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Research article

Complex Lipids and Sterols in the Leaves of *Eucalyptus macrorhyncha* (Myrtaceae) in the Context of Feeding by an Unnamed Gall-Inducing Species of *Glycaspis* (*Synglycaspis*) (Hemiptera: Psylloidea: Aphalaridae)

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Abstract

This paper reports complex lipids and sterols in the leaves of *Eucalyptus macrorhyncha*, the dedicated host of an unnamed species of *Glycaspis* (*Synglycaspis*) (hereafter referred as *Synglycaspis*), exploring their role in *Synglycaspis*'s specificity to leaves of *E. macrorhyncha*. Seeking an answer to the above, we evaluated the levels of lipids and sterols in the leaves of *Eucalyptus rossii* and *Eucalyptus dives* the chosen "closest" relatives of *E. macrorhyncha* from Orange region in the South-eastern Highlands Bioregion of New South Wales. Low levels of mono- and di-glycerides in the leaves of *E. macrorhyncha* appear to be a key factor in regulating the choice of *E. macrorhyncha* by *Synglycaspis*. An LC–MS/MS analysis revealed incidence of sitosterol, ergosterol, and stigmasterol. Sitosterol and certain undetermined sterols with apparent molecular weights corresponding to 354, 382, and 440 g mol^{-1} occur maximally in the leaves of *E. macrorhyncha*. Increased levels of ergosterol in galls housing the second, third, and fourth instars and its decreased levels in galls with fifth instars of *Synglycaspis* indicate the production of this sterol particularly during gall growth and possible utilization by the developing instars of the gall-inducing species of *Synglycaspis*. The 440 g mol^{-1} sterol was highly represented in young leaves of *E. macrorhyncha* as against in the leaves of *E. rossii* and *E. dives*, and this sterol could be a key factor in the preference of young leaves of *E. macrorhyncha* by the gravid female of *Synglycaspis* for oviposition and ensuing gall development.

Key words: galactolipid, triglyceride, sitosterol, stigmasterol, ergosterol

Plant lipidomes include eukaryotic phospholipids, chloroplast-specific membrane glucolipids, and sterols. Many of them, e.g., phospholipids and sterols, store energy in cell membranes (Schmid and Ohlrogge 2002). In insects, cholesterol is an essential requirement in reinforcing cell-membrane integrity, synthesizing moulting hormones (e.g., 20-OH ecdysone), and in regulating genes involved in growth. Plant-feeding insects, in general, cannot synthesize cholesterol without a steroid precursor, which is usually obtained from hosts (Douglas 2009). This inadequacy, in high probability, directs their reliance on hosts. Plant sources generally are low in cholesterol levels (e.g., 50 mg/kg of total lipids), which is, in principle, inadequate for the development of a plant-feeding insect (Behrman and Gopalan 2005). Nevertheless, insects extract either the available plant cholesterol or the steroid precursors—which exist as sterols—to metabolize cholesterol (Behmer and Nes 2003).

At least 100 plant sterols are known presently, the quantities and kinds of which vary with plant species. Campesterol, sitosterol, and stigmasterol are some of the well-known plant sterols. During biosynthesis of sterols in animals, mevalonate—a critical precursor in sterol biosynthesis—is metabolized via squalene to form sterols, such as cholesterol via lanosterol (Nes 2011), whereas in plants, squalene is metabolized to cholesterol via cycloartenol (Ohyama et al. 2009). The Psocoptera, Thysanoptera, Coleoptera, Diptera, Lepidoptera, Hymenoptera, and Isoptera realize their cholesterol needs from fungi. For example, *Scolytus rugulosus* (Mueller) (Coleoptera: Curculionidae), utilizes *Ambrosiella Brader* ex von Arx et Hennebert

(Ascomycota: Ceratocystidaceae) to fulfil its cholesterol needs. Predatory insects, e.g., *Vespula maculifrons* (Buysson) (Hymenoptera: Vespidae) obtain cholesterol from animal diets, e.g., *Forficula auricularia* L. (Dermaptera: Forificulidae). Plant-feeding insects depend on plants, e.g., *Manduca sexta* L. (Lepidoptera: Sphingidae) on *Nicotiana tabacum* L. (Solanaceae) (Behmer and Nes 2003). In the Sternorrhyncha (e.g., *Schizaphis graminum* (Rondani), Hemiptera: Aphididae) and Auchenorrhyncha (e.g., *Laodelphax striatellus* (Fallen), Hemiptera: Delphacidae; Noda and Koizumi 2003), the presence of cholesterol in their tissues suggest that these Hemiptera have the ability to convert plant sterols into cholesterol (Behmer and Nes 2003). Occasionally, plant-synthesized cholesterol is also utilized by the Sternorrhyncha (Behmer et al. 2011). Sterol consumption by many plant-feeding Hemiptera depends on endosymbionts, such as the dependence of Myzus persicae (Sulzer) on Buchnera Munson, Baumann et Kinsey (Enterobacteriales: Enterobacteriaceae) (Douglas 2009) for the biosynthesis of cholesterol from the consumed plant sterols. Cholesterol and other plant sterols translocate via vascular tissues of plants, from which the adult Hemiptera source their liquid food. However, the cholesterol contents in leaf tissues (~16% of total sterols) is much lower than that occurs in phloem (~90% of total sterol; Behmer et al. 2011). Consumption of sterols of either plant or fungal source at specific concentrations regulates feeding in Hemiptera, determining their nature as either a generalist or a specialist (Behmer and Nes 2003).

A majority of gall-inducing insects remain restricted to a single host-plant species displaying a high level of fidelity (Raman 1996, 2009; Raman et al. 2005). In the gall-inducing *Taxomyia taxi* (Inchbald) (Diptera: Cecidomyiidae)–*Taxus baccata* L. (Coniferales: Taxaceae) interacting system, Lovett (1980) demonstrated that gall tissues synthesized the hormones, viz., ponasterone-A and ecdysterone, that are essential for the inhabiting larvae to grow into adults. When host fidelity of plant-feeding insects and the steroid precursor-production capacity in plants are viewed in alignment, the question whether the steroid-precursor production capacity in plants is the key factor in regulating host fidelity arises. To address this question, in this study, we examined complex lipids and steroid precursors using an unnamed, gall-inducing species of *Glycaspis* (*Synglycaspis*) Moore (hereafter referred as *Synglycaspis*), which interacts with *Eucalyptus macrorhyncha* F. Muell. ex Benth.

The pouch galls induced by this species of *Synglycaspis* on *E. macrorhyncha* are spherical with defined ostioles and include abundant sugary filaments (Sharma et al. 2015a). The insects were examined by both Daniel Burckhardt (Basel, Switzerland) and Gary Taylor (Adelaide, Australia). Due to time constraints, they could not describe the species, which they suspect as new. Several males and females of this species are deposited with Gary Taylor (The Australian Centre for Evolutionary Biology and Biodiversity, School of Earth and Environmental Sciences, The University of Adelaide, Adelaide, Australia) for formal taxonomic description. The galls are externally similar to those induced by *Synglycaspis cameloides* (Moore) on leaves of *Eucalyptus obliqua* L'Hér in South Australia (Taylor 1987). Intense field work done at the Mullion Range State Conservation Area, Orange region of the South-eastern Highlands Bioregion of New South Wales in October 2013–March 2014 showed that this gall-inducing species of *Synglycaspis* occurs only on *E. macrorhyncha* and not on any other species of *Eucalyptus* in spite of high proximity between trees. We, therefore, sought answers for 1) how do complex lipid and sterol profiles change in *Synglycaspis*-infested leaves of *E. macrorhyncha* through gall development and 2) how do complex lipids and sterols in young and mature leaves of *Eucalyptus macrorhyncha*, the dedicated host of *Synglycaspis*, vary from the two determined relatives of *E. macrorhyncha*, viz., *Eucalyptus rossii* R.T.Baker & H.G.Sm. and *Eucalyptus dives* Schauer?

Natural History of *Synglycaspis* and the Rationale for the Choice of *E. macrorhyncha*'s Relatives

The unnamed gall-inducing species of *Synglycaspis* lives on the leaves of *E. macrorhyncha* inducing pouch galls (Figs. 1-3). It completes two generations in a year. The first instar initiates the gall on a young leaf. The first and second instars feed on gall parenchyma, whereas the third, fourth, and fifth instars feed on gall phloem (Sharma et al. 2015a). [Terms "immature" and its plural form "immatures" and "instars" have been used in this paper instead of the previously used "nymphal instars," following the new terminology proposed by Burckhardt et al. (2014).].

Eucalyptus rossii and *E. dives* were chosen as the "closest" relatives of *E. macrorhyncha* in Orange region, after consulting several *Eucalyptus* specialists in Australia. This choice was also based on a recent boot-strap phylogenetic study of *Eucalyptus* (Woodhams et al. 2013), which treats *E. macrorhyncha* and *E. rossii* under Section Capillula and *E. dives* under Aromatica, all of which are treated under *Eucalyptus* subgenera *Eucalyptus* + *Primitiva* (Brooker 2000). Moreover, trees of *E. macrorhyncha*, *E. rossii*, and *E. dives* (±30 yr of age) occur as mixed populations in the Mullion Range State Conservation Area (33° 07′ S, 149° 07′ E; 870 m; Orange region of the South-eastern Highlands Bioregion of New South Wales) described as the "Red Stringybark—Inland Scribbly Gum Open Forest" (Central Tablelands Landcare 2008).

Materials and Methods

Study Site, Sampling Periods, and Sample Numbers

Galls ($n = 980$) induced by *Synglycaspis* on *E. macrorhyncha* leaves were collected from the Mullion Range State Conservation Area in October 2013–March 2014. The collected galls were morphologically examined. Because all of the five developmental stages live within galls (Sharma et al. 2015a), the galls were grouped into five populations using the dimensions and developmental stage of the instar inhabiting the gall ($n = 100$, each developmental stage). Young and mature leaves ($n = 50$ each category) were defined applying linear measurements (from the leaf base to apex) and color. Shades of leaf color were physically matched with the "named" color tiles in the TAUBMANS colour chart (http://www.taubmans.com.au/colours/colour-chart) to distinguish variations in green, which are indicated in parentheses here. Young (1–3 cm; "frogmore green") and mature (7–11 cm; "green adventure") leaves of *E. macrorhyncha* bearing galls and those without galls occurring closest to gall-bearing leaves (control) were used in this study. As control, comparable portions from similar-aged leaves were used. Care was exercised in avoiding damaged materials. Young (1–4 cm; "bonton green") and mature (9–14 cm; "green kelp") leaves of *E. rossii*, and young (1–4 cm; "Mediterranean green") and mature (7–12 cm; "Irish stone") leaves of *E. dives* were also collected from the Mullion Range State Conservation Area and used in these assays.

To assay lipids, galls on young and mature leaves were excised at their junctions with the leaf blade. The selected galls were vertically slit using a sterile-razor blade. The inhabiting immatures were flushed using a hand-held distilled-water jet and the slit gall materials were treated as detailed below.

Sample Preparation and Assays Used for Complex Lipids and Sterols

Leaf samples were collected in polyethylene bags, rapidly transported to Charles Sturt University (Orange) research laboratory, and stored in a deep freezer at −80 °C. Leaf samples were lyophilized for 6–12 h (Labconco Freeze Drier, Labconco, KS), ground (Micro Hammer and Cutter Mill, Glen Creston Ltd., London, UK), and stored in labeled 3-cm glass vials. Lyophilized samples were transported to and analyzed at the Department of Biochemistry, University of Nebraska, Lincoln, NE.

The lipid-storing tri-acylglyceride (TAG) and chloroplast-membrane bound mono- and di-galactosyldiacylglycerides (MGDG and DGDG) were analyzed using an LC–MS tandem MS system (LC–MS/MS) consisting of a SCIEX Q-Trap 4000 and Shimadzu UFLC-XR. A Poroshell 120 EC-18 4.6 × 50 mm (2.7 mμ) column equipped with an Eclipse Plus-C18 narrow bore 2.1 × 12.5 mm (5 mμ) guard column (Agilent Technologies, Santa Clara, CA) was used in both analyses. For lipids, extracts of 20 mg lyophilized leaf samples were obtained following the procedures of Bligh and Dyer (1959) with minor modifications, as explained below. The lyophilized samples were hydrated with 1 ml of ice-cold water for 10 min and centrifuged at 1,290 × *g* (2,500 rpm) for 5 min and the supernatant was removed. Five milliliter aliquots of methanol–chloroform (2:1) containing 0.01% butylated hydroxytoluene (Sigma-Aldrich) were added to samples. After replacing the headspaces of the glass vials with Ar, the samples were stored in PTFE-lined screw-cap vials. Samples were vortexed for 60 min before further analysis. Lipids were isolated by solvent partitioning using 2 ml aqueous KCl solution (0.08%) and 2 ml chloroform. After vortexing for 10 s, the samples were centrifuged at 1,290 × *g* for 5 min. The organic-solvent layer was separated and evaporated under a stream of N_2 gas at 45 °C. Saturated MGDG and DGDG were used as internal standards (Matreya LLC, Pleasant Gap, PA) and 20 μg of each of these internal standards were added to each sample. Triheptadecenoin (17:1; 10 μg/sample, Nu-Check Prep, Inc., Elysian, MN) was added as the TAG internal standard and its conversion to triheptadecanoin (17:0) used to monitor the efficiency of the catalytic hydrogenation reaction. Galactolipids were analyzed in a positive mode by multiple-reaction monitoring (MRM) of the ammoniated molecular ions and the product ions from the neutral loss of the galactolipid-head groups. A binary solvent system (95:5 methanol–water) in 1.6 mM NH_4 formate–0.7 mM formic acid (Solvent A) and methanol–1.6 mM NH_4 formate–0.7 mM formic acid (Solvent B) was used for LC separation. The gradient was raised from 0 to 100% Solvent B in 6 min and retained at that point for 4 min, then lowered to 0% in 0.5 min and thus retained for 1.5 min. The column temperature was set at 30°C. TAGs were analyzed using the catalytic hydrogenation LC–MS/MS method (Allen et al. 2014). Briefly, the samples were hydrogenated with 1 mg of PtO_2 (IV) (Sigma-Aldrich) in crimp-top 2-ml vials with 0.5 ml of chloroform by replacing the head space with H_2 and vortexing in a multivial vortexer at 22°C for 30 min. Dilutions (1:10) in methanol were analyzed directly in an LC–MS/MS with the same column used for galactolipid analyses. The solvent system and LC method used were as described by Allen et al. (2014). Two technical replicates were analyzed.

Moieties of free sterols, sterol-esters, and sterol-glucosides were isolated as nonsaponified fractions of extracts. Samples were saponified by evaporating under an N_2-gas stream at 45 °C using a N_2-gas evaporator, adding 1 ml of 0.5 M methanolic KOH and heating to 100 °C for 15 min. Saponified compounds were removed and sterols isolated by solvent partitioning as described in the preceding paragraph. Hexane was removed by evaporation and sterols were derivatized using betainyl chlorobetaine following Wewer et al. (2011). Betainyl chlorobetaine (c. 10 mg) was added to samples dissolved in 1 ml of chloroform and 50 μl of pyridine and were heated to 50 °C for 4 h. Contaminants and nondissolved betainyl chlorobetaine were removed by solvent partitioning. Chloroform (0.5 ml), methanol (0.5 ml), and deionized water (1 ml) were added and the mixture was centrifuged at 748 *g* for 5 min. The betainyl-chloride sterol esters were extracted with the chloroform fraction, evaporated under N_2 stream, and suspended in 1 ml of methanol. The samples were analyzed using electrospray ionization in LC–MS/MS by direct infusion of methanol/1.6 mM ammonium formate/0.7 mM formic acid (1:10 dilution) using a SCIEX Q–Trap 4000 LC–MS/MS in a positive mode. Sterols were detected using product-ion scans of 118.1 m/z (Wewer et al. 2011). Two technical replicates were analyzed.

Lipid Maps Database (LMD; Fahy et al. 2009) was consulted to determine the characterized sterols. Those sterols, which could not be determined using LMD, are referred as "Sterol" followed by the numerals pertaining to the determined molecular weight, e.g., "Sterol 312.3," "Sterol 410.2."

Data Analysis

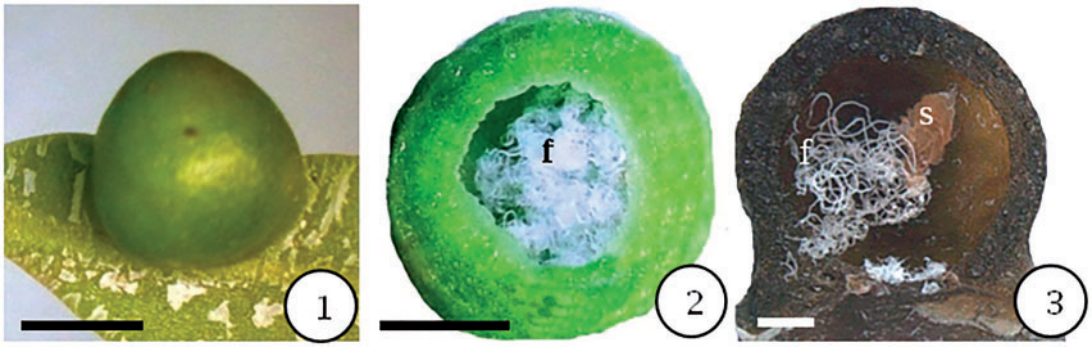
Data pertaining to complex lipids in young and mature, non-gall-bearing and gall-bearing leaves of *E. macrorhyncha* (host of *Synglycaspis*), and young and mature leaves of *E. rossii* and *E. dives* were analyzed applying one-way ANOVA to verify variations in the quantity and quality of complex lipids. Tukey's separation enabled perceiving comparability in the quantity and quality of complex lipids and sterols in the leaves of *E. macrorhyncha* (during *Synglycaspis* development) and those of *E. rossii* and *E. dives*. Data pertaining to sterols in the young and mature uninfested and infested leaves of *E. macrorhyncha*, *E. rossii*, and *E. dives* have been presented as mole%. All analyses were carried out in GenStat (VSN International 2013).

Results

Complex Lipids and Sterols in *Synglycaspis*-Infested Leaves of *E. macrorhyncha* Through Gall Development

Complex Lipids

The difference in mass of total galactolipids inclusive of MGDG's and DGDG's was significant ($P < 0.001$; $F_{6,4} = 540.8$) in uninfested young and mature leaves of *E. macrorhyncha* (Table 1). The total mass of galactolipids was greater in infested leaves of *E. macrorhyncha* than in uninfested young leaves, but lower (0.04% dry weight [DW]) than in uninfested mature leaves (0.13% DW) of *E. macrorhyncha* (Fig. 4). In the infested leaves of *E. macrorhyncha*, the values of galactolipids remained stable throughout the development time of immatures of *Synglycaspis* in galls (Fig. 5). The levels of galactolipids and triglycerides were greater in uninfested, mature leaves of *E. macrorhyncha* than in uninfested young leaves (Fig. 4). The total mass of triglycerides was greater in infested leaves of *E. macrorhyncha* than in uninfested young leaves but lower than in uninfested mature leaves (Fig. 4). The levels of triglycerides were high in leaves infested by first instars of *Synglycaspis*, but declined in leaves hosting later instars (Fig. 5). Triglyceride values did not differ significantly between young and mature leaves of *E. macrorhyncha*.



Figs. 1–3. Galls induced by *Synglycaspis* on the leaves of *E. macrorhyncha*.

1. Mature gall [bar = 5 mm].

2. Cross-sectional view of young gall (10–20 d old): f, sugary filaments [bar = 5 mm].

3. Median vertical-longitudinal section of mature gall (40 d old) showing the inhabiting immature *Synglycaspis* (s) and sugary filaments (f) (bar = 1 mm).

MGDG levels were 0.01% DW in young, uninfested leaves. With maturation, the uninfested leaves included MGDG levels of 0.06% DW. On the contrary, MGDG levels remained at 0.01% DW when gall development occurred. Levels of DGDG were greater (0.03% DW) during the inhabitation of the first and second instars of *Synglycaspis* than those of the third, fourth, and fifth instars (0.02% DW; Fig. 5).

Sterols

Sterols 354.1, 382.1, 410.2, 440.3, and sitosterol (414.3) occurred maximally in both uninfested and infested, young and mature leaves (Table 2). Mole percent of Sterols 414.3, 426.2, and 454.2 was higher in infested leaves of *E. macrorhyncha* than the uninfested leaves, whereas levels of Sterols 326.4, 354.1, 382.1, 396.3, 410.2, and 440.3 were higher in uninfested leaves of *E. macrorhyncha* than infested leaves. Stigmasterol (412.4) increased in galls housing the second instar (0.4%), but decreased in galls housing the third (9%) and fourth (1.4%) and increased again in galls housing fifth (1%) instars. Sterol 426.2 decreased in the gall that housed the second (7.8%), third (15.6%), and fourth (13%) instars, but increased in the gall that included the fifth (23.35%) instars. Sterol 534.3 shows a variable pattern among galls that include from the first instar to the fifth instar with notable swings in values.

Maximum change in levels occurred in Sterol 440.3, which decreased in mature *E. macrorhyncha* leaves by 84.3%. Sterols 410.2 and 534.3 showed intense variation in the leaves of *E. macrorhyncha*: Sterol 410.2 increased by 400% in mature leaves, whereas Sterol 534.3 decreased by 100% in mature leaves compared with those in young leaves of *E. macrorhyncha*. Sterol levels were generally high in galls that included the third and fourth instars, and declined dramatically during the habitation time of the fifth. Sitosterol (414.3) showed a striking pattern and occurred maximally in galls of the fifth instar (0.1851 mole%) at a much greater level than those in uninfested young (0.1130 mole%) and mature (0.1244 mole%) leaves of *E. macrorhyncha*. Ergosterol (396.3) and Sterol 424.4 increased in their levels in galls induced by the first–fourth instars, but decreased during the fifth-instar stage.

Complex Lipids and Sterols in Young and Mature Leaves of *E. macrorhyncha*, *E. rossii*, and *E. dives*

Complex Lipids

The total mass of galactolipids in young leaves of *E. rossii* was significantly ($P < 0.001$; $F_{6,4} = 540.8$) lower than that in mature leaves, whereas the levels of triglycerides were higher in young leaves than in mature leaves (Fig. 6; Table 1). In *E. dives* leaves, the total mass of galactolipid levels were lower in young leaves than in mature leaves (Fig. 7). Levels of MGDG and DGDG in young leaves of *E. macrorhyncha* were generally lower than the levels in young leaves of *E. rossii* and *E. dives*, whereas the levels of triglycerides were

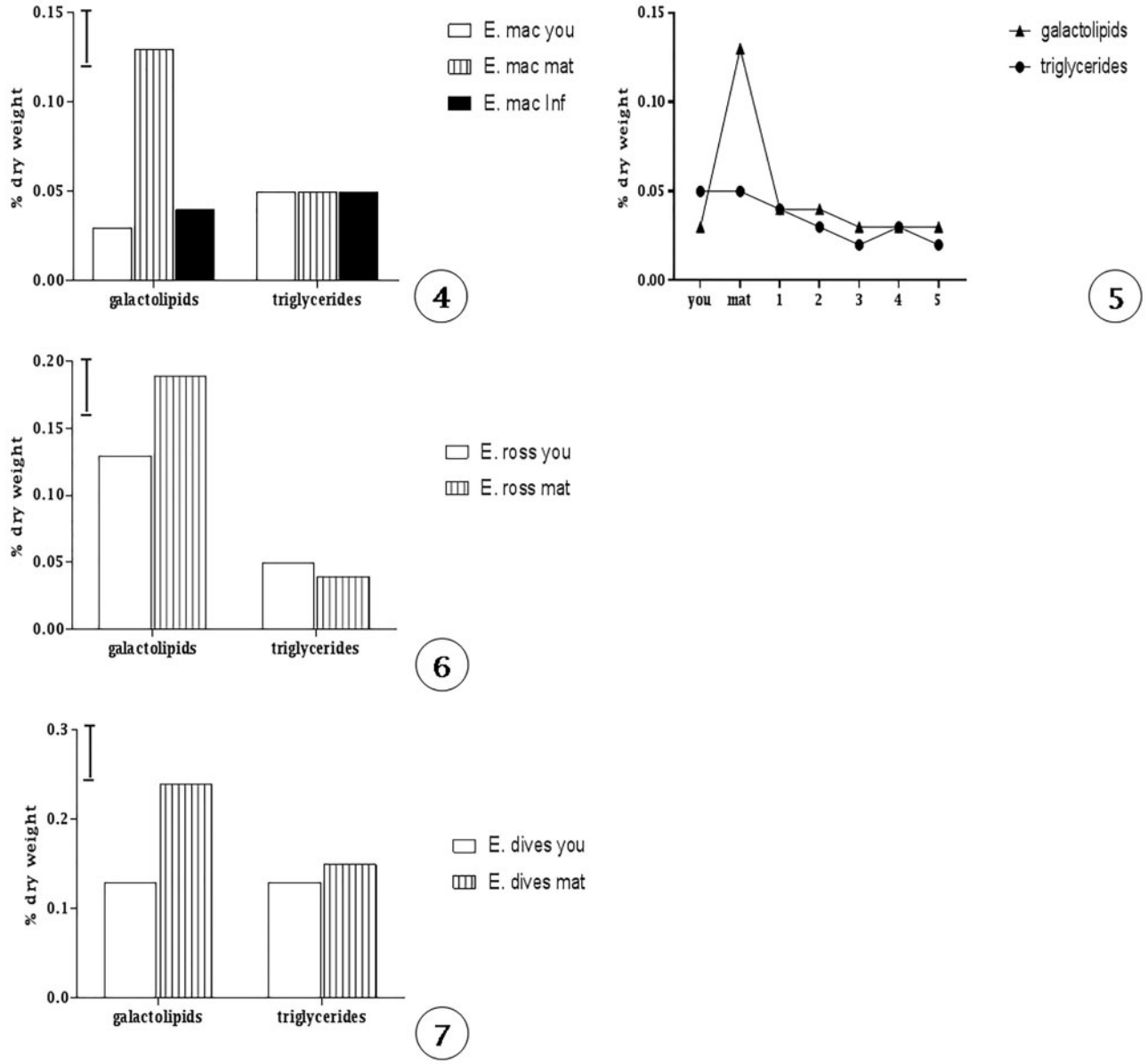
Table 1. Tukey's separation of significantly different means of MGDG, DGDG, and triglycerides (% dry weight) in *E. macrorhyncha*, the host of *Synglycaspis* sp., and *E. rossii* and *E. dives*

Host systems	Galactolipids		Triglycerides
	MGDG	DGDG	
E. macrorhyncha uninfested, young leaves	0.00687^{d}	0.02227^{d}	0.04518^{b}
E. macrorhyncha uninfested, mature leaves	0.05526^{c}	0.07599^{c}	0.05180^{b}
E. macrorhyncha infested leaves[a]	0.00985^{d}	0.02574^{d}	0.02716^{b}
E. rossii uninfested young leaves	0.05694^{c}	0.07186^{c}	0.04582^{b}
E. rossii uninfested mature leaves	0.08332^{b}	0.10979^{b}	0.03683^{b}
E. dives uninfested young leaves	0.05450^{c}	0.07605^{c}	0.13211^{a}
E. dives uninfested mature leaves	0.10552^{a}	0.13551^{a}	0.14616^{a}

[a]Leaves infested with five developmental stages are pooled.

Superscripted letters "a," "b," "c," and "d" represent Tukey's grouping; mp (mean significance) = 0.05.

The same superscript alphabet in the same column indicates that values do not differ significantly.



Figs. 4–7. Lipids (pooled mean values) in infested and uninfested leaves of *E. macrorhyncha* and young and mature leaves of *E. rossii* and *E. dives*. Bars represent standard error. ($n = 50$ in each category).

4. Galactolipids and triglycerides in uninfested young (□), uninfested mature (▥), and infested (■) leaves of *E. macrorhyncha* infested by *Synglycaspis* sp.

5. Galactolipids (▲) and triglycerides (•) in the uninfested leaves and infested leaves of *E. macrorhyncha* by *Synglycaspis* sp. [x axis: the five developmental stages of *Synglycaspis* sp.]

6. Galactolipids and triglycerides in young (□) and mature (▥) leaves of *E. rossii*.

7. Galactolipids and triglycerides in young (□) and mature (▥) leaves of *E. dives*.

nearly the same in both young and mature leaves of *E. macrorhyncha*, *E. rossii*, and *E. dives*.

Compared with young and mature leaves of *E. rossii* and *E. dives*, the total mass levels of galactolipids in *E. macrorhyncha* were low. Levels of triglycerides were nearly the same in young and mature leaves of *E. macrorhyncha* and *E. rossii* but were higher, at least by 180%, in young and mature leaves of *E. dives*. The level of increment in galactolipids from young to mature leaves of *E. macrorhyncha* was 78.6% (from 0.03% DW in young to 0.14% DW in mature), whereas in young and mature leaves of *E. rossii* and *E. dives*, the increment was about 70%. In the context of MGDG levels in uninfested leaves of *E. macrorhyncha*, the increment from young (0.01% DW) to mature (0.06% DW) was 83.3%, whereas in *E. rossii* and *E. dives* the increment was modest at 40%. In the context of DGDG levels, the increment rate from young (0.02% DW) to mature (0.08% DW) in uninfested leaves of *E. macrorhyncha* was 75%, whereas in *E. rossii* and *E. dives* the increment was 100%. The levels of triglycerides did not change in uninfested young and mature leaves of *E. macrorhyncha* (0.05% DW), whereas in the young and mature leaves of *E. rossii* triglyceride levels decreased by

Table 2. Sterols present in uninfested and galled leaves of *E. macrorhyncha* and leaves of *E. rossii* and *E. dives*

Sterol molecular weight	*E. macrorhyncha–Synglycaspis* sp. system (sterols in mole%)							*E. rossii* (sterols in mole%)		*E. dives* (sterols in mole%)	
	0		I					0		0	
	Y	M	1'	2'	3'	4'	5'	Y	M	Y	M
326.4	0.0233	0.0265	0.0183	0.0219	0.0263	0.0253	0.0233	0.0173	0.0077	0.0317	0.0213
354.1	0.0788	0.1129	0.0690	0.0738	0.0797	0.0689	0.0581	0.1398	0.0756	0.1639	0.1309
382.1	0.1287	0.1987	0.1358	0.1648	0.1676	0.1764	0.1196	0.4003	0.3181	0.2641	0.3679
396.3	0.0054	0.0177	0.0063	0.0094	0.0103	0.0136	0.0095	0.0091	0.0085	0.0034	0.0059
410.2	0.0489	0.2454	0.0595	0.1181	0.1245	0.1787	0.1333	0.0832	0.1315	0.0173	0.0248
412.4	0.0500	0.0458	0.0495	0.0497	0.0452	0.0488	0.0500	0.0244	0.0172	0.0332	0.0246
414.3	0.1130	0.1244	0.1244	0.1649	0.1801	0.1569	0.1851	0.2109	0.2407	0.2034	0.1726
424.4	0.0028	0.0147	0.0052	0.0055	0.0070	0.0120	0.0075	0.0032	0.0085	0.0070	0.0054
426.2	0.0627	0.0496	0.0801	0.0738	0.0676	0.0696	0.1045	0.0393	0.0485	0.0607	0.0548
440.3	0.3768	0.0591	0.3349	0.2324	0.1668	0.1442	0.1735	0.0225	0.0284	0.1439	0.1065
454.2	0.0164	0.0285	0.0180	0.0253	0.0319	0.0255	0.0537	0.0022	0.0065	0.0177	0.0357
534.3	0.0227	0.0002	0.0329	0.0072	0.0222	0.0033	0.0067	0.0004	0.0004	0.0025	0.0007

0: uninfested leaves, I: infested leaves.

Y: young uninfested leaves, M: mature uninfested leaves.

1', 2', 3', 4', 5': galls harboring populations of the first, second, third, fourth, and fifth instars ($n = 50$ each category).

20% from 0.05% DW (young) to 0.04% DW (mature). In *E. dives* leaves triglyceride levels increased by 13% (from 0.13% DW young to 0.15% DW mature).

Sterols

Twenty-two sterols in varying quantities were determined in *E. macrorhyncha*, *E. rossii*, *and E. dives* (Table 2). Of these, the sterol of molecular weight 396.3 could be determined as ergosterol, 412.4 as stigmasterol, and 414.3 as sitosterol. Sterols 312.3, 340.3, 368.5, 428.4, 462.4, 472.3, 486.4, 498.4, 520.4, and 576.4 were also detected but because of their <0.01 mole% values they have not been considered further. Out of the remaining 12 (Table 2), three varied significantly between species, viz., Sterol 354.1 ($P = 0.002$; $F_{10,\ 4} = 30.47$), Sterol 382.1 ($P = 0.004$; $F_{10,\ 4} = 24.34$), and Sterol 412.4 ($P < 0.001$; $F_{10,\ 4} = 179.27$; Table 3).

Sterol 354.1 was 0.0788 mole% in young, uninfested leaves of *E macrorhyncha* whereas it was 0.0690 mole% in galls that included first instars of *Synglycaspis*. This sterol was 44% greater in the young leaves of *E. rossii* (0.1398 mole%) and 52% greater in those of *E. dives* than its counterparts of *E. macrorhyncha*. Sitosterol (414.3) was 0.1130 mole% in young, uninfested leaves of *E macrorhyncha*, whereas it was 0.1244 mole% in galls that included the first instar of *Synglycaspis*. Sitosterol was greater by 46% in young leaves of *E. rossii* and 44% in *E. dives*. Ergosterol (396.3) was 0.0054 mole% in young, uninfested leaves of *E. macrorhyncha*, whereas it was 0.0063 mole% in galls that included the first instar of *Synglycaspis*. Ergosterol was 41% greater in leaves of *E. rossii* and 37% lower in *E. dives*. Stigmasterol (412.4) was 0.0500 mole% in young, uninfested leaves of *E. macrorhyncha*, whereas it was 0.0495 mole% in galls that included the first instar of *Synglycaspis*. Stigmasterol was 50% lower in leaves of *E. rossii* and 33% lower in those of *E. dives*.

Sitosterol (414.3) was 0.1244 mole% in mature, uninfested leaves of *E. macrorhyncha*, whereas it was 0.1851 mole% in galls that included the fifth instar of *Synglycaspis*. Sitosterol was greater by 48% in comparable leaves of *E. rossii* and 28% in those of *E. dives*. Ergosterol (396.3) was 0.0177 mole% in mature, uninfested leaves of *E. macrorhyncha*, whereas it was 0.0095 mole% in galls that included the fifth instar. Ergosterol was 51% lower in leaves of *E. rossii* and 67% lower in *E. dives*. Stigmasterol (412.4) was 0.0458 mole% in mature, uninfested leaves of *E. macrorhnycha*, whereas it was 0.05 mole% in galls that included the fifth instar of *Synglycaspis*. Stigmasterol was 62% lower in leaves of *E. rossii* and 46% lower in *E. dives* (Table 2).

Discussion

Differences in evolutionary pressures on insects in utilizing plant metabolites, especially lipids and sterols, influence them to behave as either a generalist or a specialist (Behmer and Nes 2003). The best

Table 3. Tukey's separation of sterols in *E. macrorhyncha*, the host of *Synglycaspis* sp., and *E. rossii* and *E. dives*

Host systems	Sterols		
	354.1	382.1	412.4 (stigmasterol)
E. macrorhyncha uninfested, young leaves	0.07882^{abcd}	0.1287^{ab}	0.04998^{d}
E. macrorhyncha uninfested, mature leaves	0.11286^{bcde}	0.1988^{abcd}	0.04580^{cd}
E. macrorhyncha infested leaves[a]	0.06991^{ab}	0.1528^{abc}	0.04862^{d}
E. rossii uninfested young leaves	0.13982^{de}	0.4003^{e}	0.02443^{ab}
E. rossii uninfested mature leaves	0.07566^{abc}	0.3181^{bde}	0.01718^{a}
E. dives uninfested young leaves	0.16393^{e}	0.2641^{abcde}	0.03323^{bc}
E. dives uninfested mature leaves	0.13090^{cde}	0.3679^{de}	0.02457^{ab}

[a]Leaves infested with five developmental stages are pooled.

Superscripted letters represent Tukey's grouping.

examples among specialist plant-feeding insects are gall-inducing insects (Raman 2011). Lipids in insect-induced galls are indicated as critically relevant in the host relations of gall-inducing insects (Bronner 1992). Gall tissues include sterols, which are utilized by the inhabiting larval stages to metabolize hormones (Lovett 1980, Jing et al. 2013). Some plant-feeding insects lack the ability to introduce Δ^7-bond into the plant-derived sterols (Behmer and Grebenok 1998, Morgan and Moynihan 2000). Therefore, they rely on sterols with pre-existing Δ^7 or $\Delta^{5,7}$ bonds (e.g., spinasterol and ergosterol) for synthesizing moulting hormones. This dependence of gall-inducing insects for sterols with Δ^7 or $\Delta^{5,7}$ bonds, in high likelihood, narrows their host range, thus making them operate as high-fidelity organisms.

Complex Lipids and Sterols in *Synglycaspis*-Infested Leaves of *E. macrorhyncha* Through Gall Development

Quantity of membrane-bound lipids (e.g., DGDG) in galls occupied by the first- and second instars of *Synglycaspis* increases markedly with gall growth, indicating that maximal acquiring of lipids by the inhabiting instar from gall tissues occurs. Moreover, at the time of ecdysis of the second instar to the third, shift in feeding site from mesophyll parenchyma to phloem occurs (Sharma et al. 2015a). The quantity of DGDG increases during the inhabitation of first and second instars, but the levels of linolenic acid—an unsaturated fatty acid—drops abruptly (Sharma et al. 2015b). *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae), while feeding on *Triticum aestivum* L. (Poaceae), has been found to manipulate *T. aestivum*'s lipid metabolism to enhance its survival and growth options (Zhu et al. 2008), where the rise in DGDG levels is explained as a factor mediating compatibility between *T. aestivum* and *M. destructor.* In *Synglycaspis–E. macrorhyncha* interaction, rise in levels of DGDG, especially during infestation times of the first and second instars indicates a similar outcome prompting a compatibility between them, which could be one underpinning factor in *Synglycaspis*'s commitment to *E. macrorhyncha*. Decline in linolenic acid values complements increase in DGDG levels. This inverse relationship between DGDG and linolenic acid values is yet another reinforcing factor in the relationship between *Synglycaspis* and *E. macrorhyncha*.

Quantity of triglycerides is greater during the inhabitation time of the first and second instars than those of the third, fourth, and fifth stages of *Synglycaspis*. Plant triglycerides are known as feeding stimulants in the biology of plant-feeding insects (Nawrot and Czaplicki 1978). Such triglycerides are nonvolatile compounds, yet they play a role in attracting and stimulating insects by their derived volatiles particularly when plant tissues are irritated during feeding and oviposition (O'Donnell et al. 1983, Arrese and Soulages 2010). Levels of triglycerides in *E. macrorhyncha* during gall initiation by the neonates of *Synglycaspis* presuppose the role played by triglyceride-derived volatiles in attracting the neonates to choose young leaves and trigger gall development. A high level of triglycerides (0.04% DW) in young uninfested leaves, matching with the settling time of first instars, supports this inference. Triglycerides are necessary for later-stage larval metamorphosis (Nestel et al. 2003). In the present study, DGDG and triglyceride levels drop during the development of the third instar of *Synglycaspis*, indicating the greater level of utilization of triglycerides and DGDG by *Synglycaspis*, especially during the shift from mesophyll parenchyma to phloem. The increment in consumption rate points to greater utilization of triglycerides in the metamorphosis of later instars.

Ergosterol, a $\Delta^{5,7}$-bonded sterol, is known to occur scantily in flowering plants (Guo et al. 1995). Insects generally show robust growth when raised on synthetic diets that include ergosterol. The larvae of the Anobiidae (Coleoptera) grow better on ergosterol-based synthetic diets because in natural circumstances, the Anobiidae are associated with fungal mycelia that include ergosterol. Similarly, the larvae of various species of the Pyralidae (Lepidoptera) consume ergosterol (Behmer and Nes 2003). *Manduca sexta* utilizes 100% of ergosterol and stores ~7% of it in body tissues (Svoboda et al. 1995) pre-empting that 93% of ergosterol is used in its metabolism. Ergosterol levels increase in *E. macrorhyncha* galls concurrently with larval development, until the development of the fourth instar. During the inhabitation time of fifth instars of *Synglycaspis*, these values drop, indicating that fourth instars utilize ergosterol intensely. This also indicates its greater requirement for younger instars of *Synglycaspis* preparing to moult into the nonfeeding fifth instars and later into adults.

Sitosterol occurs at higher levels in gall tissues of *E. macrorhyncha* occupied by all developmental stages of *Synglycaspis* than in uninfested leaves of *E. macrorhyncha*. However, it maximally occurs in galls occupied by fifth instars, which could be because of greater rate of utilization by first–fourth instars and nearly none utilization by the fifth instar, which increases the quantity of this sterol in gall tissue. Utilization of sitosterol by developing stages of *Synglycaspis* is not surprising since Festucci-Buselli et al. (2008) have categorically demonstrated that plant-feeding insects obtain sterols mainly as sitosterol from their diet sources and dealkylate sitosterol to produce cholesterol.

Stigmasterol ($\Delta^{5,22}$ sterol, molecular weight 412.4) occurs in pteridophytes and flowering plants, although usually at lower levels than those of sitosterol. Behmer and Nes (2003) indicate stigmasterol is not a "usable" sterol by insects. However, when larvae of *Plutella xylostella* L. (Lepidoptera: Plutellidae) were raised on stigmasterol-including artificial diets, their second generation showed "some" degree of adaptability (Behmer and Grebenok 1998). The level of stigmasterol increases, rather gradually, in *Synglycaspis*-infested leaves of *E. macrorhyncha*, indicating that *Synglycaspis* does not utilize stigmasterol, similar to what Behmer and Nes (2003) had suggested.

Complex Lipids and Sterols in Young and Mature Leaves of *E. macrorhyncha*, the Host of *Synglycaspis*, and in *E. rossii* and *E. dives*

Triglyceride quantities do not differ markedly in young and mature leaves of *E. macrorhyncha*, but galactolipids occur at lower quantities in young leaves of *E. macrorhyncha* than in mature leaves. Low levels of galactolipids in young leaves—the preferred sites for oviposition by gravid *Synglycaspis* and for gall induction by the neonate instars—are the possible drivers influencing the preference and selection by *Synglycaspis*. From the detected 22 sterols in young and mature leaves of *E. macrorhyncha*, Sterol 382.1 and sitosterol occur maximally. However, lanosterol ($\Delta^{8,24}$ sterol, molecular weight 426.3) is the nearest known (Fahy et al. 2009) to Sterol 440.3 detected in high quantities in young leaves of *E. macrorhyncha*. The level of Sterol 440.3 in young leaves is nearly five times greater than that in the mature leaves of *E. macrorhyncha*. High levels of Sterol 440.3 in young leaves of *E. macrorhyncha* possibly direct metamorphosis of *Synglycaspis*. Lanosterol is a precursor for the steroid necessary in the metabolism of *M. sexta* (Lepidoptera: Sphingidae), a specialist plant feeder, which selectively feeds on species of Solanaceae (Svoboda et al. 1995). The emerging picture is that in the feeding biologies of both *Synglycaspis* and *M. sexta*—specialist

plant feeders—near-similar sterols occur in their respective host tissues.

Galactolipid levels in *E. macrorhyncha* are lower than those in *E. dives* and *E. rossii*. Low levels of galactolipids are a key factor in plant susceptibility to insects and microbial pathogens (Scala et al. 2013). Low levels of these lipids in *E. macrorhyncha*, therefore, possibly play a regulatory role in the preference and selection of *E. macrorhyncha* by *Synglycaspis* as against the leaves of *E. dives* and *E. rossii*, the populations of which occur in near-equal frequency in the Mullion Range State Conservation Area.

Lower levels of MGDG and DGDG in *E. macrorhyncha* leaves than those in *E. rossii* and *E. dives* point to an effect on the growth and development of *Synglycaspis*, similar to that shown in *Mamestra configurata* Walker (Lepidoptera: Noctuiidae) where more MGDG, DGDG, and phospholipids are utilized while feeding on *Brassica napus* L. (Brassicaceae) (Bracken 1982). Drop in MGDG and DGDG levels, especially during later stages of gall development (means later stages of instars), matches with the pattern of lipid metabolism shown in galls induced by the host-specific *Dryocosmus quercuspalustris* (Osten Sacken) (Hymenoptera: Cynipidae) on the leaves of *Quercus rubra* L. (Fagaceae) (Bayer 1994).

Levels of Sterol 382.1 in *E. dives* and *E. rossii* have been much higher than those that occur in *E. macrorhyncha*. Given that the gall-inducing *Synglycaspis* is tied to leaves of *E. macrorhnycha*, it could be inferred that when levels of plant sterols exceed a "threshold level" (Behmer and Elias, 2000; Jing et al. 2014), *Synglycaspis* selectively avoids leaves of *E. rossii* and *E. dives*. Sterol 440.3, which occurs maximally in young leaves of *E. macrorhyncha*, about 15 times greater than that in young leaves of *E. rossii* and two times greater than that in young leaves of *E. dives*, also plays a role in the selection of *E. macrorhyncha* by *Synglycaspis* against *E. rossii* and *E. dives*.

In conclusion, young and mature leaves of *E. macrorhyncha* show variations in levels of complex lipids and sterols. The predominant sterols in the three selected species of *Eucalyptus*, belonging to "*Eucalyptus* + *Primitiva*," are sitosterol and Sterol 382.1. Due to gall induction, changes in complex-lipid and sterol profiles manifest. Membrane-bound lipids (MGDG and DGDG) show a greater level of variation than the storage-lipids (triglycerides) in gall tissues, reinforcing distinct membrane modifications that occur in plant tissues during gall development. Because several sterols detected in *E. macrorhyncha* could not be determined due to paucity of information in databases, we can neither confirm nor reject Lovett's (1980) hypothesis on the necessity of a Δ^7 bond for a host-specific insect. Nonetheless, the levels of ergosterol (396.3), a $\Delta^{5,7}$ bond-including sterol, increase in galls that include the second, third, and fourth instars and decrease in galls that include the fifth instars of *Synglycaspis*. This indicates that this sterol is produced in *E. macrorhyncha* during gall development and is minimized with the insect preparing to moult into adult and exit the gall. Impressively high levels of Sterol 440.3 in young leaves of *E. macrorhyncha*, the leaf stage on which gall induction occurs, and its significantly low levels in mature leaves, and similar context of being low in young and mature leaves of *E. rossii* and *E. dives*, indeed point to this sterol being critical in host selection by *Synglycaspis*.

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