All eukaryotes contain sterols, which serve as structural components in cell membranes, and as precursors for important hormones. Plant vegetative tissues are known to contain mixtures of sterols, but very little is known about the sterol composition of phloem. Plants are food for many animals, but plant-feeding arthropods (including phloem-feeding insects) are unique among animals in that they have lost the ability to synthesize sterols, and must therefore acquire these essential nutrients from their food, or via endosymbionts. Our paper starts by providing a very brief overview of variation in plant sterol content and how different sterols can affect insect herbivores, including those specializing on phloem. We then describe an experiment, where we bulk collected phloem sap exudate from bean and tobacco, and analyzed its sterol content. This approach revealed two significant observations concerning phloem sterols. First, the phloem exudate from each plant was found to contain sterols in three different fractions – free sterols, sterols conjugated to lipids (acylated), and sterols conjugated to carbohydrates (glycosylated). Second, for both plants, cholesterol was identified as the dominant sterol in each phloem exudate fraction; the remaining sterols in each fraction were a mixture of common phytosterols. We discuss our phloem exudate sterol profiles in a plant physiology/biochemistry context, and how it relates to the nutritional physiology/ecology of phloem-feeding insects. We close by proposing important next steps that will advance our knowledge concerning plant phloem sterol biology, and how phloem-sterol content might affect phloem-feeding insects.

Keywords: aphids, bean, cholesterol, hemiptera, insect nutritional physiology, phytosterols, tobacco
in the phloem sap where they accounted for 76% of the phloem sterol content (in the vegetative tissues they represented 43% of the total sterol profile). Bouvaine et al. (2012) also showed that in the fava bean, phytosterols comprised >99% of the vegetative tissue sterol profile, but that cholesterol was the dominate sterol in the phloem (43%; sitosterol and stigmasterol were present at 17.3 and 39.6%, respectively). To date, though, all phloem sterol profile analyses have been limited to sterols that occur in the free-form; in this form they are likely transported in the aqueous phloem by a lipid-binding protein (Guelette et al., 2012). However, as in other organisms, a proportion of the sterol may be conjugated – for example, attached to a lipid (acylated), or to a carbohydrate (glycosylated; Moreau et al., 2002; Aguirre et al., 2012; Schrick et al., 2012). Aciylated sterols, like free-sterols, would require a lipid-binding protein to move in the phloem. In contrast, glycosylated sterols would likely be soluble in the phloem. Unfortunately, we currently know virtually nothing about the extent to which the phloem contains sterols in free, acylated, and glycosylated forms.

Many animals, including insects, feed on plants, but all insects (in fact all arthropods) are metabolically impoverished relative to most other organisms in one crucial respect: they lack the capacity for de novo synthesis of sterols (Grieneisen, 1994; Behmer and Nes, 2003). Cholesterol is the most abundant sterol recovered from most insects, including those feeding on plants (Behmer and Nes, 2003), and most insects have an absolute requirement for cholesterol as the precursor for ecdysteroid synthesis (Grieneisen, 1994). Plant-feeding insects typically generate cholesterol by metabolizing phytosterols. For example, caterpillars can readily convert sitosterol and stigmasterol to cholesterol (Svoboda and Weinrich, 1995; Svoboda, 1999; Jing et al., 2012b). However, not all insects share the same sterol metabolic capabilities. Grasshoppers, for instance, cannot convert stigmasterol to cholesterol (Behmer and Elias, 1999, 2000). Other sterol metabolic constraints are more general – no plant-feeding insect can insert a double bond at the C5-position in the sterol nucleus, and there is little evidence that insects modify, other than for metabolic purposes (Brown et al., 2009), the position of double bonds in the sterol nucleus (reviewed by Behmer and Nes, 2003). When chewing insect herbivores ingest large quantities of sterols that cannot be converted into cholesterol, they often suffer reduced performance (Behmer and Grebenok, 1998; Behmer and Elias, 1999, 2000; Jing et al., 2012a,b). Additionally, unsuitable sterols can reduce feeding (Behmer et al., 1999).

Although most insects derive their sterol requirement from the diet, some insects that feed on foods of very low nutritional quality (e.g., wood) obtain sterols from yeast symbionts (Noda and Koizumi, 2003). Early studies (Ehrhardt, 1968) also implicated microbial symbionts in the sterol nutrition of plant-sap feeding insects, especially aphids, but improved analytical techniques have refuted these interpretations (Campbell and Nes, 1983). It is now appreciated that the yeast symbionts borne by a few plant sap-feeding insects (a minority of planthoppers and aphids) provide their insect hosts with sterols (Noda et al., 1979; Noda and Koizumi, 2003). In contrast, bacterial symbionts cannot provide sterols, because bacteria cannot biosynthesize sterols (Douglas, 2009). Therefore, most phloem feeding insects are dependent on the phloem sap for their sterol requirements. We have recently demonstrated that the aphid Myzus persicae feeds poorly and
MATERIALS AND METHODS

EXPERIMENTAL PLANTS

Two plant species were used: Phaseolus vulgaris and Nicotiana tabacum (var. Xanthi). Seedlings were germinated under transparent plastic drape (to maintain high humidity); upon establishing, the plastic drape was replaced with a 6-inch transparent plastic dome. When plants outgrew the plastic domes, they were grown under tents of transparent plastic drape. All plants were maintained at 23°C with 12L:12D at 120 μmol m⁻² s⁻¹ PAR; they were watered as needed and fertilized once per week.

COLLECTION OF PHLOEM SAP

Samples for sterol analysis were obtained from multiple plant leaves (located at least 3 leaves below the apical meristem at the day of harvest) using the EDTA exudation technique developed by King and Zevaart (1974), and later modified by Karley et al. (2002). Briefly, leaves were excised and the petioles inserted immediately into an Eppendorf tube containing 1 ml 10 mM Na⁺ EDTA solution, pH 7.5. The samples were then incubated for 60 min in the dark in a sealed chamber equilibrated at 23–25°C; the inside of the chamber was lined with water saturated paper towels to maintain high humidity.

The number of leaves harvested/plant, and the number of plant leaves used to generate each sterol exudate sample varied based on the plant. For free sterol analysis, 50% of the hexane fraction was taken, conjugated, and analyzed by GC–MS. For acylated sterol analysis, the remaining 50% of the hexane fraction was reconstituted in 100 μl of clean hexane and 8 ml of 70% MeOH-water containing 5% KOH was added, and then incubated in a shaking water bath (225 rpm) at 55°C for 2.5 h. This replaces the lipid moiety at C3 with a free hydroxyl group. The MeOH-water fractions were resuspended in 8 ml 100% methanol containing 10% HCl and then incubated in a shaking water bath (225 rpm) at 55°C for 2.5 h, to remove the carbohydrate moiety present at C3; it was replaced with a free hydroxyl group. Subsequently, all fractions contained free sterols, which were extracted from the chemically treated samples with water-equilibrated hexane; the hexane layer was then washed to neutrality with hexane-equilibrated water. The recovery rate of our internal standard (cholestane) was 92 ± 5%. The level of detection for GC–MS was tens of nanograms; detection at this low level was made possible using selected ion chromatography software, and selected ion monitoring software (GC–MSD ChemStation [Agilent Technologies]).

The sterols contained in the three fractions were converted to their respective trimethylsilyl ether (TMS) derivatives, to ensure the inerterness of the free C3 hydroxyl, by overnight incubation with a 2.1 excess volume of BSTFA + TMCS, 99:1 (Sylon BFT; Supelco Inc. Bellefonte, PA, USA). All conjugated sterols were processed by gas chromatography–mass spectroscopy (GC–MS), using an Agilent 6890N GC coupled with a 5973 mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA). The GC–MS was equipped with a fused capillary EC–5 column (30 m; Alltech, Nicholasville, KY, USA) with a 0.25 mm internal diameter and 0.25 μm film thickness. The running conditions were: inlet 280°C, transfer line 280°C, column 80°C (1 min), ramp at 10°C min⁻¹ to 240°C, 240 to 300°C, ramp of 5°C min⁻¹, with helium (1.2 ml min⁻¹) as carrier gas. The Agilent 5973 mass selective detector maintained an ion source at 250°C and quadrupole at 180°C. Sterols were identified and quantified by GC–MS using selected ion monitoring (SIM) protocols for each sterol identified (Rabin and Benveniste, 1995). Authentic sterol standards were purchased commercially [from Sigma Chemical (St. Louis, MO, USA), and Steraloids Inc. (Newport, RI, USA)].
RESULTS
Phloem sap was collected from two different plant species (P. vulgaris and N. tabacum), and for each plant we identified and quantified the abundance of free, acylated and glycosylated sterols. Both plants contained sterols in all three forms (Table 1), but the amounts of each sterol form recovered from the phloem sap differed between the two plant species. For example, P. vulgaris contained large pools of glycosylated sterols, intermediate pools of acylated sterols, and relatively small pools of free sterols. In N. tabacum, glycosylated sterols were again the largest sterol pool, but in contrast to P. vulgaris, N. tabacum contained free sterols in rather large amounts, while acylated sterols were the smallest pool.

For both plant species, cholesterol dominated each of the sterol fractions (Table 1). For P. vulgaris, cholesterol was the only sterol recovered from the glycosylated-sterol fraction, and the only sterol recovered from 3 of the 4 free-sterol fractions, and from 4 of the 5 acylated-sterol fractions. A single free-sterol sample contained cholesterol and sitosterol (87 and 13%, respectively), and a single acylated-sterol sample contained cholesterol, sitosterol and campesterol (53, 28 and 19%, respectively). In the case of N. tabacum, the sterol fractions generally contained multiple sterols (2–4), but cholesterol was always the most abundant. There were, however, some trends with respect to the non-cholesterol sterol profiles of the different tobacco sterol fractions. Generally, small amounts of sitosterol and stigmasterol, but not campesterol, were recovered in both the free- and acylated-sterol fractions. In contrast, campesterol, but not sitosterol or stigmasterol, was recovered in the glycosylated-sterol fraction.

DISCUSSION
The collection of phloem sap in the presence of EDTA is a standard technique for isolating phloem sap from whole plants (King and Zevenaar, 1974; Costello et al., 1982; Kutley et al., 2002). Madey et al. (2002) used this technique to isolate and characterize the lipids found in canola phloem sap, and confirmed that lipid exudates collected in the presence of EDTA originated from the phloem, not the xylem. To date, the presence of phytosterols in the phloem of higher plants has been demonstrated both indirectly (e.g., from aphid honeydew; Behmer et al., 2011), and directly (e.g., via phloem sap collection in the presence of EDTA; Behmer et al., 2011; Bourrain et al., 2012), as has the capacity for the movement of sterols within the phloem (Rebhenok and Adler, 1993). However, in each of these instances the focus was entirely on free sterols, for two primary reasons. First, in the case of phloem and honeydew, only small amounts of material could be collected, which precluded a more thorough investigation into the presence of conjugated sterols. Second, with respect to sterol movement in the phloem, analysis for sterol conjugates was simply not considered. In the current study we employed a bulk collection technique, where petioles of multiple leaves were placed in EDTA containing solutions, to ensure that a sufficient quantity of phloem sap could be collected for analysis of sterol form, as well as sterol composition. Our data demonstrate, for the first time, that sterols in the phloem of higher plants are maintained as free sterols, acylated sterols and sterol glycosides. Additionally, our results show that the percentage of these different sterol forms can vary between plants, and that cholesterol was the most abundant sterol for each sterol fraction.

On average, about half of the phloem sterol pool (47–60%) was glycosylated; the remaining half of sterol was a mixture of free- and acylated forms. Considering that the phloem stream is aqueous, it perhaps should not be surprising to find relatively large pools of glycosylated sterol in the phloem. Glycosylation makes sense, as it affords sterols greater solubility in the phloem. Sterols are known to be important signaling molecules, in both animals (Icardona and Eaton, 2008) and plants (Cassese, 2002), and given the description of the phloem acting as an “information superhighway” in plants (Lucas and Wolf, 1999), sterols in a glycosylated form could move relatively unencumbered in the phloem, and in this form interact more readily with potential receptor sites on individual cells (in a fashion similar to hormones, which also operate at low concentrations). On the other hand, movement of free- and acylated sterols would require a shuttle system. Animals, including insects, use a protein shuttle system to transport sterols in their blood (Arrese and Loulages, 2010), and although putative proteins that provide such a role have been recently identified in plants (Gautelle et al., 2012), no data exist to link the association of sterols with a phloem resident protein. Alternatively, free and

Table 1 | Phloem sap sterol content for Phaseolus vulgaris and Nicotiana tabacum.

| Plant                | Median total phloem sterol amount (μg) | Sterol content (adjusted median % (w/w) of total in each sterol form) |
|----------------------|----------------------------------------|---------------------------------------------------------------------|
|                      |                                        | Cholesterol | Sitosterol | Campesterol | Stigmasterol |
| (a) Phaseolus vulgaris |                                        |             |            |             |             |
| Free sterols (4)     | 28.0                                   | 100.0       | -          | -           | -           |
| Acylated sterols (5) | 60.1                                   | 100.0       | -          | -           | -           |
| Glycosylated sterols (4) | 130.0                               | 100.0       | -          | -           | -           |
| (b) Nicotiana tabacum |                                        |             |            |             |             |
| Free sterols (3)     | 76.3                                   | 97.8        | 0.9        | -           | 1.3         |
| Acylated sterols (6) | 35.1                                   | 96.2        | 1.7        | -           | 0.1         |
| Glycosylated sterols (3) | 1028.6                              | 86.3        | -          | 11.7        |             |

For each plant three different sterol forms were collected: (i) free, (ii) acylated (lipid conjugated) and (iii) glycosylated (sugar conjugated). The number of replicate samples for each sterol form, from each plant, is indicated in parentheses.
Although these functions need not be mutually exclusive, exer-
Aguirre, M. R., Ruiz-Mendez, M. V.,
dealkylation efficiency (Campbell and Nes, 1983), having access
test how available these sterols are to phloem-feeding insects,
ever, given that a high proportion of sterols in the phloem can
be synthesized in the phloem. Considering the presence of
enzymes are associated with the phloem, and suggested that sterols
accumulate in the phloem. If cholesterol in the phloem is
not acting as a signaling molecule, or providing direct physiological
function, perhaps it is present as a precursor to important plant
steroid hormones, such as brassinosteroids. Insects require very
small pools of cholesterol as the precursor to the steroid molting
hormones (Gilbert et al., 2002), and in plants cholesterol is a close
analogue to 24- methyle cholesterol (Benveniste, 2002), which is an
intermediate in the synthesis of camptosterol, the direct precursor
to brassinosteroid (Noguchi et al., 1999). Alternatively, the differ-
ent chemical forms of cholesterol in the phloem stream might be
associated with different targets, potentially facilitating multiple
outcomes. There is also a question of where phloem sterols origi-
nate. Devarenne et al. (2002) demonstrated that sterol biosynthetic
enzymes are associated with the phloem, and suggested that sterols
can be synthesized in the phloem. Considering the presence of
both sterol biosynthetic enzymes and cholesterol in the phloem, it
is also possible that structural sterols are synthesized in the phloem
and transported where they are needed (Devarenne et al., 2002).
Although these functions need not be mutually exclusive, exer-
iments focused on the growth, development and physiological
response of the plant to various abiotic factors (e.g., temperature
extremes, light conditions, humidity levels and volatile chemicals),
coupled with the collection of phloem exudate from select leaves,
will help clarify these relationships.

For phloem-feeding insects, which perform best on diets con-
taining cholesterol (Bouwaine et al., 2012), and which have low
dealkylation efficiency (Campbell and Nes, 1983), having access
to a food source rich in cholesterol is very advantageous. How-
ever, given that a high proportion of sterols in the phloem can be
in a conjugated form, it will be important to experimentally
test how available these sterols are to phloem-feeding insects,
such as aphids. At a minimum, phloem-feeding insects would
require active gluco sides and/or acylases in the midgut. Sterol
form may also have implications related to experiments using
chemically-defined diets. One intriguing aspect about aphids
reared on chemically-defined diets is that performance relative to
plants is often greatly reduced, especially with respect to repro-
duction (Douglas, 2003). The chemically-defined diets typically
used in these studies are presented to aphids as aqueous solu-
tions contained within a Parafilm packet/sachet (Kunkel, 1976;
Douglas, 1988; Bouvaine et al., 2012), with the sterols either sol-
ubilized, or incorporated into liposomes, and then added to the
aqueous solution. Unfortunately we know little about how evenly
sterols, delivered using either of these methods, are dis-
tributed in the chemically-defined diet, or the extent to which
they are ingested. Knowing that phloem sap contains a signifi-
cant proportion of glycosylated sterols, it would be illuminating to
re-evaluate aphid performance on diets containing free-, acylated-,
and glycosylated cholesterol. A simple hypothesis is that per-
formance would be superior on diets containing glycosylated
sterols.

The data we present, and the story we tell, is in many ways
very preliminary as we have no information on the identity of the
acylated/glycosylated groups attached to phloem sterols, no infor-
mation on the absolute amounts of each different sterol form and
type present in the phloem, and no clear understanding of what
biotic or abiotic factors influence the level and types of sterols
present in the phloem sap. Additionally, while our collective data
show that the sterol profile of the phloem is dominated by choles-
terol, we still see phloem sterol profile differences between closely
related plants (e.g., the common bean and fava bean). Another
key outstanding issue is the extent to which cholesterol is the
dominant phloem sap sterol in all plants. For instance, all the
plants so far studied (with respect to phloem sap sterols) accumu-
late Δ5-sterols, but some plants accumulate Δ7-sterols in the
vegetative tissue (e.g., spinach and Solidago). In these plants is
cholesterol (a Δ7-sterol) the dominant phloem sap sterol? Unpub-
lished preliminary data involving spinach, and sterol profiles from
hemipterans feeding on Solidago (Janson et al., 2009), suggest
this might be the case. Comparative studies, sampling phloem
sap exudate from a variety of taxonomically diverse plants, and
combined with sterol tissue analysis of phloem feeding insects
(work currently underway), will help shed light on the extent to
which cholesterol is widely occurring, and the dominant phloem
sap sterol in plants. Such studies will also provide insight into
the potential of modifying plant sterol profiles as a novel way to
manage hemipteran pest populations (Behmer et al., 2011).

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