in D. punctata corpora allata. However, the possible involvement of a pentose pathway needed to be tested in view of the demand for NADPH for JH synthesis, in particular by HMG-CoA reductase and methyl farnesoate epoxidase (17). We have shown here that about one-third of the glucose degraded to acetyl-CoA for JH III synthesis was metabolized through a pentose pathway. These calculations of pentose pathway contribution are subject to a number of assumptions (13, 14) but it nonetheless appears that the pentose phosphate pathway is very important in corpora allata cells. Its contribution to glucose metabolism is higher in corpora allata than in most vertebrate tissues including rat adipose tissue that is specialized in lipid synthesis (14). It is still unknown whether the share of total glucose metabolism contributed by the glycolytic and nonglycolytic pathways is dependent on glucose concentration or on the physiological age of the glands. We have also shown that glucose, when present alone, was not able to maintain an optimal rate of JH III synthesis over a long term incubation. In fact, considerable dilution of glucose-derived acetyl-CoA was observed. An optimal rate was obtained only in the presence of glucose and amino acids.

These observations suggested that amino acids might provide a substantial source of carbon for JH III synthesis. Incorporation of radioactivity from [4,5-3H]leucine into JH III and the demonstration that amino acids sustained a low, but significant level of JH synthesis support this view. Conversion of some amino acids to acetyl-CoA (directly or through pyruvate), acetoacletyl-CoA, and 3-hydroxy-3-methylglutaryl-CoA (leucine) that can be used in JH III biosynthesis thus appears to be an important aspect of corpus allatum biochemistry that is linked to the oxidative degradation of amino acids.

Our study does not address the possible role of lipids as a source of energy or as an acetyl-CoA precursor. The available lipid source in the hemolymph is a lipoprotein called lipophorin. Cockroach lipophorin carries about 50% of its weight as lipids, mainly as hydrocarbons, diacylglycerol, and phosphatidylyceroline (18). In preliminary experiments, we have purified lipophorin from D. punctata using a KBr gradient flotation technique (19). This lipophorin preparation did not stimulate JH synthesis by corpora allata incubated in the absence of glucose and amino acids. Equally unsuccessful were incubations with palmitic acid, dipalmitin, or dipalmitoyl-phosphatidylcholine. Future experiments with a lipophorin preparation loaded with radiolabeled lipids should more clearly establish whether lipophorin can provide precursors of acetyl-CoA for JH III synthesis.

In addition to carbohydrates, amino acids (and lipids?) as sources of "early" precursors or intermediates, plant-derived farnesol may be found in the hemolymph of phytophagous species. Farnesol has been shown to stimulate JH III biosyn-
thesis by *D. punctata* corpora allata *in vitro* (8), but it is likely that farnesol from plant sources would be carried in the hemolymph by lipophorin, and that the concentrations achieved would be too low to affect JH III synthesis *in vivo*.

Exogenous acetate has been recognized as a precursor for JH biosynthesis in *in vitro* systems (1), and stoichiometric incorporation of [2-14C]acetate into JH III could be observed in the absence of any other exogenous carbon source, although rates of JH III synthesis sustained in those short term experiments were abnormally low (10). Here we show that acetate should be considered as an artificial substrate capable of entering the JH III biosynthetic pathway *in vitro*. Acetyl-CoA synthetase although present in the corpora allata, did not appear to play an important role in JH III biosynthesis under normal, *in vivo* conditions. Indeed, acetate alone did not support JH III synthesis beyond a few hours *in vitro* and even in the presence of adequate levels of glucose, acetate did not stimulate the rate of JH synthesis over long term incubations.

Just as acetate has been used as an acetyl-CoA precursor, propionate has been used as propionyl-CoA precursor for synthesis of the higher homologs of JH III in *Lepidoptera*, the phylogenetically ancient cockroaches are relevant to the importance of amino acid metabolism in JH synthesis suggested by the results obtained with free acetate, we think that it is not free propionate but rather propionyl-CoA that is the normal substrate for synthesis of JH II and JH I in *Lepidoptera*.

Our studies on the precursor supply for JH III synthesis in the phylogenetically ancient cockroaches are relevant to the evolution of JH biosynthesis. The demonstration of the importance of amino acid metabolism in JH III synthesis suggests that these pathways have been conserved in insects and specialized in *Lepidoptera*. Indeed the metabolism of threonine, isoleucine, methionine, and valine may lead in several steps to propionyl-CoA. Another potential source of propionyl-CoA, such as succinyl-CoA via methylenalacetyl-CoA is probably minor if operating at all, in view of the very low levels in insects of vitamin B12, the cofactor of methylenalacetyl-CoA mutase (21, 22).

Exogenous mevalonate has been shown to serve as JH III precursor in the corpora allata of several species including *D. punctata* (1, 8). Stimulation of low activity glands and lack of stimulation of high activity glands by exogenous mevalonate was observed previously (8) and has been confirmed, in part, by the present study. We show here that low activity corpora allata from day-5 virgin females were not stimulated by mevalonate, whereas a significant stimulation was observed with low activity glands from day-9 mated females. The stimulation of JH III synthesis by exogenous precursors such as farnesol and farnesoate gives useful insights into the regulation of the corpora allata (1, 2, 7) because it is indicative of the degree of saturation of the enzymes (dehydrogenases, methyl transferase, and epoxidase) situated beyond the entry of those precursors. However, the use of exogenous mevalonate as a probe of the physiology of the corpora allata is made difficult by the limited penetration of mevalonate into the gland cells. We showed that mevalonate was not able to restore JH III synthesis to normal levels even when it was virtually the sole precursor available for synthesis (i.e. when endogenous mevalonate synthesis was blocked by the HMG-CoA reductase inhibitor mevinolin). In spite of this low penetration of mevalonate, JH III synthesis is stimulated in corpora allata from day-9 insects. Thus, the enzymes of JH biosynthesis situated after HMG-CoA reductase have a lower overall elasticity coefficient (23) with respect to mevalonate in day-5 mated or virgin females when compared to day-9 mated females.

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