A Dietary Test of Putative Deleterious Sterols for the Aphid Myzus persicae

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Abstract

The aphid Myzus persicae displays high mortality on tobacco plants bearing a transgene which results in the accumulation of the ketosteroids cholestan-3-one and cholest-4-en-3-one in the phloem sap. To test whether the ketosteroids are the basis of the plant resistance to the aphids, M. persicae were reared on chemically-defined diets with different steroid contents at 0.1–10 μg ml⁻¹. Relative to sterol-free diet and dietary supplements of the two ketosteroids and two phytosterols, dietary cholesterol significantly extended aphid lifespan and increased fecundity at one or more dietary concentrations tested. Median lifespan was 50% lower on the diet supplemented with cholest-4-en-3-one than on the cholesterol-supplemented diet. Aphid feeding rate did not vary significantly across the treatments, indicative of no anti-feedant effect of any sterol/steroid. Aphids reared on diets containing equal amounts of cholesterol and cholest-4-en-3-one showed fecundity equivalent to aphids on diets containing only cholesterol. Aphids were reared on diets that reproduced the relative steroid abundance in the phloem sap of the control and modified tobacco plants, and their performance on the two diet formulations was broadly equivalent. We conclude that, at the concentrations tested, plant ketosteroids support weaker aphid performance than cholesterol, but do not cause acute toxicity to the aphids. In plants, the ketosteroids may act synergistically with plant factors absent from artificial diets but are unlikely to be solely responsible for resistance of modified tobacco plants.

Introduction

Sterols are an essential constituent of eukaryotic membranes and contribute to other functions, notably hormones of animals (e.g. mammalian estrogens, insect ecdysteroids) and plants (e.g. brassinosteroids) [1,2]. Despite the broadly equivalent function of sterols in animals and plants, the sterol profile differs between these two groups: animal sterols are generally dominated by cholesterol, while plants contain multiple sterols, known as phytosterols (e.g., sitosterol, stigmasterol), but generally very small amounts of cholesterol [3]. The composition of phytosterols varies among plant species, a trait that is of special significance for phytophagous insects for two linked reasons. First, insects, unlike most other animals, cannot synthesize sterols and are, therefore, dependent on a dietary supply of these nutrients; and, second, phytophagous insects vary in their capacity to utilize different phytosterols [4,5]. A mismatch between the plant sterol content and the sterol utilization traits of a particular insect is predicted to confer plant resistance to the insect of interest [1] thereby limiting the plant range of the insect [4].

The basis of this study is an innovative approach to modify the phytosterol content of plants. Specifically, plants transformed with the bacterial gene choM (sterol oxidase) have a dramatically altered sterol profile, dominated by oxidized ketosteroids instead of phytosterols [6,7]. Three lepidopterans [8,9] and the aphid Myzus persicae [10] display depressed performance on tobacco plants bearing the choM transgene (“modified” plants) relative to plants transformed with the empty vector (“control” plants). The central importance of the ketosteroids in the resistance of modified plants against lepidopteran caterpillars is indicated by the very poor performance of the lepidopteran Heliothis zea on diet supplemented with cholest-3-one, a dominant ketosteroid in the modified plants, relative to diets with no sterol or sterols found in control plants [9].

This study exploited the ease with which the aphid M. persicae can be reared on chemically-defined diets to investigate the effect of ketosteroids on aphid performance. The phloem sap of the modified tobacco plants is dominated by cholest-4-en-3-one and also contains cholestan-3-one (Figure 1); these two ketosteroids are undetectable in the control tobacco plants [10]. Given the negative effects of these two sterols on Lepidoptera, this study tested the hypothesis that the aphids would also display very high larval mortality and sharply curtailed fecundity on diets containing these ketosteroids, relative to diets with sterols detected in control plants (cholesterol and the phytosterols sitosterol and stigmasterol).
Results

Aphid Performance on Single-sterol Diets

To investigate the effect of the individual sterols and steroids on the performance of \textit{M. persicae}, 2-day-old aphids born on sterol-free diets were transferred to chemically-defined diets supplemented with single sterols/steroids previously identified in the phloem sap of control or modified tobacco [9]. Each sterol was tested at one of three concentrations: 0.1, 1 and 10 μg ml$^{-1}$. As a control, we also tested aphids on a sterol-free diet. The insects attached and fed readily across all the diets, and developed to adulthood over 10 days.

Fecundity, lifespan, and larval relative growth rate (Table 1) were similar in all treatments on the lowest dietary sterol/sterol concentration (0.1 μg ml$^{-1}$). At the higher dietary concentrations, significant differences in fecundity and lifespan were observed (Table 1). At 1 μg dietary sterol ml$^{-1}$, thehighest fecundity was obtained with dietary cholesterol; this was also the only treatment for which dietary sterol ml$^{-1}$, fecundity was equivalent on diets containing cholesterol, sitosterol and cholestan-3-one treatments, but the median fecundity was zero for aphids on sterol-free diet and diets containing 10 μg ml$^{-1}$ of either sterol. The effect of diet on the lifespan of the aphids was also concentration-dependent. At 1 μg ml$^{-1}$, dietary cholesterol promoted longer lifespan than other sterol/sterol supplements; and at 10 μg ml$^{-1}$, lifespan did not differ among treatments, apart from the reduced lifespan of aphids on diets containing choles-terol. Median lifespan on the cholesterol and no-sterol control did not differ significantly across all three concentrations. Growth rate differences were observed between treatments with 1 μg ml$^{-1}$, but not 10 μg ml$^{-1}$ dietary sterol (Table 1). However, on the 1 μg ml$^{-1}$ treatments, differences relative to cholesterol were all non-significant.

Interaction between Cholesterol and Cholest-4-en-3-one

This experiment investigated whether cholest-4-en-3-one, a sterol that did not promote aphid reproduction, influenced the effect of dietary cholesterol on aphid performance (Table 2). \textit{M. persicae} was reared on diets containing different concentrations of cholesterol (0, 0.1 and 10 μg ml$^{-1}$), with or without cholest-4-en-3-one (10 μg ml$^{-1}$). Significant differences in reproductive output were observed across the six diets (Table 2) so specific statistical comparisons were made. First, as observed previously (Table 1), reproductive output was significantly higher on the high cholesterol diet (10 μg ml$^{-1}$) compared to diet with 10 μg cholesterol ml$^{-1}$ plus 10 μg cholest-4-en-3-one ml$^{-1}$. Next we tested if dietary cholest-4-en-3-one interacts with cholesterol use. Aphid reproductive output did not differ significantly between diets containing 10 μg cholesterol ml$^{-1}$ and 10 μg cholesterol plus 10 μg cholest-4-en-3-one ml$^{-1}$. Furthermore, supplementing low cholesterol diets (0.1 μg ml$^{-1}$) with 10 μg cholesterol did not rescue reproduction relative to the high cholesterol diet. We concluded that cholest-4-en-3-one does not interact with the effect of cholesterol on aphid performance.

Aphid Performance on Sterol Mixes Reproducing Normal and Modified Tobacco

The final experiments tested the performance of \textit{M. persicae} on chemically-defined diets containing a sterol mix representing the...
Table 1. Aphid performance on diets containing different dietary sterols.

| Dietary sterol         | Relative growth rate (g g⁻¹ day⁻¹) Median (range) | Lifespan (days) Median (range) | Number of reproducing aphids/total | Number of offspring aphid⁻¹ Median (range) |
|------------------------|---------------------------------------------------|-------------------------------|------------------------------------|---------------------------------------------|
|                        | 0.1 µg ml⁻¹ | 1 µg ml⁻¹ | 10 µg ml⁻¹ | 0.1 µg ml⁻¹ | 1 µg ml⁻¹ | 10 µg ml⁻¹ | 0.1 µg ml⁻¹ | 1 µg ml⁻¹ | 10 µg ml⁻¹ | 0.1 µg ml⁻¹ | 1 µg ml⁻¹ | 10 µg ml⁻¹ |
| None                   | 0.258 (0.205–0.303) | 0.273 (0.213–0.305) | 0.275 (0.220–0.319) | 24 (10–32) | 23 (11–30) | 23.5 (11–32) | 4/10 | 4/10* | 4/10* | 0 (0–8) | 0* (0–9) | 0* (0–8) |
| Cholesterol-3-one      | 0.294 (0.185–0.344) | 0.254 (0.215–0.287) | 0.253 (0.149–0.309) | 21 (13–31) | 27 (17–33) | 27.5 (23–30) | 6/10 | 10/10 | 7/7 | 4 (0–13) | 11.5 (8–17) | 9 (8–12) |
| Sitosterol             | 0.269 (0.225–0.336) | 0.286 (0.258–0.306) | 0.257 (0.212–0.309) | 13.5 (12–30) | 14.5 (11–32) | 26.5 (11–32) | 0/10 | 4/10* | 9/10 | 0* (0) | 0* (0–15) | 9.5 (0–17) |
| Stigmasterol           | 0.268 (0.206–0.312) | 0.238 (0.169–0.272) | 0.232 (0.164–0.286) | 26 (11–31) | 15* (8–31) | 20 (11–28) | 4/10 | 3/10* | 2/9* | 0* (0–9) | 0* (0–8) | 0* (0–10) |
| Cholestan-3-one        | 0.269 (0.185–0.337) | 0.281 (0.214–0.331) | 0.294 (0.255–0.342) | 20 (12–31) | 11.5* (8–30) | 26 (13–30) | 2/10 | 1/10* | 8/10 | 0 (0–8) | 0* (0–3) | 8 (0–15) |
| Cholesterol-4-en-3-one | 0.271 (0.222–0.313) | 0.239 (0.173–0.269) | 0.245 (0.193–0.293) | 17 (11–32) | 11* (8–28) | 12.5* (10–28) | 2/10 | 2/9* | 1/10* | 0 (0–7) | 0* (0–8) | 0* (0–6) |

Kruskal-Wallis results are reported for each column, with critical probability after Bonferroni correction for three tests (lifespan, relative growth rate) = 0.016, and for six tests (reproductive indices) = 0.008, with statistically significant values indicated in bold; *indicates significant reduction compared to cholesterol (p<0.05).

doi:10.1371/journal.pone.0086256.t001

Discussion

The genetic basis of plant function is complex, such that simple sterol transgenic plants that have been modified with sterol transgenes can affect a range of plant traits, including the availability of dietary sterol. The transformation of modified tobacco plants with sterol transgenes did not express aphid performance on diets containing cholesterol, cholesterol-3-one, and cholesterol-4-en-3-one. These results suggest that the impact of dietary sterol could be modified by the availability of dietary sterol. The same sterol/mixed diet did not depress aphid performance on diets containing cholesterol, cholesterol-3-one, and cholesterol-4-en-3-one. The modified plant sap did not significantly affect lifespan and had variable effects on reproductive output (Table 3). The modified plant sap indicated that the steroid composition of the modified plant sap had no significant effect on aphid performance on diets with sterol profiles matching the composition of the modified plant sap. The availability of dietary sterol over this period of time [12].

The results of this study indicate that the sterol composition of the modified plant sap, modified plant sap, and modified plant sap can affect a range of plant traits, including the availability of dietary sterol over this period of time [12]. The modified plant sap, modified plant sap, and modified plant sap can affect a range of plant traits, including the availability of dietary sterol over this period of time [12]. The modified plant sap, modified plant sap, and modified plant sap can affect a range of plant traits, including the availability of dietary sterol over this period of time [12]. The modified plant sap, modified plant sap, and modified plant sap can affect a range of plant traits, including the availability of dietary sterol over this period of time [12].
discrepancies between the results obtained for diet- and plant-reared aphids cannot readily be attributed to concentration differences between phloem sap and the diets. Although their absolute concentration in the tobacco phloem sap remains to be determined, the phloem-mobile sterols of other plants attain 0.3–3 μg ml⁻¹ [13,14], which lies within the range (0.1–10 μg ml⁻¹) adopted for our dietary analysis.

The most parsimonious interpretation of these results is that the ketosteroids are poorly utilized by the aphids, but not acutely toxic. This is especially true when ketosteroids are paired with a minimal amount of cholesterol. Consequently, the exceptionally poor performance of the aphids on modified tobacco plants [9] cannot be attributed exclusively to the ketosteroids. This result contrasts with the evidence that dietary cholestan-3-one causes a substantial reduction in developmental rate of the lepidopteran Heliothis zea, which feeds on bulk plant tissue, not plant sap [9]. It would, however, be premature to conclude that the different insects vary in their susceptibility to ketosteroids because the concentration of the dietary steroids used in the study of H. zea [9] was 170 μg ml⁻¹, an order of magnitude greater than used in our study on M. persicae. These data suggest that the plant resistance mechanism in the modified plants containing the choM transgene may comprise a synergistic interaction between the ketosteroids and other plant constituents that are absent from the diet. Further progress in

**Table 2.** Aphid reproduction on diets containing cholesterol, plus or minus cholest-4-en-3-one.

| Diet                                      | Number of reproducing aphids/total | Number of offspring aphid⁻¹ Median (range) |
|-------------------------------------------|-----------------------------------|------------------------------------------|
| Sterol-free                               | 11/20*                            | 1* (0–12)                                |
| Cholesterol (0.1 μg ml⁻¹)                 | 10/20*                            | 0.5* (0–13)                              |
| Cholesterol (10 μg ml⁻¹)                  | 16/18                             | 9 (0–17)                                 |
| Cholest-4-en-3-one (10 μg ml⁻¹)           | 4/19*                             | 0* (0–11)                                |
| Cholesterol (0.1 μg ml⁻¹) + cholest-4-en-3-one (10 μg ml⁻¹) | 7/18*                            | 0.5* (0–15)                              |
| Cholesterol (10 μg ml⁻¹) + cholest-4-en-3-one (10 μg ml⁻¹) | 14/20                            | 7.5 (0–16)                               |

χ² = 18.82, df = 6, p < 0.001

Kruskal-Wallis results are reported for each column, with critical probability = 0.025 after Bonferroni correction for two tests. Statistically significant values of χ² are shown in bold. * indicates a significant reduction compared to 10 μg cholesterol ml⁻¹ (p < 0.05).
elucidating the underlying mechanisms will depend on a greater understanding of the fate of ketosteroids ingested by the aphids, including the extent to which these compounds are assimilated across the gut wall and their subsequent accumulation or metabolic transformations in the insect tissues.

### Materials and Methods

#### Experimental Sterols and Diets

The sterols/steroids were purchased from Sigma Chemical (St. Louis, MO, USA) or Steroids Inc. (Newport, RI, USA); the other diet constituents were purchased from Sigma Chemical (St. Louis, MO, USA). The purchased sterols were tested for purity by HPLC against standards: cholesterol, stigmasterol, cholestan-3-one, and cholest-4-en-3-one were 99% pure, and sitosterol (from Sigma Chemical), which was 60% pure, and was brought to >99% purity by HPLC. Chemically-defined diets were prepared as described previously [15]. Each diet contained 0.15 M amino acids and 0.5 M sucrose, and sterols/steroids were added following published methods [11]. Briefly, they were dissolved in chloroform (1 mg ml$^{-1}$), and added to diets at concentrations between 0.1 and 10 μg sterol ml$^{-1}$.

#### Experimental Aphids

The green peach aphid *Myzus persicae* Sulzer clone SB10/1 was derived from a single parthenogenetic female from a long-term laboratory colony maintained and cultured on preflowering tobacco (*Nicotiana tabacum*) cv Samsun. Routine cultures and all experiments were conducted at 20°C with 18L:6D light regime at 100 μmol m$^{-2}$ sec$^{-1}$ PAR. The experimental insects were larvae deposited onto sterol-free diet over 24 h by adult apterous aphids collected from routine culture on plants. When 2-days-old, the larvae were transferred individually to their test diet in 2.5 cm diam. cages, maintained at 75% relative humidity; all diets were changed every third day. For each treatment, 10 replicate larvae were reared singly. The weight of each larval aphid at day 2 and day 7 was determined to an accuracy of 1 μg; for these two time periods, and the relative growth rate (RGR) for each insect was calculated as: RGR = log$_e$(final weight/initial weight)/number of days. The insects were monitored daily until death, and for each aphid lifespan and reproductive output was recorded.

### Statistical Analysis

All data sets were checked for normal distributions by the Anderson Darling test, and homogeneity of variances by the Levine and Bartlett tests. All the aphid performance data (lifespan, reproduction, RGR) followed non-normal distributions, and were analyzed using nonparametric tests. Kruskal-Wallis tests were used for analysis of lifespan, number of offspring/aphid, and RGR; where significant differences were detected, and when there were more than 2 treatments, post-hoc comparisons were performed with a specified control treatment, using the Dunn Method for Joint Ranking [18]. The number of aphids reproducing was analyzed using Proportion tests, and Tukey-type multiple comparisons were performed to identify which treatments differed from one another [19]. Food uptake followed a normal distribution and was analyzed with ANOVA.
Author Contributions
Conceived and designed the experiments: SB RG STB AED. Performed the experiments: SB MLF. Analyzed the data: SB STB AED. Contributed reagents/materials/analysis tools: RG. Wrote the paper: SB STB AED.

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