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The physiology of sterol nutrition in the pea aphid *Acyrthosiphon pisum*

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ABSTRACT

The phloem sap of fava bean (*Vicia faba*) plants utilized by the pea aphid *Acyrthosiphon pisum* contains three sterols, cholesterol, stigmasterol and sitosterol, in a 2:2:1 ratio. To investigate the nutritional value of these sterols, pea aphids were reared on chemically-defined diets containing each sterol at 0.1, 1 and 10 μg ml^{-1} with a sterol-free diet as control. Larval growth rate and aphid lifespan did not vary significantly across the diets, indicating that sterol reserves can buffer some performance indices against a shortfall in dietary sterol over at least one generation. However, lifetime reproductive output was depressed in aphids on diets containing stigmasterol or no sterol, relative to diets supplemented with cholesterol or sitosterol. The cholesterol density of embryos in teneral adults was significantly higher than in the total body; and the number and biomass of embryos in aphids on diets with stigmasterol and no sterols were reduced relative to diets with cholesterol or sitosterol, indicating that the reproductive output of the pea aphid can be limited by the amount and composition of dietary sterol. In a complementary RNA-seq analysis of pea aphids reared on plants and diets with different sterol contents, 7.6% of the 17,417 detected gene transcripts were differentially expressed. Transcript abundance of genes with annotated function in sterol utilization did not vary significantly among treatments, suggesting that the metabolic response to dietary sterol may be mediated primarily at the level of enzyme function or metabolite concentration.

1. Introduction

A plant presents multiple barriers that a phytophagous insect must overcome for successful feeding, growth and reproduction. These barriers include physical traits (toughness, water content etc.), chemical properties (including toxins and phenolics), and unbalanced or inadequate nutrient content (Price et al., 2011). A potentially important, but relatively neglected, nutritional barrier is the sterol content and composition of plant tissues. The basis for this interaction is threefold. First, sterols, especially cholesterol, are a required constituent of all animals, including insects, as a structural component of membranes and substrate for steroidal hormone synthesis (e.g. the insect ecdysteroids). Second, insects and other arthropods, uniquely among animals, cannot synthesize sterols *de novo*, and depend on an exogenous source, usually the diet. Third, plants produce >100 sterols, requiring metabolic transformation to cholesterol or utilization without transformation by phytophagous insects (Behmer and Nes, 2003). Furthermore, sterol nutrition may contribute to the complex patterns of plant utilization by phytophagous insects because the plant sterol content varies among species and with tissue type, and the capacity to utilize different phytosterols varies among phytophagous insects. For example, the phytosterol spinasterol is apparently absent from grasses, and is toxic to the grass-feeder *Schistocerca americana* (Behmer and Elias, 1999), but it is a quantitatively-important sterol in the dicot *Solidago altissima*, which supports a diverse insect community, including some taxa that accumulate spinasterol without modification and others that metabolize spinasterol to cholesterol (Janson et al., 2009).

Nutritional factors may be a particularly important determinant of plant utilization patterns by phloem-feeding insects because many mechanisms of chemical protection available to other plant tissues are not compatible with this intracellular compartment (Douglas, 2006). Most research on nutritional barriers has concerned sugars and amino acids, which are the two most abundant classes of phloem-mobile nutrients (Douglas, 2003), and very little is known about the sterol nutrition of phloem-feeding insects. A minority of phloem-feeding insects, including some planthoppers, derive sterols from fungal symbionts (Noda and Koizumi, 2003), but the great majority of phloem-feeders bear bacterial symbionts that cannot synthesize sterols. Therefore, most aphids, whitefly,

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psyllids, mealybugs and leafhoppers are predicted to meet their sterol requirements exclusively from phloem sap (Behmer and Nes, 2003; Douglas, 2003). The sterol profile of phloem sap can differ markedly from bulk plant tissues, and contain both phytosterols and cholesterol (Shigematsu et al., 1982; Behmer et al., 2011). Aphids are reported to have limited capacity to dealkylate phytosterols to cholesterol (Campbell and Nes, 1983), but their capacity to utilize different phytosterols has not been investigated systematically.

The physiological study of aphids is being transformed by the availability of genomic data, especially the fully-sequenced genome of the pea aphid *Acyrthosiphon pisum* (IAGC, 2010). Nevertheless, genomic resources can be applied most productively where base-line information on the insect traits of interest is available. In this context, the aims of this study were twofold: (1) to establish the sterol utilization traits of the pea aphid by analysis of aphids reared on diets supplemented with phloem-mobile sterols and (2) to determine the impact of dietary sterols on aphid gene expression.

2. Material and methods

2.1. The aphids

A parthenogenetic line of the pea aphid *A. pisum* Harris (clone CWR09/18) was used in this study. It was derived from a single female collected in 2009 from alfalfa crop in Freeville, New York, USA. Aphids were maintained on preflowering fava bean (*Vicia faba*) cv. Windsor. All plant and aphid cultures were reared at 20 °C under a 18 L: 6D light regime, at 100 μmol m^{-2} sec^{-1} PAR.

2.2. Sterol sample collection and analysis

Samples of leaf tissue and phloem sap exudates were collected from *V. faba*, the pea aphid rearing plant (Behmer et al., 2011). The top fully-expanded leaf from three plants was excised and dried at 50 °C for 3 days. The equivalent leaf from replicate plants was excised, and its petiole was immediately inserted into 0.2 ml 5 mM EDTA solution, pH 7.5, and incubated for 90 min in the dark in a sealed chamber equilibrated at 25 °C with a dish of saturated KH_2PO_4 to maintain high humidity. This protocol for EDTA exudation of phloem sap (Karley et al., 2002) minimizes nonspecific metabolite leakage from plant cells. The EDTA samples were frozen at −20 °C prior to analysis. For analysis of sterols in aphid honeydew, the honeydew produced by aphids was collected using absorbent paper (Nuc-wipes, National Diagnostic), dried for 50 °C for 3 days, and then processed as for plant tissues above.

The procedure for sample preparation, sterol extraction and analysis followed the method of Behmer et al. (2011) exactly. Briefly, the plant and insect samples were disrupted with 30 ml 95% ethanol with #5 glass beads (Sigma, St Louis, MO, USA). Next, sterols were extracted through a series of ethanol, chloroform, methanol and methanol:water-equilibrated hexane washes, and then separated by HPLC (Waters) with sterol standards. Peaks with the elution profile predicted for sterols were identified and quantified by gas chromatography–mass spectroscopy (Agilent 6850N GC coupled with a 5973 mass selective detector (Technologies, Inc., Santa Clara, CA, USA).

2.3. Aphid performance studies

Aphid performance was recorded on chemically-defined diets prepared following the procedure and composition of Prosser and Douglas (1992), with 0.5 M sucrose and 0.15 M amino acids (formulation A). Diets were either sterol-free or supplemented with cholesterol (cholest-5-en-3β-ol), sitosterol (5,22-cholestadiene-24β-ethyl-3β-ol), or stigmasterol (5,22-cholestadien-24β-ethyl-3β-ol). All diet constituents were purchased from Sigma Chemical (St. Louis, MO, USA). The purchased sterols were tested for purity by HPLC against standards: cholesterol and stigmasterol were >99% pure, but the sitosterol was 60% pure and was brought to >99% purity by TLC and HPLC. Each sterol was dissolved in chloroform (1 mg ml^{-1}), and added to diets at one of three concentrations: 0.1, 1.0, and 10 μg sterol ml^{-1} diet. Air at ambient temperature was blown across the surface of all diets to remove the chloroform, and then administered to the aphids. The diet-reared aphids were maintained at 75% humidity under the same light and temperature conditions as on plants, and all diets were changed every third day.

To determine aphid lifespan, age to first reproduction, and daily fecundity, adult apterae were transferred from routine culture on plants to sterol-free diet, and allowed to larviposit over 24 h. When 2 days-old, 10 larvae were transferred individually to cages (2.5 cm dm) of sterol-free diet and diets containing 0.1, 1.0 or 10 μg ml^{-1} of one sterol, and were monitored daily to death. In a separate experiment, 10 replicate insects for each diet treatment were weighed at day-2 and day-7, to the nearest μg, using a Mettler Toledo (MX5) microbalance, and the relative growth rate (RGR) for each experimental insect was calculated (RGR = [$\log_e$(final weight/initial weight)]/number of days).

As alternative indices of reproductive potential, the number of embryos with pigmented eyespots, and the protein content and cholesterol content of aphid embryos were quantified. Embryos were dissected using fine pins at ×40 magnification. To determine the contribution of embryo protein to total aphid protein of 10 day-old teneral apterae, the protein content of the whole body (8 replicates) and embryos (15 replicates) was determined. Each sample was homogenized in 800 μl ice-cold buffer A (35 mM Tris, 25 mM KCl, 10 mM $MgCl_2$, pH 7.5), and centrifuged for 1 min at 18,000 g. The protein content of 5 μl supernatant was determined by the DC protein assay (Biorad; following the manufacturer's instructions), using a Biorad X-mark spectrophotometer, and 2–100 μg ml^{-1} bovine serum albumin (BioRad) standards. All samples and standards were assayed in triplicate. The cholesterol density in the whole body and embryos of four replicate samples of 7 day-old larvae was assayed using the cholesterol/cholesteryl ester quantification assay (AbCam), following the manufacturer's instructions. The cholesterol contents were normalized to protein content.

2.4. Radiotracer studies

Food uptake by aphids was quantified by the radioisotope technique of Wright et al. (1985) and Karley et al. (2002). Twenty-five 7-days-old aphid larvae, reared from birth on a sterol-free diet, were transferred individually to a Perspex ring (3.5 cm dm, 0.5 cm height) with diet containing 16 μCi [^{14}C] inulin ml^{-1} (Sigma), either supplemented with 10 μg non-radioactive sterol (cholesterol, sitosterol or stigmasterol), or no sterol. Honeydew produced by the aphid was deposited onto a 3.5 cm circle of absorbent paper (Nuc-wipes, National Diagnostic) placed under each ring. After 48 h, the paper circle was transferred to 5 ml Ecoscint (National Diagnostic) and counted in a scintillation counter (Beckman LS6500), with a preset ^{14}C window and quench curve. The mean of three replicate aphids and paper circles on non-radioactive diets of the same formulation was subtracted from the experimental values. Control experiments confirmed that the radioactivity in the aphid carcass was consistently <10% of the radioactivity recovered from the honeydew, confirming that the inulin is not assimilated.

In a separate experiment, the recovery of radioactivity in honeydew from aphids feeding on dietary ^{14}C-cholesterol was deter-

Table 1
The sterol content of *V. faba*.

Plant tissue	% sterol content (adjusted median)			
	Campesterol	Cholesterol	Sitosterol	Stigmasterol
Total leaf	21.3	0.3	33.9	44.5
Phloem sap	Undetectable	43	17.3	39.4

mined using the same protocol, but with aphids fed on diets containing 0, 1, 5 or 10 μg cholesterol, each combined with 40 μCi [U-^{14}C] cholesterol (Sigma) per ml diet.

2.5. RNA-seq analysis

Each sample comprised the RNA of 7-day-old final larval instar aphids that had been feeding since birth on either plants (20 aphids per sample) or diets (25 aphids per sample) supplemented with no sterol or with cholesterol or sitosterol at 0.1, 1 or 10 μg ml^{-1}. The RNA was extracted using the RNeasy Mini kit (Qiagen) following manufacturer's instructions, and treated with DNase using DNA-Free kit (Ambion); RNA quality and integrity was confirmed using a Bioanalyzer 2100 (Agilent). About 2 μg of resulting RNA was subjected to multiplex library construction performed using Illumina mRNA sequencing sample preparation kit following manufacturer's instructions (Illumina). The aphid samples were multiplexed using adaptors prepared by Cornell University Life Sciences Core Laboratories, and sequenced by Illumina HiSeq2000 Platform or Illumina Genome Analyzer II platform. FastxToolkit (http://hannonlab.cshl.edu/fastxtoolkit/) was applied to sort libraries, remove barcode tags and adaptor sequences, and trim the sequence read length to 50 bp for subsequent alignment. The expression score of each transcript represented in RPKM (reads per kilobase of transcript per million mapped reads) (Mortazavi et al., 2008) was computed by the TopHat (Trapnell et al., 2009) and Cufflinks (Trapnell et al., 2010) pipeline. The TopHat program aligned the 50 bp sequence reads to reference indexes of pea aphid OGS 1.0 mRNAs (Official Gene Set, representing 34,821 mRNA transcript sequences of 34,604 gene models) (IAGC, 2010; Legeai et al., 2010), plus the 13 protein-coding mitochondria genes. The RPKM value of each gene transcript was computed by Cufflinks, based on the alignment resulting from TopHat, and a GTF file providing coordinates of gene transcript boundaries. The mapping of reads to genes was visualized using Integrative Genomics Viewer v. 1.2 (http://www.broadinstitute.org/igv/v1.2). Pairwise comparisons of RPKM values of samples were obtained using Tophat and Cuffdiff pipeline (Trapnell et al., 2010). To detect genes with differential expression between treatments, the criteria of FDR $\leqslant 0.05$ (estimated by Cuffdiff) and difference in RPKM that is both $\geqslant$two-fold and $\geqslant 1$ units were applied. Hierarchical cluster analysis of gene expression profiles was obtained after $\log_2$ transformation of the RPKM values, using the Hierarchical Clustering module, via the Gene Pattern genomic analysis platform (Reich et al., 2006). Specifically, a linear correlation between every pair of transcripts in each sample was calculated. This was then converted into a distance measure, and a linkage map based on the average distance among transcript pairs was constructed to obtain the hierarchical cluster dendrogram.

2.6. 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. Performance and nutritional data meeting these criteria were analyzed by ANOVA, with Duncan's posthoc test for pair-wise comparisons. Datasets with non-normal distributions were analyzed with the Sheirer-Ray-Hare test. The % composition of plant sterols were not normally distributed, and the median values that did not sum to 100. Adjusted median values, comprising raw medians divided by the total content of all sterols in that tissue type (Behmer et al, 2011), are displayed.

3. Results

3.1. The sterol content of V. faba leaves and phloem sap

Stigmasterol was the most abundant sterol recovered from *V. faba* leaf tissue, followed by sitosterol then campesterol (Table 1). In contrast, cholesterol was the most abundant sterol recovered in the phloem, closely followed by stigmasterol (Table 1); the phloem also contained sitosterol, but in lesser amounts. In supplementary analyses, stigmasterol was the only sterol detected in the honeydew of three replicate colonies of pea aphids (mixed ages) feeding on *V. faba* (data not shown).

Table 2
Performance indices of aphids feeding on sterol-free and sterol-supplemented diets, with statistical analysis by Schierer-Ray-Hare test. The critical probability after Bonferroni correction for four tests is 0.0125.

Sterol concentration (μg ml^{-1}) diet	Dietary sterol			No dietary sterol	Schierer-Ray-Hare test	
	Cholesterol	Sitosterol	Stigmasterol		Sterol type	Sterol concentration
(a) Median relative growth rate (g g^{-1} day^{-1})						
0.1	0.366	0.346	0.352	0.365		
1	0.325	0.336	0.358	0.350	0.64	0.70
10	0.305	0.317	0.370	0.342		
(b) Median age to first reproduction (days)						
0.1	13	14	15	14.5		
1	14	13	13.5	15	1.96	0.11
10	13	13	16	15		
(c) Median life-time fecundity (number of offspring)						
0.1	4.5	8.5	0	0		
1	17	14	2.5	3	26.82*	15.63*
10	15.5	16	0.5	1		
(d) Median lifespan (days)						
0.1	21.5	20	21.5	17.5		
1	15	27	24	15.5	3.54	1.73
10	28	27	21	16.5		

* $p < 0.0125$.

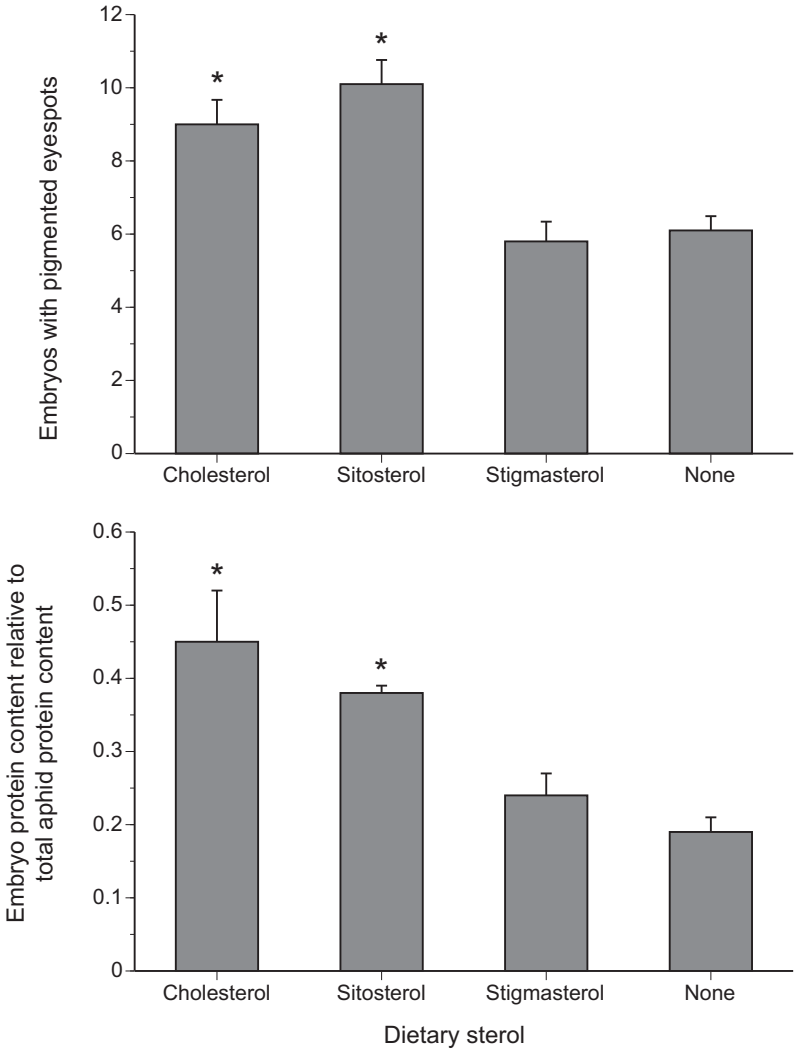

Fig. 1. The embryo content of 10 day old pre-reproductive adult apterous aphids. (a) Number of embryos with red-pigmented eyes (ANOVA $F_{5,3} = 17.5$, $0.001 < p < 0.01$). (b) Embryo protein content (ANOVA, $F_{5,3} = 6.924$, $0.01 < p < 0.05$). Values are means + SEM. *indicates a statistically significant difference from the sterol-free diet ($p < 0.05$).

3.2. Aphid responses to dietary sterols

The 2-day-old larvae fed readily from diets containing each sterol, at each of the three concentrations tested, and the control sterol-free diet, as indicated by sustained honeydew production. To test more precisely whether dietary sterols influence aphid feeding rate, diet consumption by 25 aphids on sterol-free diet and diet supplemented with one of cholesterol, sitosterol and stigmasterol at 10 μg ml^{-1} was assessed. Over the 2 day experiment, each 7–9-day-old aphid consumed 0.2–0.4 μl diet, with no significant variation across the diets (ANOVA $F_{3,29} = 2.11$, $p > 0.05$).

Sterol type and concentration did not significantly affect larval RGR, age to first reproduction or lifespan, but did have a significant effect on lifetime fecundity (Table 2). At every sterol concentration tested, more aphids reproduced on diets supplemented with cholesterol or sitosterol than on sterol-free diets and, at 1 and 10 μg ml^{-1} diet, these sterols supported a median of >10 offspring per aphid. The reproductive output of aphids on stigmasterol-supplemented diets and sterol-free diet were not significantly different ($p > 0.05$).

To investigate the basis of the variation in reproductive output with dietary sterols, the embryo complement of 10 day-old apterous adults (at this age, the aphids are pre-reproductive on all diets) reared on sterol-free diet and diets with 10 μg sterol ml^{-1} was studied. All aphids examined contained embryos that appeared structurally intact and without abnormalities (evident at ×30 magnification), and the embryo strings included embryos with pigmented eyespots, indicative of stage 18–22 of embryo development (Miura et al., 2003). Both the number of embryos with pigmented eyespots and the contribution of embryo protein to the total protein content of the aphids were significantly higher in aphids reared on diets with cholesterol and sitosterol than the stigmasterol-supplemented diet and sterol-free diet (Fig. 1).

Table 3
Cholesterol content of 7 day-old larval pea aphids.

Aphid tissue	μg cholesterol mg^{-1} protein (mean ± SEM, $n = 4$)	
	Sterol-free diet	10 μg cholesterol ml^{-1} diet
Total body	15 ± 2.9	12 ± 2.7
Embryos	26 ± 5.8	25 ± 4.7
ANOVA		
Diet	$F_{1,16} = 0.365$, $p > 0.05$	
Aphid tissue	$F_{1,16} = 12.7$, $0.01 < p < 0.05$	
Interaction	$F_{1,16} = 0.14$, $p > 0.05$	

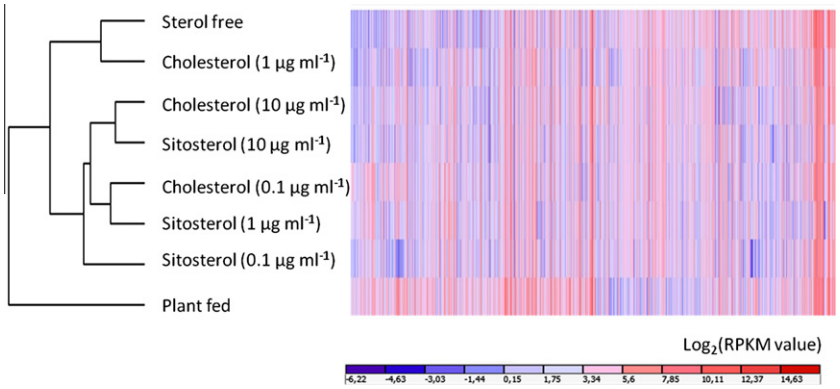

Fig. 2. Hierarchical cluster analysis of gene expression profiles of 2281 genes differentially expressed between the 8 aphid samples. The color scheme bar represents values of $\log_2$ (RPKM).

The cholesterol content of embryos was significantly elevated relative to the total aphid body in insects reared on both sterol-free diet and diet supplemented with 10 µg cholesterol ml^{-1} (Table 3), but this nutritional index was not significantly affected by diet.

The final physiological experiment quantified the fate of radiolabel in ^{14}C-cholesterol administered over 2 days, in 7–9-day-old aphids. In all experiments using diets with 3 µg ^{14}C cholesterol ml^{-1} and 0–13 µg non-radioactive cholesterol ml^{-1}, ^{14}C was recovered from the aphids, but not from honeydew. From the detection limit of the assay, it was calculated that the aphids assimilate dietary cholesterol with at least 90% efficiency.

3.3. Transcriptome analysis

Between 2.9 and 30 million reads were obtained from multiplexed RNA-seq analysis of pea aphids reared on plants, sterol-free diet and diet supplemented with cholesterol or sitosterol (Supplementary Table 1). Of these, 71–75% could be mapped to the aphid genome, representing 14,631–19,172 genes, including 11,575 genes (60–79%) with RPKM > 1 in all samples (Supplementary Table 2). Gene Ontology analysis showed very similar distributions of gene functions across all samples (Supplementary Fig. 1).

The expression of 2281 (7.6%) of the 17,417 genes detected in the transcriptome analysis with RPKM > 1 differed significantly between at least two treatments, and hierarchical cluster analysis of these genes divided the samples into two groups: plant-reared aphids and the aphids reared on the 7 diets (Fig. 2). This difference was driven largely by the 326 genes detected only in plant-reared aphids and the 120 genes detected in aphids on all diets but not in the aphids on plants (Supplementary Table 3). Most of these genes have no annotated function and RPKM < 10 units; exceptionally, 12 genes with expression unique to plant-reared aphids have RPKM > 100, including ACYPI144068, the second most highly expressed gene, with RPKM of 107,339 units. The predicted functions of genes with annotations (17% and 9% of genes specific to plant- and diet- reared aphids, respectively) are diverse (Supplementary Table 2).

The transcriptomes of diet-reared aphids were analyzed for genes with predicted function in sterol utilization and metabolism: (1) Δ-24-sterol reductase (ACYPI009020), predicted homolog of the enzyme activity implicated in the last step of the dealkylation of the phytosterols, including sitosterol, to cholesterol in the insect *Manduca sexta* (Svoboda and Weirich, 1995); (2) Niemann Pick protein NPC1 (ACYPI004532), homolog of the mammalian NPC1 (Carstea et al., 1997) and the NPC1a in *Drosophila melanogaster* which is ubiquitously expressed and promotes the intracellular trafficking of cholesterol (Fluegel et al., 2006); and (3) the halloween genes mediating the transformation of cholesterol to ecdysteroid hormones (Christiaens et al., 2010). Transcripts of all these genes were detected, with no significant variation in RPKM across the diets (by the criteria of FDR $\leqslant$ 0.05 and difference in RPKM that is both $\geqslant$twofold and $\geqslant$1 units). We also scored the cytochrome P450 genes, which include members of the clade CYP2 involved in fatty acid and sterol metabolism (Feyereisen, 1999; Ramsey et al., 2010). Fifty-four of the 83 annotated P450 genes were expressed (RPKM $\geqslant$ 1) in the diet-reared aphids. The mean RPKM varied between 0.9 and 610, and just one CYP2 gene (ACYPI003371) had higher expression in aphids feeding on 0.1 µg and 10 µg cholesterol ml^{-1} than on sterol-free and sitosterol-supplemented diets, but the difference was not significant for aphids on 1 µg cholesterol ml^{-1} diet.

Subsequent analysis of the diet-reared aphids addressed the full transcriptome data. The pattern of variation did not match to the identity or concentration of dietary sterol by cluster analysis (Fig. 2) and other classification techniques applied (not shown). These results matched to our visual inspection of the data, which revealed no genes up/down-regulated in aphids on the sterol-free diet relative to every cholesterol- or sitosterol-supplemented diet. As a supplementary approach, we determined the genes with significantly different expression between aphids reared on 0.1 versus 1 µg cholesterol ml^{-1}, and on 1 versus 10 µg cholesterol ml^{-1} diet. Of the 704 genes identified in these analyses, just one gene ACYPI24182 (with no annotated function) was significantly downregulated at the higher cholesterol concentration in both comparisons, and three genes [ACYPI008868 (annotated as lipase-like), ACYPI004672 (a candidate omega-amidase) and ACYPI009984 (no annotated function)] were upregulated in both tests. The pattern for aphids on sitosterol-supplemented aphids was similar. Of the 745 differentially-expressed genes, all effects were unique to one comparison apart from three genes consistently downregulated with increasing sitosterol concentration [ACYPI006282 (serine protease); ACYPI005037 and ACYPI45829 (no annotated function)], and four genes consistently upregulated [ACYPI007329 (cuticle protein); ACYPI008608, ACYPI009401 and ACYPI47859 with no annotated function].

4. Discussion

The profile of sterols in plant phloem sap is central to our understanding of the sterol nutrition of aphids. Although phytosterols dominate the sterol content of bulk plant tissues, cholesterol has previously been identified as a major component of phloem sap sterols in Chinese cabbage and tobacco plants (Behmer et al., 2011). The data presented here for the fava bean extends the dis-

tribution of phloem cholesterol to leguminous plants, confirming that cholesterol, traditionally viewed as an animal sterol, can represent an important dietary sterol for phloem-feeding insects. Furthermore, the pea aphid can utilize dietary cholesterol very efficiently, as indicated by the undetectable cholesterol in honeydew produced by insects reared on plants or diets containing cholesterol.

Research on chewing phytophagous insects, including the orthopteran *Schistocerca* and lepidopteran *Plutella xylostella* (Behmer and Grebenok, 1998; Behmer and Elias, 1999), demonstrate that phytosterols vary in their nutritional value to insects. This first systematic analysis of phytosterol utilization in an aphid extends this generality to phloem-feeding insects. Specifically, of the two major phytosterols in the phloem sap of the fava bean host plant, only sitosterol and not stigamasterol supports performance superior to the sterol-free diet. Two factors may contribute to this difference. Aphids may have a greater capacity to metabolize sitosterol than stigmasterol to cholesterol, and sitosterol may be utilized without dealkylation to cholesterol for some functions, including membrane biogenesis; there is evidence that sitosterol is superior to stigmasterol in supporting cell membrane architecture (Hodzic et al., 2008). The exclusive recovery of stigmasterol in the honeydew of pea aphids reared on plants indicates that variation in net assimilation of different dietary sterols may contribute to the variation in their support of insect growth and reproduction. It might also suggest that aphids, in a fashion analogous to vertebrates, may selectively pump particular sterol structures back into the gut lumen (Berge et al., 2000).

Linked to the inability of insects to synthesize sterols *de novo*, the deleterious effects of sterol-free diet are generally apparent within a single insect generation. The sterol requirement of the pea aphid was revealed as reduced reproductive output on the sterol-free diet relative to diet supplemented with cholesterol; high mortality during larval development or at eclosion, as reported in some other insects on sterol-free diets (Nes et al., 1997), was not observed, suggesting that the maternal allocation of sterol to offspring is substantial in the pea aphid, as has also been suggested for other insect herbivores (Behmer and Grebenok, 1998). The significantly higher sterol density of embryos than the whole aphid body (Table 3), which has also been observed in other hemimetabolous insects (Behmer et al., 1999), is consistent with this conclusion. Furthermore, the uniform sterol density of embryos in aphids on sterol-free and cholesterol-supplemented diets indicates that sterol allocation to reproduction is strongly regulated. This allocation strategy, in which sterol deficiency leads to reduced number of offspring without compromising the sterol content of each embryo (Fig. 1 and Table 3), suggests that sterol provisioning of offspring is selectively important, with the implication that the pea aphid may have evolved in the context of variable, and often limiting, levels of utilizable sterols in its natural diet of plant phloem sap.

An important challenge for insect nutritional physiology is to relate physiological responses at the whole-insect level to molecular phenotype. Variation in gene expression with diet offers a potentially fruitful route to identify relevant genes, and interpretation of the RNA-seq data in this study is facilitated by minimal variation in overall condition of the insects on the different diets. For example, where insects under different diet treatments vary widely in growth rate, size or general health, it can be difficult to discriminate whether transcriptional differences arise from the direct effects of diet or from diet-dependent effects on insect condition. This issue is illustrated by the difference between the transcriptomes of plant-reared and diet-reared aphids (Fig. 2). The difference can be attributed partly to differences between the two food substrates. Phloem sap has a more complex and variable composition that potentially includes defensive compounds as well as nutrients. Additionally, the different chemical and mechanical cues in plants and diet sachets may affect gene expression patterns in specific systems of the aphid (e.g. nervous system, salivary glands, alimentary tract). However, aphid performance is superior on plants than diets; plant-reared aphids are generally larger and have substantially greater investment in embryos than diet-reared aphids. Consequently, some transcriptional differences can be attributed to these physiological differences. Further research, especially using aphids reared on plants for short periods, are required to discriminate between the transcriptional changes attributable to food source and down-stream effects of food source on insect physiology.

These constraints on our capacity to interpret transcriptional differences between plant- and diet-reared aphids are much less significant for the comparisons across diets with different sterol contents, because the performance differences among the aphids were relatively slight. The multiple statistically-significant differences in transcript abundance among aphids reared on the different diets did not included genes with annotated function related to sterol acquisition or metabolism and, furthermore, they could not be linked to the identity or concentration of the dietary sterol. These results indicate that the aphid metabolic response to variation in the dietary content or composition of dietary sterols may be dominated by processes operating at the level of enzyme activity or pool size of specific metabolites.

Taken collectively, our data raise the possibility that the physiology of sterol nutrition at the whole-insect and molecular level in aphids may have evolved to function in the context of variable sterol levels in plant phloem sap. This interpretation is consistent with the evidence that other phloem nutrients, particularly sugars and amino acids, also vary temporally and spatially at multiple scales, e.g. with time of day, plant developmental age and season, among plants, with position on plant and even between adjacent sieve elements (Pate et al., 1998; Karley et al., 2002; Douglas et al., 2006; Gattolin et al., 2008). Our data indicate that testing this hypothesis will require coordinated analysis of aphid responses to phloem sterols from the perspectives of the level and activity of key sterol-metabolizing enzymes and sterol metabolites, and their interactions with reproduction and embryogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2012.07.014.

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