# OXIDIZABLE PHENOLIC CONCENTRATIONS DO NOT AFFECT DEVELOPMENT AND SURVIVAL OF *Paropsis atomaria* LARVAE EATING *Eucalyptus* FOLIAGE

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**Abstract –** Insect folivores can cause extensive damage to plants. However, different plant species, and even individuals within species, can differ in their susceptibility to insect attack. Polyphenols that readily oxidize have recently gained attention as potential defenses against insect folivores. We tested the hypothesis that variation in oxidizable phenolic concentrations in *Eucalyptus* foliage influences feeding and survival of *Paropsis atomaria* (*Eucalyptus* leaf beetle) larvae. First we demonstrated that oxidizable phenolic concentrations vary both within and between *Eucalyptus* species, ranging from 0 to 61 $mg.g^{-1}$ DM (0 to 81 % of total phenolics), in 175 samples representing 13 *Eucalyptus* species. Foliage from six individuals from each of ten species of *Eucalyptus* were then offered to batches of newly hatched *P. atomaria* larvae, and feeding, instar progression and mortality of the first and second instar larvae were recorded. Although feeding and survival parameters differed dramatically between individual plants, they were not influenced by the oxidizable phenolic concentration of leaves,

suggesting that *P. atomaria* larvae may have effective mechanisms to deal with oxidizable phenolics. Larvae feeding on plants with high nitrogen (N) concentrations had higher survival rates and reached third instar earlier, but N concentrations did not explain most of the variation in feeding and survival. The cause of variation in eucalypt herbivory by *P. atomaria* larvae is therefore still unknown, although oxidizable phenolics could potentially defend eucalypt foliage against other insect herbivores.

**Key Words –** Plant secondary metabolites, Insect herbivore, *Eucalyptus*, Tannins, Oxidation, Nutrition.

## INTRODUCTION

Plant leaves are commonly eaten by herbivores, reducing the ability of plants to photosynthesize. A common way that plants defend themselves from herbivores is by synthesizing compounds that make them less appetizing. Traditionally, these are known as plant secondary metabolites (PSMs). Among the thousands of PSMs, tannins and related polyphenolics comprise one of the most ubiquitous groups in woody plants, and can represent as much as 30 % of leaf biomass (e.g. Fox and Macauley 1977). Tannins are a diverse group of compounds that can bind to protein (Hagerman 2012), chelate metal (Zhang et al. 2016), and readily oxidize (Hagerman et al. 1996). However, they display great diversity in molecular structure and in their bioactivities (Salminen and Karonen 2011).

The protein-binding effects of tannins have been extensively studied, and protein precipitation capacity has often been used as a proxy for total tannin concentrations (Salminen and Karonen 2011). Plants with a high capacity to precipitate proteins have been repeatedly shown to negatively affect mammalian herbivores, such as by reducing protein digestibility and deterring feeding (Cooper and Owensmith 1985; Marsh et al. 2003; McArt et al. 2009). However, tannins do not appear to affect protein digestion in insect herbivores due to unfavorable gut conditions (Barbehenn and Constabel 2011; Fox and Macauley 1977; Martin et al. 1987). The lack of correlation between insect feeding and tannin concentrations led to the hypothesis that foliar protein was limited, and so insects should feed in such a way as to maximize their intake of protein (Slansky and Feeny 1977). This remained the favored hypothesis for many years, even in the face of studies that found that nitrogen (N) was more than adequate for growth and maintenance of insect populations (Ohmart et al. 1987).

More recently, it has been proposed that tannins and other phenolics affect insect-plant interactions via redox-mediated mechanisms. Phenolics can be oxidized to produce semiquinones, which are damaging free radicals that could cause oxidative stress in the midgut and its tissues (Appel 1993; Barbehenn et al. 2008). The initial approach to testing this idea was to supplement artificial diets with isolated tannins of different classes. The results were variable, but overall supportive of the "pro-oxidant" hypothesis of tannin action. For instance, galloyl glucoses (a group of hydrolysable tannins) appeared to be oxidised in the digestive tract of larvae of the moth *Epirrita autumnata* and decreased feeding in the second to fourth instars (Salminen and Lempa 2002).

Although studies with isolated tannins are useful for illustrating the types of mechanisms by which these compounds affect insects, they are not reflective of what would happen in intact plants. A living intact leaf contains many different types of polyphenols that vary between and within species (Barbehenn and Constabel 2011; Moore et al. 2014), and may be compartmentalized within the leaf, limiting some reactions. Furthermore, each of these polyphenols may have a different effect; some polyphenols oxidize more readily than others (Salminen and Karonen 2011). Artificial diets cannot replicate the complexity of mixtures or the structure of the leaf. The better option is to use conspecific plants with different polyphenol concentrations.

To test the oxidative stress hypothesis, it is important to be able to measure the portion of polyphenols that readily oxidize. Without the ability to measure oxidizable polyphenol concentrations separately from total polyphenols in intact plants, insect performance on plants could not be attributed to the effects of oxidizable polyphenols. In 2011, Salminen and Karonen (2011) introduced an assay that measured the oxidizable phenolic concentration of a plant sample. The method is based on the idea that the Folin-Ciocalteau reagent can be used to

78 measure total phenolic hydroxyl groups before and after oxidation, using mild base (sodium
79 carbonate) to promote the oxidation step (Salminen and Karonen 2011). This assay means that
80 relationships between oxidizable phenolic concentrations and feeding in insects can be
81 analyzed with intact leaves, rather than artificially altered diets.

82 The dominance of eucalypts in Australian ecosystems, as well as the widespread damage to
83 eucalypt forestry plantations caused by insect folivores (Paine et al. 2011), makes it important
84 to understand what drives insect feeding preferences on *Eucalyptus*. *Eucalyptus* foliage has
85 long been known to have significant concentrations of polyphenols, as well as significant
86 variation between species in the types of polyphenols present (e.g. Hillis 1966). Observations
87 of insects from several different orders feeding on *Eucalyptus* leaves have revealed that not all
88 trees of the same species are eaten (Fox and Morrow 1981). Although chemical explanations
89 have been frequently advanced to explain these differences, there have been few convincing
90 demonstrations of the cause of these feeding differences (Paine et al. 2011). Matsuki et al.
91 (2011) showed that a formylated phloroglucinol compound (FPC), sideroxylonal, explained
92 variation in host preferences of Christmas beetles (*Anoplognathus* spp, Scarabaeidae) for
93 different individuals of *Eucalyptus melliodora* and *Eucalyptus sideroxylon*. Likewise, damage
94 to *Eucalyptus tricarpa* by Christmas beetles was related to the sideroxylonal concentration of
95 foliage (Andrew et al. 2007). In contrast sideroxylonal and related FPCs did not appear to
96 explain variations in the feeding rates of *Eucalyptus* leaf beetles (*Paropsis atomaria*) (Henery
97 et al. 2009) or autumn gum moths (*Mnesampela privata*) (Östrand et al. 2008).

98 Given the availability of new methods of chemical analysis, the aim of this project was to
99 examine the importance of oxidizable phenolics in the feeding ecology of *P. atomaria*. There
100 were three specific hypotheses to be tested in this work: (1) That oxidizable phenolic
101 concentrations vary intra-specifically in the leaves of selected *Eucalyptus* species; (2) That

variations in oxidizable phenolic concentrations significantly affect the growth rate, survival and food intake of *P. atomaria* larvae feeing on *Eucalyptus* foliage; and (3) That oxidizable phenolics are more important than total nitrogen in explaining variation in survival and feeding on *Eucalyptus* leaves.

These hypotheses were tested first with chemical assays of plants collected in the field, and subsequently by the quantification of performance (intake rate, development time, and mortality) of *P. atomaria* larvae on cultivated plant material. *P. atomaria* is widespread across south-eastern Australia, easily obtainable and can be readily cultured. They have a varied diet that includes many different eucalypt species (Nahrung et al. 2008), and have shown unexplained feeding preferences for specific individuals within species (Henery et al. 2008a), making them an ideal candidate for this study.

## METHODS AND MATERIALS

*Composition of Field-Collected* Eucalyptus *Leaves*. We collected mature leaves from 175 trees representing 13 *Eucalyptus* species from south-eastern New South Wales and the Australian Capital Territory, Australia (Supplementary Table 1). Upon collection, leaves were immediately placed into a portable freezer, and were transported to The Australian National University (ANU), Canberra, where they were freeze-dried, and ground in a Foss Cyclotec 1093 mill (Foss, Höganäs, Sweden) to pass a 1 mm sieve. We used the method described in Salminen and Karonen (2011) to determine the total phenolic and oxidizable phenolic concentrations of the leaves. Briefly, 10 ± 0.5 mg of each freeze-dried and ground plant sample was extracted three times in 800 µL 7:3 acetone:water for 2 hr per extraction. The supernatants from the three extractions were combined and then freeze-dried. The freeze-dried extracts were redissolved in a known volume (initially 500 µL but later adjusted to 300 µL) of distilled water and filtered using a 0.45 µm PTFE filter.

126 To measure the total phenolic concentration, 20 µL of the reconstituted extract was combined
127 with 280 µL of carbonate buffer: formic acid mixture (9 parts 0.05 M carbonate buffer at pH
128 10: 5 parts 0.6 % formic acid), and 50 µL of this solution was placed in the well of a 96-well
129 plate with 50 µL 1N Folin-Ciocalteu Reagent (Sigma) and 100 µL 20 % (m/v) sodium
130 carbonate solution. After incubating for 1 hr at 25 °C, the absorbance of the samples was read
131 at 730 nm on a Tecan Infinite M1000 pro spectrophotometer (Tecan, Männedorf,
132 Switzerland). A series of gallic acid standards (0 – 2 $mg.mL^{-1}$) were treated in the same way in
133 order to determine the concentration of total phenolics as gallic acid equivalents (GAE).

134 To measure the concentration of oxidizable phenolics, sample extracts were first diluted to
135 give an absorbance of 1.0 ± 0.1 (Salminen and Karonen 2011). Following this 20 µL sample
136 extract was added to 180 µL 0.05 M carbonate buffer (pH 10) and incubated at 25 °C. After
137 exactly 90 min, 100 µL 0.6 % formic acid was added to each sample to stop the oxidation
138 process. In the well of a 96 well plate, 50 µL of oxidized mixture, 50 µL 1N Folin-Ciocalteu
139 Reagent and 100 µL 20 % (w/v) sodium carbonate solution were combined and incubated at
140 25 °C for one hour, after which the absorbance at 730 nm was read. The concentration of
141 oxidizable phenolics was calculated in GAE as the percentage difference between the pre- and
142 post-oxidation concentrations, multiplied by the total phenolic concentration of each sample.
143 Both chemical analyses were done in triplicate on each reconstituted extract. All samples with
144 coefficients of variance > 10 % between triplicates were excluded from the final data set,
145 leaving 165 samples.

146 The total nitrogen concentration of all 175 samples was measured using a Dumas combustion
147 procedure on a Leco Truspec CN determinator (Leco Corporation, St Joseph, MI, USA).

148 The ANOVA function in Genstat v17 (VSN International Ltd., UK) was used to test whether
149 mean concentrations of N, total phenolics and oxidizable phenolics differed between

*Eucalyptus* species. The regression function in Genstat was used to test for correlations between foliar concentrations of N, total phenolics and oxidizable phenolics.

*Feeding Experiments*. A breeding colony of *P. atomaria* was established at the ANU from adults, larvae and eggs collected from the Jerrabomberra Wetlands and Black Mountain Peninsula, Canberra, Australia. The beetles were kept indoors in three enclosures placed next to a window, and raised on a diet of *Eucalyptus elata* and *E. stellulata* leaves collected from mature trees. Fresh leaves were placed in the enclosures every three days.

Previously established *Eucalyptus* seedlings (approx. 12 months old) that were growing in a shade house at the ANU were used in the insect feeding experiments (Supplementary Table 2). Ten species were chosen based on the published feeding preferences of *P. atomaria* and personal observation of the presence of adults and larvae in the wild (Table 1). Six individuals of each plant species were used. Within each species, all of the individuals were at least half-siblings. As it was impossible to feed all plants to larvae at the same time (the timing was dependent on when larvae hatched), each plant was assigned a number from 1 to 60, and the order of plants used in the experiments was randomized using the statistical software GenStat 15.1.

As eggs were laid, they were removed from the colony and placed in a plastic container. All leaves were removed to prevent larval feeding before feeding trials commenced. The mixing of eggs and larvae within the container effectively randomized the cohorts. As batches of larvae hatched, 20 newly hatched larvae were placed on a branchlet taken from their allocated experimental tree. Each branchlet was placed with the stem poking into a Parafilm-covered conical flask filled with water. The Parafilm served as a barrier to prevent larvae from crawling into the water. The feeding experiments were conducted in a temperature-controlled room set at 25 °C on a 12h:12h light: dark cycle, with a half hour fade to simulate dawn and

dusk. The total area of the branchlets was measured before and after the feeding trials, via averaging triplicate digital scans of the branchlets from a Konica Minolta photocopier, using the software package ImageJ v1.47 (Rasband 1997-2016). In order to confirm that total leaf area did not change in the absence of insect herbivory, total leaf areas were recorded at day 0, 2, 4, 6 and 8 on a separate set of branchlets that were not exposed to herbivory. The regression function in Genstat showed that there was no change in leaf area over eight days after branchlets were cut from plants ($F_{1,91} = 0.18$, $P = 0.673$). Any change in leaf area in the presence of larvae was therefore assumed to be due to consumption by the larvae.

Frass produced by the larvae were collected at 1000 h and 1730 h every day for the duration of each feeding trial. At the same time, the number of larvae of each instar remaining in each cohort was also recorded. Feeding experiments stopped either when 50% of the remaining larvae had reached 3rd instar, or when all larvae died. Previous work has shown that the early instars are the most likely to show differences in feeding and mortality on resistant plants (Henery et al. 2009).

The nutritional composition of the leaves eaten by larvae was determined using leaves taken from the same position on another branchlet of the experimental plant. These leaves were frozen at -20 °C and then freeze dried. Once dry, a Qiagen TissueLyser™ was used to grind the leaves into powder for chemical analysis. Total nitrogen, total phenolic and oxidizable phenolic concentrations were determined in the same manner as described for the field-collected plants.

*Leaf Area to Leaf Mass Conversion*. For each plant used in the feeding experiments, three leaves of the same age as those eaten by larvae were collected. Their areas were measured using the software package ImageJ v1.47. The leaves were then dried in an oven at 40 °C for 48 h and weighed to the nearest 0.1 mg to obtain their dry mass. The area: mass ratios for each

plant were used to calculate dry matter intake (DMI) for insect larvae from the area of leaf eaten.

*Statistical Analysis of Feeding Experiments*. The ANOVA function in Genstat was used to test whether mean concentrations of N, total phenolics and oxidizable phenolics differed between *Eucalyptus* species. The regression function in Genstat was used to test for correlations between foliar concentrations of N, total phenolics and oxidizable phenolics.

Insect feeding experiments were analyzed with Residual Maximum Likelihood (REML) linear mixed models in Genstat. Separate models tested how leaf composition affected the number of days taken to reach second instar, number surviving to second instar, day cohort reached third instar, number surviving to third instar, total frass produced, and mass of leaf eaten. In all models, the fixed effects were the N concentration, total phenolic concentration and oxidizable phenolic concentration of leaves, as well as the *Eucalyptus* species and all interaction terms. Final models were made by progressively removing non-significant terms until the final model contained only significant terms ($P < 0.05$). Non-significant terms reported in the results are from the full model, while significant terms are from the final model.

## RESULTS

*Composition of Field-Collected* Eucalyptus *Leaves*. There were significant differences between *Eucalyptus* species in total foliar phenolic concentrations ($F_{12,174}=15.92$, $P<0.001$) and oxidizable phenolic concentrations ($F_{12,162}=3.51$, $P<0.001$; Table 2). There was also variation within species. *Eucalyptus globoidea* showed the largest range of total phenolic concentrations between individuals, whereas *E. pauciflora* had the smallest range (Table 2). The greatest range of oxidizable phenolic concentrations between individuals within a species was in *E. elata*. The smallest range was in *E. pauciflora* (Table 2).

There was no relationship between the N and oxidizable phenolic concentrations of leaves ($F_{1,173}$ = 1.60, $P$ = 0.207). In contrast, there was a positive correlation between the concentrations of total phenolics and oxidizable phenolics ($F_{1,173}$ = 127.39, $P$ < 0.001, $r^2$=0.42), and a negative correlation between total phenolics and N ($F_{1,185}$ = 62.67, $P$ < 0.001, $r^2$=0.25).

*Feeding Experiments.* The nutritional composition of *Eucalyptus* foliage used for the feeding experiments varied substantially (Table 2), despite the fact that all six plants within each species were at least half siblings. N concentrations ranged from 0.4 to 1.9% DM, while total phenolic and oxidizable phenolic concentrations ranged from 27 to 192 and 0 to 72 mg.GAE $g^{-1}$ DM, respectively (Table 2). There was a positive correlation between the total and oxidizable phenolic concentrations of leaves ($F_{1,58}$ = 139.47, $P$ < 0.001, $r^2$ = 0.70). In contrast, there was no relationship between the foliar N concentration and either the total phenolic ($F_{1,58}$ = 1.80, $P$ = 0.18) or oxidizable phenolic concentrations ($F_{1,58}$ = 2.69, $P$ = 0.11).

*Paropsis atomaria* larvae showed very different growth and survival patterns on different plants (Figure 1). Larvae that ate more food produced more frass ($F_{1,58}$ = 124.09, $P$ < 0.001, $r^2$ = 0.68), a result that mimics other studies of insects feeding on eucalypt leaves (Henery et al. 2009; Murray et al. 2013). This relationship did not differ between *Eucalyptus* species ($F_{9,50}$ = 1.43, $P$ = 0.202). The oxidizable phenolic concentration, total phenolic concentration, and species of eucalypt being fed did not have a significant effect on most of the dependent variables measured in the feeding trials (i.e. day cohort reached second instar, number alive at second instar, day cohort reached third instar, number alive at third instar, total frass produced and leaf mass eaten; $P$ > 0.138 in all analyses). However, cohorts of larvae consistently reached third instar earlier when being fed some species of *Eucalyptus* ($F_{7,12}$=4.55, $P$=0.011), and there was a trend for more larvae in each cohort to survive to second instar when the

oxidizable phenolic concentrations of plants were higher ($F_{1,58}$ = 3.24, $P$ = 0.077). In contrast, at higher N concentrations cohorts of larvae ate more ($F_{1,58}$=7.13, $P$ = 0.01; Figure 2a), produced more frass ($F_{1,58}$=7.03, $P$ = 0.01), reached third instar earlier ($F_{1,12}$=4.98, $P$ = 0.046), and more larvae in the cohort survived to third instar ($F_{1,58}$=24.30, $P$ < 0.001; Figure 2b). Cohorts for which no larvae survived to third instar still survived longer if they were feeding on a plant with a higher N concentration ($F_{1,37}$=5.10, $P$ = 0.03). There were no significant interaction terms, such as between species and nutritional composition, or between N and oxidizable or total phenolic concentrations ($P$ > 0.113 in all analyses).

## DISCUSSION

Variation in the chemical composition of leaves is an important determinant of feeding and fitness traits for both insect and mammalian herbivores (Moore et al. 2014). This study made three key findings that contribute to the body of work in this area, and that are discussed in more detail below. First, there is substantial variation in oxidizable phenolic concentrations between and within species of *Eucalyptus*. Second, the first and second instar larvae of *Paropsis atomaria* show vastly different survival and growth rates on different plants, confirming that some plants are more suitable food for them than others. Finally, contrary to our original hypothesis, the oxidizable phenolic concentration of *Eucalyptus* plants is not a significant factor in determining plant suitability. Foliar N concentration was the only significant factor that could explain the measured performance aspects of the larvae; larvae that were fed leaves with higher N concentrations had higher growth and survival rates.

### *Oxidizable phenolic concentrations vary*

The results of the field survey indicate strong variation in oxidizable phenolic concentrations between *Eucalyptus* individuals. Some individuals have more than ten times higher oxidizable

phenolic concentrations than other individuals within the same species. The concentrations of oxidizable phenolics measured in *Eucalyptus* species are similar to those measured in 12 plant species from Finland and Uganda by Vihakas et al. (2014). In their study, between 0 and 57 mg GAE $g^{-1}$ DM of leaf phenolic extracts were oxidized at pH 10, while between 0 and 61 mg GAE $g^{-1}$ DM were oxidizable in eucalypts.

Genetic factors have been shown to contribute to variation in the concentrations of some secondary metabolites in eucalypts, although tannin concentration (measured as amount of dry matter able to bind to polyethylene glycol) did not exhibit a genetic relationship (Andrew et al. 2005). However, environmental factors, such as soil nutrients and light availability, can affect tannin concentration (Close et al. 2003; Moore et al. 2004). For example, extreme light radiation can lead to photoinhibition, which limits the photosynthetic rate of plants, which in turn leads to greater allocation of carbon to secondary metabolites (Close et al. 2003). The *Eucalyptus* individuals used for this survey of oxidizable phenolic concentrations were obtained from many different locations, encompassing coastal environments to elevations greater than 1000 m (Supplementary Table 1). Environmental variation could therefore account for some of the variation in phenolic concentrations that we observed. However, the plants fed to insects also demonstrated considerable variation in oxidizable phenolic concentrations even though they were grown together in a shade house. In some plants, foliar chemical defenses, including tannins, also increase directly as a result of defoliation by herbivorous insects (Schultz and Baldwin 1982). There is little evidence for short-term induction of defenses in *Eucalyptus* (Henery et al. 2008b; Rapley et al. 2007), but repeated herbivory reduced the performance of *Thyrinteina arnobia* (Lepidoptera: Geometridae) on *E. cloeziana* over multiple generations (De Oliveira et al. 2010).

A strong negative correlation was found between total phenolic and N concentrations of leaves collected in the field. This is in keeping with previous findings in *Eucalyptus* (Moore et al. 2004). There are many proposed reasons for why phenolic and N concentrations appear to be inversely linked. Low nutrient availability has been shown to lead to higher carbon:nitrogen (C:N) ratios in leaves (Lawler et al. 1997). The Carbon-Nutrient balance hypothesis suggests that when there is less N available relative to C, the C:N ratio of leaves increases, and the 'excess' carbon available to the plant can be allocated to secondary metabolites (Coley et al. 1985). Lawler et al. (1997) also found that increasing the atmospheric $CO_2$ increased concentrations of some secondary metabolites in *Eucalyptus*. This idea can be taken further; it has been suggested that any factor which slows plant growth more than photosynthesis (whose rate is directly linked to carbon availability) will lead to increased levels of secondary metabolites, as the carbon cannot be used for any other purpose (Herms and Mattson 1992). Herms and Mattson (1992) also suggested that plants invest more in secondary metabolites in low-nutrient conditions for another reason; when nutrients available to replace losses to herbivory are low, herbivory is more costly for a plant, so plants defend their leaves more strongly.

Oxidizable phenolics accounted for between 0 and 81 % of the total phenolic concentration of samples, although there was an overall positive correlation between oxidizable phenolic and total phenolic concentrations. It is possible that this correlation would not always be consistent, as Salminen et al. (2004) found that oak leaves showed high seasonal variation in the composition of individual tannins, and that total phenolic concentration did not predict this variation well. Maple leaves also follow seasonal patterns in tannin concentration and profile (Barbehenn et al. 2013). Although eucalypts are not deciduous, Close et al. (2001) observed temporal variation in phenolic profiles of *Eucalyptus nitens*, which they attributed to

315 environmental factors that limit growth, leading to changes in the C:N ratio. The *Eucalyptus*
316 leaves for our study were all collected within a few weeks and were all mature leaves. In
317 future studies, *Eucalyptus* samples could be collected from the field at different times to
318 elucidate possible seasonal variation.

319 *Oxidizable phenolic concentrations have little effect on feeding or fitness*

320 Although growth and survival rates of *P. atomaria* larvae differed substantially between
321 individual plants within and between *Eucalyptus* species, there was no significant relationship
322 between any measure of *P. atomaria* feeding or survival and either oxidizable or total phenolic
323 concentration. Previous studies have produced mixed results, but do indicate a smaller effect
324 of tannins on herbivores that specialize in eating high-tannin leaves, and a larger effect on
325 generalists and herbivores that feed on leaves with low tannin concentrations. For instance,
326 *Acridoidea* grasshoppers, with a typical diet that does not include leaves containing tannins,
327 were fatally affected by tannin-coated leaves, with lesions in the gut that pointed towards
328 oxidative stress (Bernays et al. 1980). Likewise, vescalagin, a hydrolyzable tannin from oak
329 leaves, deterred feeding and reduced growth rates of two generalist moth species, but had little
330 effect on two moth species that specialize on oaks (Roslin and Salminen 2008). In eucalypts,
331 the total tannin concentration affected the survival of caterpillars of *Mnesampela privata*
332 moths (Rapley et al. 2007), but not *P. atomaria* (Fox and Macauley 1977). Steinbauer et al.
333 (2016) also reported that a eucalypt psyllid, *Ctenarytaina bipartita*, had higher fecundity on
334 foliage with higher concentrations of galloyl groups, which are typical components of
335 hydrolysable tannins . To date, there have only been a few studies focused specifically on the
336 effect of oxidizable phenolics. However, Barbehenn et al. (2009a; 2009b) found no significant
337 effect of increased oxidizable phenolic concentration on the fitness of gypsy moth (*Lymantria*
338 *dispar*) caterpillars, a specialist herbivore. Instead, the insects ate more material that had

higher oxidizable phenolic concentration (Barbehenn et al. 2009a). Thus, some polyphenols may actually act as feeding stimulants for specialist herbivores. Although this did not appear to be the case for *P. atomaria*, there was a trend for more larvae in a cohort to survive to second instar when the host plant contained a higher concentration of oxidizable phenolics. Given that *P. atomaria* larvae and adults both feed exclusively on eucalypt leaves, this insect may have adaptations that allows it to overcome any negative effects of ingesting large amounts of oxidizable phenolics.

In future studies it might be beneficial to modify the pH at which the oxidizable phenolic assay is performed. Fox and Macauley (1977) found that the gut pH of *P. atomaria* larvae ranged from 7.2 in the foregut to 8.5 in the mid- and hindguts, whereas the oxidizable phenolic assay is conducted at pH 10. It is therefore possible that the concentration of oxidizable phenolics that we measured is not the same as that encountered by *P. atomaria* larvae. It would be useful to how pH affects this analytical method for measuring oxidizable phenolics .

*Nitrogen concentrations affect feeding and fitness of* P. atomaria

In contrast with oxidizable phenolic concentrations, the foliar N concentration had a positive effect on both feeding and survival parameters for *P. atomaria* larvae. In particular, larvae raised on plants with higher N concentrations reached third instar earlier, and more larvae survived to third instar. Larval cohorts also ate more from plants with higher N concentrations, although this is likely because more individuals contributed to eating (i.e. more survived), rather than because each individual ate more per day. Nevertheless, the outcome is the same for the plant – a larger amount of biomass was removed from plants with higher N concentrations during the development of first and second instar *P. atomaria* larvae. The relationship between N concentration and growth and survival of *P. atomaria* has also been found previously (Fox and Macauley 1977; Gherlenda et al. 2015; Morrow and Fox 1980;

363 Ohmart and Edwards 1991; Ohmart et al. 1985), although it disappears above about 1.7 %
364 DM, where the concentration of N is no longer limiting (Miles et al. 1982; Ohmart and
365 Edwards 1991; Ohmart et al. 1985). In our study, only one plant contained more than 1.7 % N,
366 and this was the only plant on which all larvae survived to third instar. At the other end of the
367 spectrum, no larvae survived on plants containing less than 0.7 % N. Given the range of N
368 concentrations that our plants encompassed, it is not surprising that we found a relationship
369 between N concentration and larval growth and survival.

370 Despite this relationship, our study suggests that the foliar N concentration is not the only
371 factor affecting growth and survival of *P. atomaria* larvae. Between N concentrations of 0.7
372 and 1.4 % DM, survival rates were highly variable. This fits with previous suggestions that
373 other factors also influence the susceptibility of eucalypt foliage to damage by *P. atomaria*.
374 For example, Larsson and Ohmart (1988) found that *P. atomaria* larvae preferred to feed on
375 younger leaves, despite the fact that old and new leaves did not have significantly different N
376 concentrations. This was attributed to the toughness of leaves; older leaves were too tough to
377 chew. Differences in leaf toughness between plants, however, would not explain the large
378 variation in feeding and fitness traits found in our study, as all branchlets used in the feeding
379 experiments contained young, soft leaves covering a similar range of leaf ages (W. Zhou,
380 personal observation). Henery et al. (2008a) observed variation in susceptibility to insect
381 defoliation between individual *Eucalyptus* trees, even at high N concentrations, and this
382 susceptibility had a strong genetic component. Henery et al. (2008b) concluded that toxicity
383 may play an important role in distinguishing resistant from susceptible foliage, as *P. atomaria*
384 larvae feeding on resistant *E. grandis* foliage had lesions in the midgut that were absent from
385 larvae feeding on susceptible foliage. They were unable to determine the toxic component

386 responsible for these lesions, but suggested that oxidizable phenolics should be considered
387 (Henery et al. 2008b).

388 We explored whether the effectiveness of oxidizable or total phenolic concentrations was
389 moderated by the N concentration of foliage by looking for interactions between these
390 parameters. Mammalian herbivore feeding studies have shown that higher N concentrations in
391 the diet can increase tolerance for PSMs (Au et al. 2013; Nersesian et al. 2012). In other
392 words, some PSMs are more effective at deterring mammalian herbivores from feeding when
393 the N concentration of their diet is lower. In our study, the concentration of N did not affect
394 the responses of larvae to either oxidizable or total phenolic concentration. This suggests either
395 that oxidizable phenolics play little role in the defense of *Eucalyptus* foliage against *P.*
396 *atomaria* larvae, or that the way we measured these compounds is not optimized for *P.*
397 *atomaria* (e.g. anti-oxidant compounds may also be important, or the assay may need to be
398 conducted at a different pH).

399 *Conclusions*

400 This study has shown that oxidizable phenolic concentrations differ between and within
401 *Eucalyptus* species, making them a potential candidate for explaining variable feeding patterns
402 for some insect species. However, they did not explain survival and growth rates of *P.*
403 *atomaria* larvae offered leaves from a variety of *Eucalyptus* species. Instead, the results re-
404 emphasize the importance of N concentrations in determining defoliation levels, with high N
405 concentrations improving growth and survival rates of larvae. Nevertheless, *P. atomaria* is just
406 one of a large number of insect species from many orders that feed on *Eucalyptus* leaves
407 (Ohmart and Edwards 1991; Paine et al. 2011). Many of these insects are responsible for
408 significant damage in eucalypt forestry plantations (Paine et al. 2011), and differing levels of
409 damage on trees growing close to each other suggest that individuals possess different levels

410 of chemical defense (Henery et al. 2008a). It is possible that oxidizable phenolics are more
411 effective defenses against other insect pests of *Eucalyptus* than they are against *P. atomaria*,
412 and it would be valuable to test this in future studies.

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415 J-P Salminen for advice on the oxidizable phenolic assay.

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573

574

575

576 TABLE 1 The species of *Eucalyptus* used in feeding trials with *P. atomaria* larvae in this

577 study, and previous studies confirming that they are eaten by *P. atomaria*

| **Species** | **Study** |
|---|---|
| *E. camaldulensis* | Miles et al. (1982) |
| *E. fastigata* | Carne (1966) |
| *E. fraxinoides* | Fox and Macauley (1977) |
| *E. grandis* | Henery et al. (2008a) |
| *E. polyanthemos* | Carne (1966) |
| *E. radiata* | Tanton and Epila (1984) |
| *E. sideroxylon* | W. Zhou, personal observation |
| *E. sieberi* | W. Zhou, personal observation |
| *E. tereticornis* | Schutze and Clarke (2008) |
| *E. viminalis* | Morrow and Fox (1980) |

578

579

580 TABLE 2 Mean [range] nitrogen (N) and phenolic concentrations of *Eucalyptus* leaves
581 collected either from trees growing naturally in the field, or seedlings grown in a glasshouse.
582 The seedlings within each glasshouse species were at least half-siblings. The letter in brackets
583 after the species name specifies whether that species belongs to the eucalypt subgenus
584 *Symphomyrtus* (S) or *Eucalyptus* (= *Monocalyptus*; M)

| **Species** | **# of samples** | **N (% DM)** | **Total phenolics (mg GAE $g^{-1}$ DM)** | **Oxidizable phenolics (mg GAE $g^{-1}$ DM)** | **% of phenolics oxidized** |
|---|---|---|---|---|---|
| **Field collected** | | | | | |
| *E. agglomerata* (M) | 14 | 0.97 [0.86-1.10] | 65 [11-106] | 17 [4-35] | 28 [6-54] |
| *E. dives* (M) | 17 | 1.30 [1.11-1.52] | 51 [33-68] | 14 [0-22] | 29 [0-50] |
| *E. elata* (M) | 16 | 1.71 [1.26-2.15] | 34 [13-76] | 17 [6-61] | 49 [23-81] |
| *E. fastigata* (M) | 20 | 1.38 [1.16-1.66] | 49 [19-83] | 22 [3-40] | 42 [12-60] |
| *E. fraxinoides* (M) | 14 | 1.17 [0.88-1.45] | 50 [38-78] | 18 [3-30] | 37 [5-66] |
| *E. globoidea* (M) | 15 | 1.12 [0.76-1.51] | 77 [18-102] | 19 [5-43] | 26 [9-47] |
| *E. macrorrhyncha* (M) | 8 | 1.05 [0.96-1.15] | 40 [27-63] | 13 [2-25] | 31 [8-39] |
| *E. pauciflora* (M) | 9 | 1.49 [1.23-1.66] | 20 [10-32] | 7 [3-14] | 37 [13-49] |
| *E. pilularis* (M) | 5 | 0.87 [0.77-0.93] | 53 [34-65] | 12 [6-19] | 21 [14-29] |
| *E. radiata* (M) | 17 | 1.45 [1.22-1.66] | 57 [31-91] | 19 [3-31] | 34 [20-49] |
| *E. rossii* (M) | 9 | 1.06 [0.95-1.25] | 83 [56-97] | 26 [6-39] | 30 [8-40] |
| *E. sieberi* (M) | 11 | 0.99 [0.79-1.18] | 62 [43-85] | 18 [6-25] | 30 [12-43] |
| *E. stellulata* (M) | 10 | 1.25 [1.10-1.41] | 59 [28-81] | 18 [5-35] | 28 [12-43] |

| | | | | | |
|---|---|---|---|---|---|
| *F*-value | | 30.99 | 15.92 | 3.51 | 4.04 |
| *p*-value | | <0.001 | <0.001 | <0.001 | <0.001 |
| lsd | | 0.12 | 13 | 8 | 11 |
| **Glasshouse grown** | | | | | |
| *E. camaldulensis* (S) | 6 | 1.13 [0.91-1.29] | 62 [52-68] | 20 [16-24] | 33 [23-43] |
| *E. fastigata* (M) | 6 | 0.77 [0.62-1.02] | 50 [35-67] | 14 [7-23] | 29 [18-47] |
| *E. fraxinoides* (M) | 6 | 0.78 [0.61-1.06] | 66 [37-96] | 19 [4-41] | 27 [10-42] |
| *E. grandis* (S) | 6 | 0.88 [0.70-1.17] | 91 [49-192] | 22 [4-72] | 21 [6-37] |
| *E. polyanthemos* (S) | 6 | 1.17 [0.82-1.94] | 42 [27-61] | 8 [2-16] | 17 [6-26] |
| *E. radiata* (M) | 6 | 0.92 [0.62-1.27] | 58 [40-75] | 7 [0-26] | 11 [0-42] |
| *E. sideroxylon* (S) | 6 | 1.15 [0.95-1.38] | 47 [34-62] | 8 [0-18] | 17 [0-41] |
| *E. sieberi* (M) | 6 | 0.51 [0.38-0.84] | 71 [49-91] | 26 [1-59] | 32 [2-65] |
| *E. tereticornis* (S) | 6 | 0.88 [0.72-1.37] | 110 [63-254] | 33 [18-87] | 29 [24-38] |
| *E. viminalis* (S) | 6 | 0.76 [0.56-1.16] | 74 [54-86] | 19 [2-40] | 24 [2-46] |
| *F*-value | | 4.45 | 2.79 | 1.76 | 1.63 |
| *p*-value | | <0.001 | 0.01 | 0.099 | 0.133 |
| lsd | | 0.28 | 36 | 18 | 17 |

585

586 FIGURE CAPTIONS

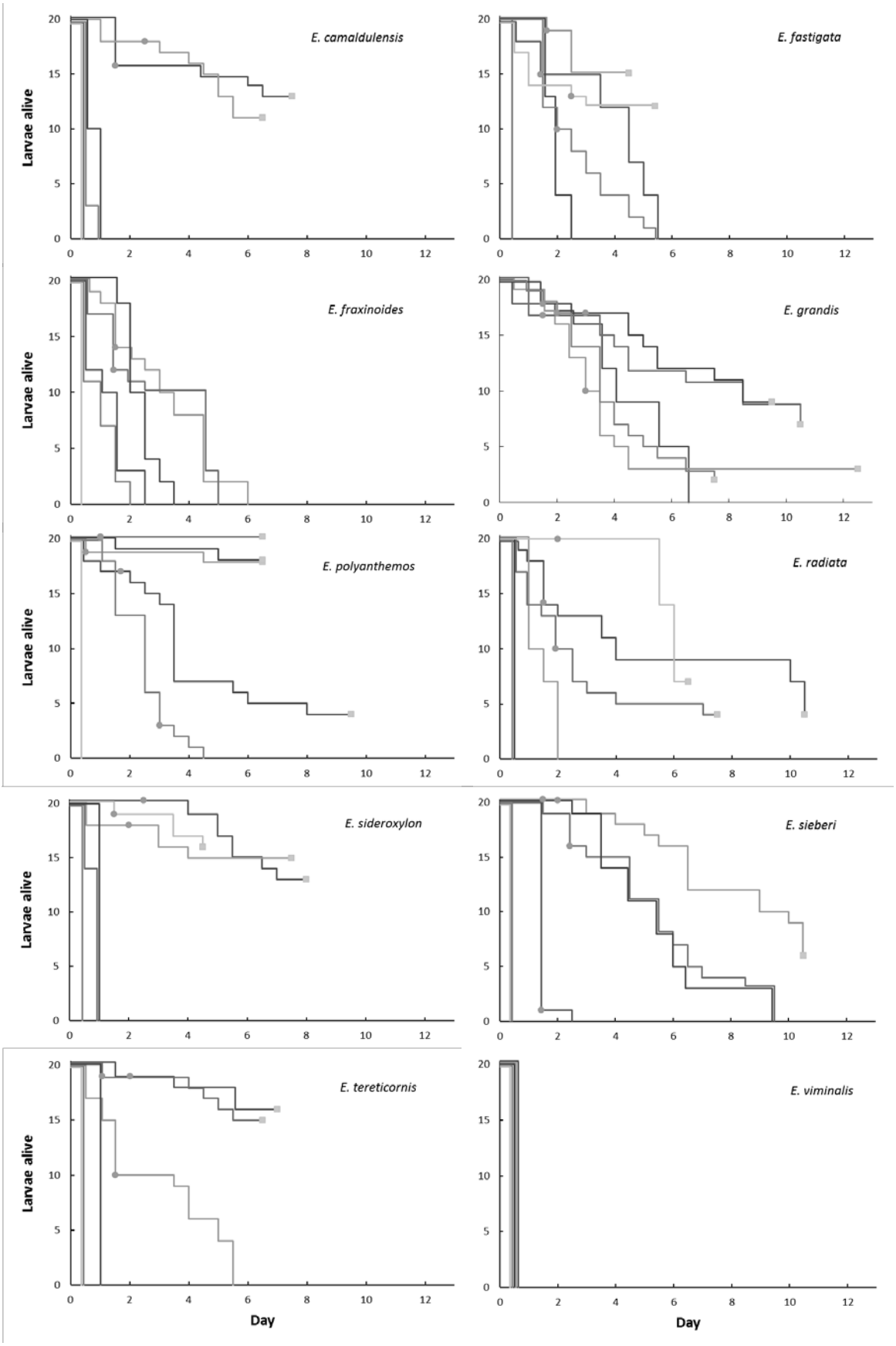
587 **FIG. 1** Survival curves for cohorts of *Paropsis atomaria* larvae on six individual plants from
588 each of ten species of *Eucalyptus*. Each line represents a separate plant, on which twenty
589 newly-hatched larvae were initially placed. Circles show the day on which at least half of the
590 surviving larvae from a cohort reached second instar, while squares show the same for third
591 instar. Lines that overlap have been offset slightly to improve clarity

592 **FIG. 2** The effect of foliar N concentration on a) the amount of leaf eaten by cohorts of larvae,
593 and b) the number of larvae from each cohort surviving to third instar. Points with the same
594 symbol are the same species of eucalypt

595

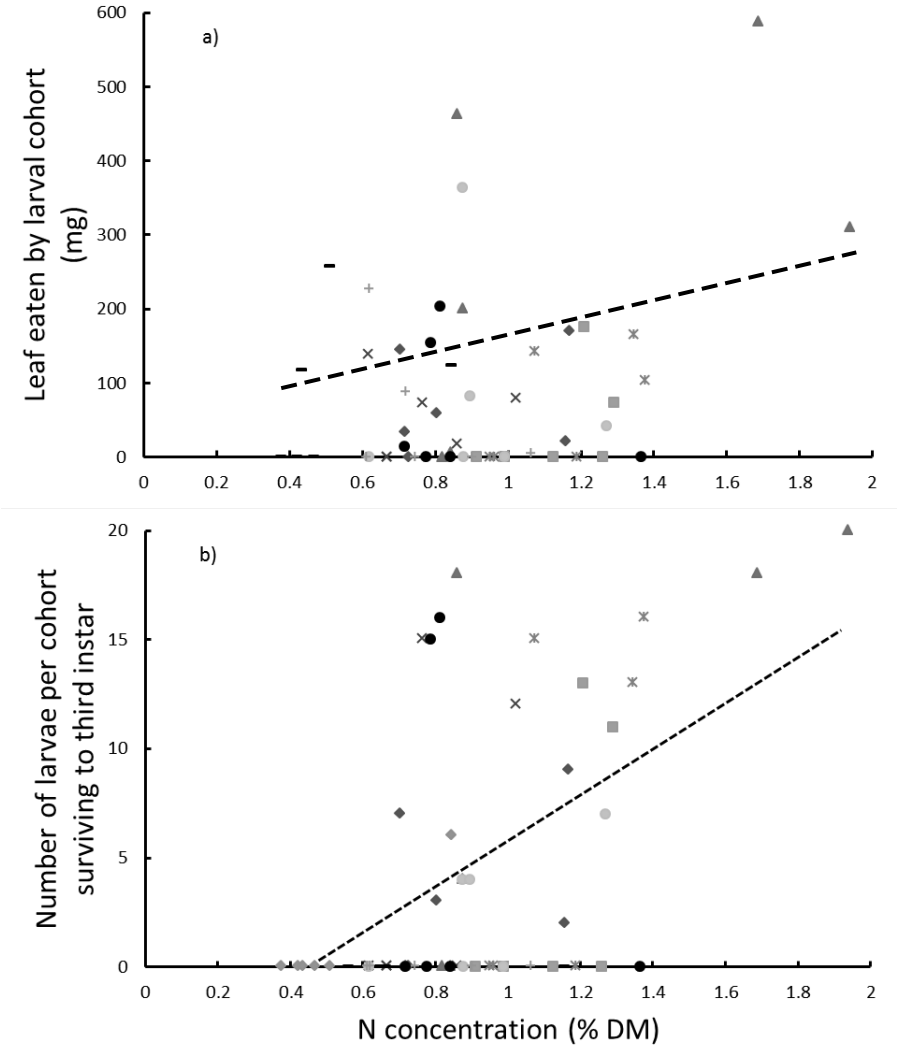
596 **FIG. 1**

597


E. camaldulensis
E. fastigata
E. fraxinoides
E. grandis
E. polyanthemos
E. radiata
E. sideroxylon
E. sieberi
E. tereticornis
E. viminalis
Larvae alive
Day

598 **FIG. 2**

599


a)
Leaf eaten by larval cohort (mg)
600
500
400
300
200
100
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
b)
Number of larvae per cohort surviving to third instar
20
15
10
5
0
N concentration (% DM)