Effects of diet and metamorphosis upon the sterol composition of the butterfly Morpho peleides

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Abstract   Whole body sterol metabolism in insects has seldom been studied. We were able to design an appropriate study at a butterfly farm in Belize. We collected six larvae of butterfly (Morpho peleides), their food (leaves of Pterocarpus bayssii), and their excretions. In addition, six adult butterflies were collected. The sterols of the diet, the larva, and the adult butterfly were analyzed by gas-liquid chromatography. The structures of these sterols were identified by digitonin precipitation, GC-MS, and NMR. Four sterols (cholesterol, campesterol, stigmasterol, and sitosterol) and a sterol mixture were found in the food, the body, and the excreta of the larva. The tissue sterol content of the larva was 326 μg. They consumed 276 μg of sterols per day. Their excretion was 185 μg per day as sterols. The total tissue sterol contents of the larva and butterfly were similar, but they had different sterol compositions, which indicated interconversion of sterols during development. There was a progressive increase in the cholesterol content from larva to butterfly and a decrease in the content of sitosterol and other plant sterols, which were likely converted to cholesterol. Our data indicated an active sterol metabolism in butterfly larva. Diet played an important role in determining its sterol composition. During metamorphosis, there was an interconversion of sterols. This is the first paper documenting the fecal sterol excretion in insects as related to dietary intakes.—Connor, W. E., Y. Wang, M. Green, and D. S. Lin. Effects of diet and metamorphosis upon the sterol composition of the butterfly Morpho peleides. J. Lipid Res. 2006. 47: 1444–1448.

Supplementary key words cholesterol • plant sterols • sitosterol • campesterol • dealkylation • larva • adult butterfly • tissue sterols • fecal sterols

Insects and vertebrates share many common metabolic pathways. In many areas of research, insects are useful models that can facilitate our general understanding of biology (1). However, unlike vertebrates, insects are unable to biosynthesize sterols and must acquire these essential nutrients from the diet (2–5). In addition to a role as a structural component of the cell membranes, cholesterol serves as the precursor of the insect molting hormone ecdysteroids (6, 7). Different aspects of sterol metabolism in insects have been studied in many species (8). Excellent reviews are available on this subject (4, 9). The butterfly of the Lepidoptera order undergoes metamorphosis from larva to butterfly. The larva feed on the leaves of plants and then spin a chrysalis. The butterfly feeds on flower nectar, which is available later in the year. We found no information in the literature about the sterol composition of the butterfly before and after metamorphosis and no information about how the diet of the larva might influence its sterol composition. In a recently published study, we found that the butterfly drastically decreases its body weight and body fat during metamorphosis (10).

On a trip to Belize, Central America, we visited the Butterfly Farm at Chaa Creek. Chaa Creek is a tourist attraction and hostel for travelers in Belize. As we have developed an interest in the evolutionary patterns of fatty acids, having previously studied snails and slugs (11), we saw the potentialities of butterfly research and discussed our ideas with the scientific director (M.G.). We decided to collaborate on fatty acid and sterol research. At the butterfly farm, the butterfly Morpho peleides, or Blue Morpho, is raised in a controlled environment. The sole food of these butterfly larva is the leaves of the rain forest tree Pterocarpus bayssii, on which the butterfly lays its eggs. Because the larva grew on this sole food source, and the butterfly spend tremendous energy for metamorphosis and later flight with limited food intake, we hypothesized that diet must play an important role in the sterol composition of the larva and that the butterfly may have similar sterol content and composition as the larva. To test these hypotheses, we measured food consumption of the larva on a daily basis and analyzed the sterol composition and content of the diet as well as of the larva. After the larva had undergone metamorphosis, we examined the sterol composition and content of the butterflies that emerged, which had a beautiful blue color. In addition, we analyzed the sterol content in the fecal excretion of the larva.

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Sterol composition in the leaves, larva, and butterflies

The tree leaves and whole larva and butterflies were freeze-dried. Lipids were extracted by grinding with chloroform and methanol (12). The sterols were analyzed by the methods described previously (13). A tracer amount of [4-14C]cholesterol was added to an aliquot of lipid extract. Sterols in the lipid extract after mild saponification were precipitated with digitonin (14). The free sterols were recovered from digitonate by dissolving the precipitate in pyridine and extracting the free sterols with diethyl ether. The sterols were identified by GC-MS and NMR. Instead, three additional sterols were identified: stigmast-7-en-3β-ol, stigmast-22-en-3β-ol, and stigmastan-3β-ol (see text).

Sterol absorption and excretion in butterfly 1445

The GLC analysis was performed on an instrument equipped with a hydrogen flame ionization detector (model 8500; Perkin-Elmer Corp., Cupertino, CA). Cholesterol was used as an internal standard. Standard solution containing cholesterol, campesterol, stigmasterol, and sitosterol was run simultaneously. Their retention times were used for identification. The peak retention times and areas were calculated with a computer (Turbochrom PE Instruments, Inc., Fullerton, CA).

In the leaves and tissues, four sterols (sitosterol, stigmasterol, campesterol, and cholesterol) were identified by GC retention time with authentic standards. Confirmation of these four sterol identifications by GC-MS and NMR was carried out with the help of W. K. Wilson of Rice University. (Methods are available upon request.) In our GC analysis, there was a peak having a similar retention time as that of isofucosterol. However, this was not confirmed by GC-MS and NMR analysis. Instead, three additional sterols were identified: stigmast-7-en-3β-ol, stigmast-22-en-3β-ol, and stigmastan-3β-ol. Because the structures of these three compounds are similar and the sum of these three compounds identified by GC-MS and NMR was similar to the amount of the unknown peak identified by GC, we tentatively designated the last peak in our GC analysis as the “mixture” of these three sterols.

Sterol neutral steroids

Fecal steroids were analyzed by the methods reported previously (16). An aliquot of 48 h excretion was weighed out and suspended in alcoholic NaOH. Traces of [4-14C]cholesterol were added to monitor the recovery. After mild saponification, the fecal neutral steroids were extracted. The sterols were precipitated

Sterol absorption and excretion in butterfly 1445

Methods

Sample collection

Each butterfly larva (M. peleides pupae) was housed in a closed environment (glass chamber) at the Chaa Creek Butterfly Farm in Belize. The larva were fed the leaves of P. bayessii, which is the preferred food source of the larva. The 48 h excretion of six larva was collected. The leaf consumption of this period was obtained from the records of leaf weight before and after the 48 h experiment. In addition, adult butterflies hatched at the Oregon Zoo were also collected at the end of their lifespan. These butterflies were of the identical species and were shipped from another Central American country, Costa Rica.

Values shown are µg/day. The mixture consisted of stigmast-7-en-3β-ol, stigmast-22-en-3β-ol, and stigmastan-3β-ol (see text). 

a No intake of food during the 2 day fecal collection.

b Excludes larva No. 2.

Table 1. Dietary sterol intake of the butterfly larva

<table>
<thead>
<tr>
<th>Larva</th>
<th>Cholesterol</th>
<th>Campesterol</th>
<th>Stigmasterol</th>
<th>Sitosterol</th>
<th>Mixture</th>
<th>Total</th>
<th>Total Sterols</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27.6</td>
<td>3.9</td>
<td>47.5</td>
<td>197.2</td>
<td>24.9</td>
<td>275.5</td>
<td>301.1</td>
</tr>
<tr>
<td>2a</td>
<td>24.9</td>
<td>3.5</td>
<td>42.8</td>
<td>177.6</td>
<td>22.4</td>
<td>246.3</td>
<td>271.2</td>
</tr>
<tr>
<td>3</td>
<td>19.9</td>
<td>2.8</td>
<td>34.2</td>
<td>142.0</td>
<td>17.9</td>
<td>196.9</td>
<td>216.8</td>
</tr>
<tr>
<td>4</td>
<td>33.9</td>
<td>4.7</td>
<td>58.2</td>
<td>241.8</td>
<td>30.5</td>
<td>335.2</td>
<td>369.1</td>
</tr>
<tr>
<td>5</td>
<td>20.4</td>
<td>2.8</td>
<td>35.1</td>
<td>145.8</td>
<td>18.4</td>
<td>202.1</td>
<td>222.5</td>
</tr>
<tr>
<td>6</td>
<td>21.1 ± 11.6</td>
<td>3.0 ± 1.6</td>
<td>36.3 ± 19.9</td>
<td>150.7 ± 82.5</td>
<td>19.0 ± 10.4</td>
<td>209.0 ± 114.3</td>
<td>239.1 ± 125.9</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>25.3 ± 5.8</td>
<td>3.4 ± 0.8</td>
<td>43.6 ± 9.9</td>
<td>180.9 ± 41.0</td>
<td>22.8 ± 5.2</td>
<td>250.8 ± 56.9</td>
<td>276.1 ± 62.6</td>
</tr>
</tbody>
</table>

Table 2. Sterol content of the butterfly larva

<table>
<thead>
<tr>
<th>Larva</th>
<th>Body Weight (Dried)</th>
<th>Cholesterol</th>
<th>Campesterol</th>
<th>Stigmasterol</th>
<th>Sitosterol</th>
<th>Mixture</th>
<th>Total</th>
<th>Total Sterols</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.499</td>
<td>86.3</td>
<td>1.1</td>
<td>25.1</td>
<td>97.1</td>
<td>44.7</td>
<td>168.0</td>
<td>254.3</td>
</tr>
<tr>
<td>2a</td>
<td>0.334</td>
<td>145.6</td>
<td>5.9</td>
<td>3.1</td>
<td>66.5</td>
<td>7.5</td>
<td>83.0</td>
<td>228.6</td>
</tr>
<tr>
<td>3</td>
<td>0.490</td>
<td>134.0</td>
<td>4.9</td>
<td>28.9</td>
<td>151.8</td>
<td>42.4</td>
<td>228.0</td>
<td>362.0</td>
</tr>
<tr>
<td>4</td>
<td>0.395</td>
<td>145.6</td>
<td>7.7</td>
<td>18.4</td>
<td>118.3</td>
<td>21.0</td>
<td>165.4</td>
<td>311.0</td>
</tr>
<tr>
<td>5</td>
<td>0.724</td>
<td>215.5</td>
<td>23.6</td>
<td>18.2</td>
<td>164.8</td>
<td>39.8</td>
<td>246.4</td>
<td>461.9</td>
</tr>
<tr>
<td>6</td>
<td>0.488</td>
<td>195.6</td>
<td>2.1</td>
<td>6.2</td>
<td>111.1</td>
<td>20.5</td>
<td>139.9</td>
<td>335.5</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>0.473 ± 0.136</td>
<td>153.8 ± 46.1</td>
<td>7.6 ± 8.2</td>
<td>16.7 ± 10.2</td>
<td>118.3 ± 36.0</td>
<td>29.3 ± 15.1</td>
<td>171.8 ± 59.5</td>
<td>325.6 ± 83.3</td>
</tr>
<tr>
<td>Means ± SDb</td>
<td>0.501 ± 0.132</td>
<td>155.4 ± 51.4</td>
<td>7.9 ± 9.2</td>
<td>19.4 ± 8.7</td>
<td>128.6 ± 28.5</td>
<td>33.7 ± 11.9</td>
<td>189.5 ± 45.3</td>
<td>344.9 ± 76.5</td>
</tr>
</tbody>
</table>

Values shown are µg/whole body, except for body weight, which is shown in g.

a No intake of food during the 2 day fecal collection.

b Excludes larva No. 2.
by digitonin as described. Digitonin-precipitable sterols were subjected to thin-layer chromatography [Florisol TLC plate with the solvent ether-heptane (55:45)] to separate sterols from stanols and stanones (bacteria-modified product). All digitonin-precipitable sterols were confined in the sterol band. No stanols or stanones were found. TMS derivatives of these sterols were analyzed by GLC as described previously.

RESULTS

Dietary intake of sterols

Based on our TLC and GC analysis with the confirmation data by NMR and GC-MS, we identified four major sterols and a mixture of sterols in the leaf (Table 1). From the amount of leaf consumption and the sterol composition of the leaf, we calculated the daily sterol intake of the larva (Table 1). One larva, No. 2, did not eat during the 48 h experimental period, as occurs during molting. The other larva consumed 217–369 mg of sterols per day. Among the five sterols in the leaf, sitosterol was the most abundant, at 65.5%; stigmasterol was 15.8%, mixture was 8.3%, and campesterol was 1.2%. Interestingly, cholesterol contributed 9.2% of the total sterols in this leaf.

Sterol content of larva

The larva weighed 0.3–0.7 g, with a mean of 0.47 g. The sterol composition of the larva is shown in Table 2. The same four sterols and the mixture of sterols found in the leaves were detected in the larva. Their total body sterols varied from 229 to 461 mg. Unlike the leaf, cholesterol contributed most of the total sterols, 45.0%; sitosterol was 37.3%, mixture was 9.8%, stigmasterol was 5.6%, and campesterol was 2.3%. It is clear that the sterol composition of the diet and the larva was very different (Fig. 1). This suggests the preferential conversion of dietary plant sterols to cholesterol in the larva (as discussed later). The tissue-diet ratio of cholesterol was 4.89, in contrast to the 0.35–1.92 ratio for the other sterols.

Sterol content of butterfly

Six adult butterflies were analyzed (Table 3). They weighed 0.1–0.5 g, with a mean of 0.28 g. The same five sterols that were found in the larva were also detected in the butterflies. The total sterol content was similar between larva and butterfly. However, the sterol composition was very different. Cholesterol constituted the most, 285 mg per butterfly. Campesterol was 16.8 mg. Little stigmasterol was detected. Sitosterol was 73.1 mg, and mixture was 3.4 mg. When the sterols of the butterfly were compared with the sterol composition of larva, cholesterol increased by 28% (Fig. 2) at the expense of sitosterol (−17%) and the mixture (−8%) during the development from larva to butterfly.

Sterol excretion

The same four sterols and the mixture of sterols found in the diet (leaf) were detected in the excreta of the larva (Table 4). Total sterol excretion ranged from 101 to 240 mg/day. The lowest value was from caterpillar No. 2, which did not eat during the 2 day experimental period. For all five larva, the sterol excretion was as follows: sitosterol, 67.9%; mixture, 14.2%; stigmasterol, 12.8%; cholesterol, 4.6%; and campesterol, 0.5% of total sterols. A comparison of the fecal sterols with the larva indicates that cholesterol had the lowest ratio compared with other sterols (0.1 vs. 0.2 for campesterol and 1.58–2.51 for the other three sterols). These differences resulted from a lower clearance and from conversion from other sterols. The greater excretion of plant sterols than cholesterol, especially sitosterol, suggests their more limited absorption from the diet.

DISCUSSION

There are several unique features of the current study. First, the larva were raised in a closed environment, so that

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**TABLE 3.** Sterol content of adult butterfly

<table>
<thead>
<tr>
<th>Butterfly</th>
<th>Body Weight (Dried)</th>
<th>Cholesterol</th>
<th>Campesterol</th>
<th>Stigmasterol</th>
<th>Sitosterol</th>
<th>Mixture</th>
<th>Total</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.39</td>
<td>404.2</td>
<td>17.6</td>
<td>—</td>
<td>99.6</td>
<td>3.3</td>
<td>120.5</td>
<td>524.7</td>
</tr>
<tr>
<td>2</td>
<td>0.47</td>
<td>402.7</td>
<td>43.6</td>
<td>7.8</td>
<td>121.5</td>
<td>3.7</td>
<td>176.6</td>
<td>579.3</td>
</tr>
<tr>
<td>3</td>
<td>0.30</td>
<td>229.2</td>
<td>8.8</td>
<td>—</td>
<td>49.0</td>
<td>5.7</td>
<td>63.5</td>
<td>292.7</td>
</tr>
<tr>
<td>4</td>
<td>0.23</td>
<td>376.6</td>
<td>18.1</td>
<td>—</td>
<td>103.4</td>
<td>2.5</td>
<td>124.0</td>
<td>500.6</td>
</tr>
<tr>
<td>5</td>
<td>0.20</td>
<td>198.2</td>
<td>6.8</td>
<td>—</td>
<td>46.5</td>
<td>2.9</td>
<td>56.2</td>
<td>254.4</td>
</tr>
<tr>
<td>6</td>
<td>0.11</td>
<td>102.1</td>
<td>5.6</td>
<td>—</td>
<td>18.4</td>
<td>2.1</td>
<td>26.1</td>
<td>128.3</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.28 ± 0.13</td>
<td>285.5 ± 126.9</td>
<td>16.8 ± 14.2</td>
<td>—</td>
<td>73.1 ± 40.6</td>
<td>3.4 ± 1.3</td>
<td>94.5 ± 55.5</td>
<td>380 ± 180.0</td>
</tr>
</tbody>
</table>

Values shown are mg/whole body, except for body weight, which is shown in g.
The food intake and fecal excretion could be quantified. Second, the sterols in the diet and tissues were identified and analyzed. Third, the sterols in the feces were measured. With three sets of data (diet, tissue, and excretion), we then examined the metabolism of individual sterols in the butterfly larva. These data can be portrayed more definitively because insects cannot synthesize sterols (2–5). All sterols present in the tissues and excreta must have been derived from the diet.

The same sterol pattern in both diet and tissue suggested active absorption of the sterols from the diet. Komnick and Giesa (17) reported the absorption of cholesterol in the dragonfly. Cholesterol was transported in the hemolymph and incorporated into the fat body and Malpighian tubules. Joshi and Hari (18) pointed out that the site of cholesterol absorption was different for different insects: foregut for omnivorous insects and midgut for phytophagous insects. Our data confirm previous findings in this respect (4).

Unlike humans and animals, the larva did not produce sterols to form cholesterol, which is important for membrane structure and hormone production. Most phytophagous insects obtain adequate amounts of cholesterol by converting the C28 and C29 phytosterols to cholesterol via dealkylation of the C24 alkyl group (9, 27). We have demonstrated the conversion of sitosterol to cholesterol in the Florida land crab (4). The pathway from 24-alkyl sterol to cholesterol has been investigated extensively in a number of insects (9, 28).

This study provides the first data about the fecal sterol excretion of insects. We have identified the five sterols that butterfly larva ingested from their diet by fecal analyses. Unlike humans and animals, the larva did not produce stanols and stanones from sterols by bacterial transformation, as occurs in humans (29). Our data confirm previous findings in this respect (4).

Significant amounts of cholesterol were found in the leaves of *P. bayessii*. We did not find ergosterol in our analyses. This excludes the possibility of fungal contamination.

The five larva (No. 2 was excluded because of not eating) consumed 275 μg of sterols per day, excreted 202 μg of sterols per day, and contained 345 μg of body sterols. The larva were growing and were accumulating membrane sterols. They were in a positive sterol balance. These data clearly indicated that there was active sterol metabolism in the larva. Individual sterols may have different turnover rates. In the normal human, sitosterol has a faster turnover than cholesterol (24, 25).

It was not surprising that the butterfly was lighter than the larva (0.28 vs. 0.47 mg). Insect flight muscle is the most metabolically active tissue known. The most efficient source of energy probably is fatty acid (26). Thus, the butterfly must lose considerable fat tissue during flight. In our recent report, we found that larva lost large amounts of fat during metamorphosis (10). However, the larva and butterfly have similar total sterol contents, 325 and 380 mg (Tables 2, 4). This indicates that the sterols were components of the membrane structure and were not metabolized. The sterol composition of the larva and the butterflies was quite different. For comparison, there was a 28% increase in cholesterol, a 17% decrease in sitosterol, and an 8% decrease in the mixture of sterols during the development from larva to butterfly. These data strongly suggested that the butterfly must dealkylate the plant sterols to form cholesterol, which is important for membrane structure and hormone production. Most phytophagous insects obtain adequate amounts of cholesterol by converting the C28 and C29 phytosterols to cholesterol via dealkylation of the C24 alkyl group (9, 27). We have demonstrated the conversion of sitosterol to cholesterol in the Florida land crab (4). The pathway from 24-alkyl sterol to cholesterol has been investigated extensively in a number of insects (9, 28).

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Significant amounts of cholesterol were found in the leaves of *P. bayessii*. We did not find ergosterol in our analyses. This excludes the possibility of fungal contamination.

**TABLE 4. Fecal neutral sterol excretion in butterfly larva**

<table>
<thead>
<tr>
<th>Plant Sterols</th>
<th>Cholesterol</th>
<th>Campesterol</th>
<th>Stigmasterol</th>
<th>Sitosterol</th>
<th>Mixture</th>
<th>Total Sterols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larva</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.4</td>
<td>0</td>
<td>36.6</td>
<td>131.7</td>
<td>10.3</td>
<td>178.6</td>
</tr>
<tr>
<td>2</td>
<td>9.1</td>
<td>1.5</td>
<td>12.0</td>
<td>62.9</td>
<td>15.5</td>
<td>91.9</td>
</tr>
<tr>
<td>3</td>
<td>6.8</td>
<td>2.6</td>
<td>35.1</td>
<td>164.4</td>
<td>24.8</td>
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</tr>
<tr>
<td>4</td>
<td>5.5</td>
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<td>137.2</td>
<td>11.9</td>
<td>178.7</td>
</tr>
<tr>
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<td>13.3</td>
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<td>84.9</td>
<td>54.1</td>
<td>151.0</td>
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<tr>
<td>6</td>
<td>9.6</td>
<td>0</td>
<td>18.1</td>
<td>172.8</td>
<td>40.2</td>
<td>213.1</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>8.6 ± 2.7</td>
<td>1.0 ± 1.2</td>
<td>23.6 ± 11.7</td>
<td>125.7 ± 43.6</td>
<td>26.1 ± 17.6</td>
<td>176.4 ± 51.6</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>8.5 ± 3.1</td>
<td>0.9 ± 1.3</td>
<td>25.9 ± 11.4</td>
<td>158.2 ± 34.5</td>
<td>28.3 ± 18.8</td>
<td>193.5 ± 34.6</td>
</tr>
</tbody>
</table>

*Values shown are μg/day.

*No intake of food during the 2 day fecal collection.

*Excludes larva No. 2.*
Furthermore, NMR and GC-MS data confirmed the presence of cholesterol, which is predominantly of animal origin. However, the presence of cholesterol in higher plants has been known for years (30, 31). Cholesterol is even the major sterol component in some plant tissues (31). The sterol methyl transferase 1 controls the level of cholesterol in plants by influencing the addition of methyl and ethyl groups to the cholesterol molecule (32). It was suggested that cholesterol may play a more important role in membrane permeability (30).

In conclusion, the diet of the larva plays an important role in determining the sterol composition of the adult M. peleides butterfly. The diet is remarkable for containing cholesterol as well as plant sterols. Metamorphosis does not change total sterol content but significantly changes its sterol composition in the direction of more cholesterol. It is likely that a plant sterol such as sitosterol is converted to cholesterol.

Adult butterflies were harvested from the butterfly exhibit at the Oregon Zoo (Portland, OR). The authors are greatly indebted to the butterfly curator, Mary Jo Andersen, for her generous assistance. The GC-MS and NMR analyses of leaf samples were performed at Rice University with the help of W. K. Wilson. This work was supported by the Oregon Health and Science University Foundation.

REFERENCES