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Authors

Mace, KC

Mills, NJ

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Response of walnut aphid populations to increasing foliar nitrogen content

Kevi C. Mace and Nicholas J. Mills

Department of Environmental Science, Policy and Management, University of California, Mulford Hall, Berkeley, CA 94720-3114, U.S.A.

- Abstract**
- 1 An understanding of how fertilizer input affects pest populations and, consequently, the need for pesticide use would allow for the development of more effective integrated management strategies.
 - 2 The present study addressed the response of walnut aphid *Chromaphis juglandicola* (Kaltenbach) (Hemiptera: Aphididae) populations to different levels of nitrogen application to walnut seedlings and the relationship between aphid density and chlorophyll content index (CCI), a non-invasive measure of foliar nitrogen, for mature trees in commercial walnut orchards.
 - 3 Although added nitrogen significantly increased soluble nitrogen content, CCI and soluble nitrogen : phosphorus ratios in the foliage of walnut seedlings, there was no effect on walnut aphid population growth. Similarly, there was no relationship between aphid density and CCI for mature trees in the field.
 - 4 A neutral response suggests that walnut aphid population growth on potted seedlings is limited by factors other than soluble nitrogen, such as other nutrients, amino acid composition or defensive compounds. Consequently, fertilizer management appears unlikely to effectively contribute to the control of *C. juglandicola* in commercial walnut orchards.

Keywords Aphid population growth, *Chromaphis juglandicola*, fertilizer, phosphorus, walnuts.

Introduction

Insect populations are influenced by top-down effects from natural enemies and by bottom-up effects from host plants in both natural and agricultural settings (Stiling & Rossi, 1997; Forkner & Hunter, 2000; Denno *et al.*, 2002). Given that the suppression of insect populations is the goal of pest management, both top-down and bottom-up processes have been explored in agricultural systems. Biological control by natural enemies has been the focus of top-down studies, although it does not operate in isolation from the bottom-up effects mediated by the host plant (Kagata & Ohgushi, 2006; Krauss *et al.*, 2007; Garratt *et al.*, 2010; Schüepp *et al.*, 2014). The use of pest resistant crop varieties has been the principal bottom-up approach for pest management and has received considerable attention in many crops (Maxwell & Jennings, 1980; Broekgaarden *et al.*, 2011; Niks *et al.*, 2011). There are, however, other important management practices, in addition to plant breeding, that can affect pest populations through bottom-up effects. For example,

short-term changes in nutrient quality of crop plants induced by the application of fertilizers can influence aphid populations (Kytö *et al.*, 1996; Zehnder & Hunter, 2009; Garratt *et al.*, 2010).

In agriculture, plant vigour is intensively managed to maximize production, principally through the application of nitrogen fertilizer. Fertilizer use can impact insect populations directly through at least two pathways in crop plants: nutritional status and plant defence. An improved nutritional status of plants can fuel insect population growth (Awmack & Leather, 2002) because nitrogen is a macronutrient known to be limiting for phytophagous insects (Denno *et al.*, 2002; Behmer, 2009). However, heavy fertilizer use has been shown to be detrimental to some phytophagous insects (Zehnder & Hunter, 2009; Hosseini *et al.*, 2010; Sauge *et al.*, 2010) possibly as a result of nutrient imbalance (Zehnder & Hunter, 2009). Nitrogen application can also change the ratio of nitrogen to phosphorus, carbon and other macronutrients (Cisneros & Godfrey, 2001; Chau *et al.*, 2005; Tao & Hunter, 2012). Most insects actively maintain close to optimal levels of macronutrients in their tissues; therefore, changing the ratio of macronutrients in food plants can affect insect feeding and performance (Sterner & Elser, 2002), thus

Correspondence: Kevi Mace. Tel.: +1 707 972 4253; fax: 510 642 5903; e-mail: kmace@berkeley.edu

affecting herbivore abundance (Awmack & Leather, 2002; Denno *et al.*, 2002; Zehnder & Hunter, 2009).

In addition to changes in nutritional status, plants with excess nitrogen may be able to produce more defensive compounds, thus hindering insect population growth (Sauge *et al.*, 2010; Vannette & Hunter, 2011; Tao & Hunter, 2012). There is some evidence, however, that fertilizer use can decrease the production of nitrogen-based defensive compounds (Sauge *et al.*, 2010). Additionally, herbivores in different feeding guilds react in fundamentally different ways to fertilizer use because they utilize different parts of the plant (Koricheva *et al.*, 1998; Huberty & Denno, 2004). Phloem-feeding insects, such as aphids, are more likely to be positively affected by increased nitrogen in the nutritionally poor phloem sap that they consume, whereas chewing insects, such as grasshoppers and moths, are more likely to be negatively affected by increased defensive compounds that they ingest with leaf tissue (Awmack & Leather, 2002; Behmer, 2009).

Aphids are important agricultural pests in many crops (van Emden & Harrington, 2007) and, based on observations from both natural and agricultural systems, their response to increased nitrogen can vary from positive (Cisneros & Godfrey, 2001; Nevo & Coll, 2001; Chau *et al.*, 2005; Noma *et al.*, 2010) to negative (Zehnder & Hunter, 2009; Hosseini *et al.*, 2010). Although most studies have been based on annual agricultural crops, aphids are also key pests in tree fruit and nut crops and far less is known about their response to increased nitrogen in tree foliage. Nonetheless, how nitrogen is managed in perennial orchards could affect the pest pressure from aphids, and thus the use of pesticides in these crops. Given the environmental and human health risks associated with increasing inputs from agricultural intensification, it is important to understand how fertilizer use can influence the potential for aphid population increase in tree crops. The diversity of insect responses to nitrogen that have been observed, and the lack of clear associated mechanisms to account for the variation (Kytö *et al.*, 1996; Gash, 2012) suggest that additional research in this area is needed to improve crop management.

Walnuts (*Juglans regia*), an important tree crop in California, historically sustained damage from the aphid *Chromaphis juglandicola* (Kaltenbach) (Hemiptera: Aphididae) until the introduction of the Iranian strain of the parasitoid *Trioxys pallidus* (Haliday) (Hymenoptera: Braconidae) in 1971 provided successful biological control (Frazer & van den Bosch, 1973). Renewed outbreaks have recently been reported (Hougardy & Mills, 2009) and growers are now sporadically using in-season insecticide treatments for the management of walnut aphids. One possible reason for the renewed outbreaks could be that intensification of fertilizer use in walnut production has led to an increased potential for aphid population growth, in which case a more holistic integration of fertilizer and pest management could help to sustain the biological control of this pest. The first part of the present study aimed to test the influence of different levels of nitrogen fertilizer on the foliar nutrient status and nutrient balance of walnut seedlings, and on the population growth rate of walnut aphids, under controlled greenhouse conditions. The second part aimed to determine the relationship between walnut aphid density and foliar nitrogen content in the field by sampling mature trees in organic and conventional commercial walnut orchards. Our hypothesis was that both aphid population

growth on seedlings in the greenhouse and aphid densities on mature trees in the field would be greater on walnuts with a higher foliar nitrogen content. If this were true, then fertilizer management could be a viable option for the integrated control of aphids.

Materials and methods

Aphid population growth on walnut seedlings

Walnut seedlings were grown from seed in individual 1-L pots with Supersoil potting soil (Scotts Miracle Gro, Marysville, Ohio). This mix contains 14% nitrogen, 9% phosphorous and 2% potassium, as well as 0.25% iron. Seedlings were kept in a greenhouse at 20–25 °C under an LD 16 : 8 h photocycle and watered weekly. Because potted walnut seedlings do not remain vigorous without regular fertilizer input, when walnut seedlings were 5–6 weeks old and had at least five full leaves, they were randomly assigned to a nitrogen treatment. The nitrogen treatments were low, standard and high, corresponding to 0.5, 1 and 2 times the standard field application rate for walnuts, which is 225 kg/ha (Ramos, 1997). Ammonium nitrate (Sigma-Aldrich Corporation, St Louis, Missouri) was used to create the nitrogen treatments. Seedlings were watered with 125, 250 or 500 ml of 0.14 g nitrogen/250 ml dH₂O solution and additional water, where necessary, for a total of 500 ml of irrigation once a week for the 3 weeks preceding an experiment. Depending on the availability of plants of the same age and size, five blocks of seedlings were established from March to August 2012 with each block consisting of six to 11 plants in each treatment ($n = 11$, 6, 10, 7 and 7 seedlings for blocks 1–5, respectively, giving a total of 41 seedlings per treatment). Plants were maintained and experiments conducted in a greenhouse at 22 °C and under an LD 16 : 8 h photocycle. Block 2 experienced temperature fluctuations as a result of air conditioner malfunction but, because it did not show any significant difference in aphid population growth compared with the other blocks, it was not excluded from the analyses.

A colony of *C. juglandicola* was maintained in a greenhouse at 20–25 °C under a LD 16 : 8 h photocycle on potted walnut seedlings. The colony was originally collected from Chico, California, in June 2009 and was maintained in the greenhouse for 3 years before use in the experiments. Additional field-collected aphids were introduced to the colony each year from a variety of locations in the Central Valley of California to maintain genetic diversity in the colony.

Three to four days after the third application of each nitrogen treatment, a healthy leaf was selected from each walnut seedling, trimmed to have only five leaflets and enclosed in a cylindrical plastic cage (diameter 7 cm, length 13 cm) with nylon sleeve ends. Removal of the bottom two leaflets was necessary to standardize the leaf area available to the aphids and was considered unlikely to affect leaf quality given that even severe pruning of peach seedlings has been shown not to affect leaf nitrogen concentration (Mediene *et al.*, 2002). Five first-instar, two-second instar, one third-instar, one fourth-instar and one adult walnut aphid, representing a stable stage distribution for *C. juglandicola* (Hougardy & Mills, 2008), were placed on each enclosed leaf and the sleeves of the plastic cages were tied closed. The aphids were left on the walnut seedlings for two generations,

255–265 degree days celsius (°DC) above a lower developmental threshold of 8.7 °C, or approximately 19 days (Hougardy & Mills, 2008). The seedlings were watered once a week during the course of the experiment, and degree days were monitored with a HOBO data logger (Onset, Bourne, Massachusetts) placed on top of a cage in the centre of the greenhouse compartment.

When experimental aphid cohorts had experienced 255–265 °DC, the cylindrical cages were carefully removed and the number of live aphids was recorded. Each instar was counted separately. This time period corresponds to approximately two aphid generations, approximately 19 days (Hougardy & Mills, 2008), and was sufficient time for the aphid populations to increase from ten individuals to 50–3000 individuals. For each walnut seedling, the instantaneous rate of increase (r) of the experimental populations of *C. juglandicola* was estimated from $r = \frac{\log\left(\frac{N_1}{N_0}\right)}{\text{degree days}}$ where N_0 = initial number of aphids and N_1 = final number of aphids (Hosseini *et al.*, 2010; Latham & Mills, 2010). This is the recommended method for estimating population growth rate when initial and final numbers are the measurement variables available (McCallum, 2000). Potted seedlings where aphids were present outside the cage and had, therefore, escaped, were excluded from analysis (leaving $n = 108$ with 36 plants per treatment).

Immediately before and after the experiment, the nitrogen status of the seedling foliage was quantified using an Apogee CCM-200 handheld chlorophyll meter (Apogee Instruments, Logan, Utah). The meter estimates a chlorophyll content index (CCI) from the transmission of light at 931 nm/635 nm, and chlorophyll content has been used previously to assess the influence of foliar nitrogen on aphid populations (Vos & Bom, 1993; Richardson *et al.*, 2002; Schüepp *et al.*, 2014). Both times, one reading was taken from each of the three terminal leaflets on the first three leaves of every seedling and the nine readings were averaged to give a single CCI estimate for each plant ($n = 108$ seedlings). For each reading, the meter was placed next to (but not over) the mid-vein.

To calibrate the CCI readings in relation to the foliar nutrient content of the walnut seedlings, foliage was analyzed from a subset of the seedlings used immediately after the experiment. For blocks 1 and 3 ($n = 57$ from three treatments $\times$ 19 seedlings from which aphids had not escaped), the first three leaves were removed from each seedling at the end of the experiment, oven-dried at 60 °C and ground for analysis of the soluble content of two key nutrients: nitrogen and phosphorus. Soluble nitrate ($\text{NO}_3\text{-N}$) was determined using the reduction to nitrite via a copperized cadmium column, diazotization with sulfanilamide followed by coupling with *N*-(1-naphthyl) ethylenediamine dihydrochloride and reading the absorbance of the product at 520 nm (Sechtig, 1992; Miller, 1998). This analytical method has a quantitative detection limit of 10 p.p.m. Soluble phosphate ($\text{PO}_4\text{-P}$) was quantified spectrophotometrically by reacting with ammonium molybdate and antimony potassium tartrate and reducing with ascorbic acid before reading the absorbance of the product at 880 nm (Prokopy, 1995). The detection limit for phosphate is 50 p.p.m. All samples were analyzed using an automated Flow Injection Analyzer (Lachat, Loveland, Colorado) and each soluble nutrient was measured as the percentage content of foliar dry weight.

Third-instar aphids from each treatment were weighed to evaluate potential differences in size. To accurately detect weights, 20 third-instar aphids were placed in a weighing dish on a scale (Sartorius AG, New York, New York). Twenty replicates of 20 aphids were weighed. The reported weights are expressed in milligrammes per 20 aphids.

Aphid density on mature trees in walnut orchards

In 2012, six organic and six conventional orchards were sampled for aphid density and foliar nitrogen status, and in 2013 the same 12 orchards were re-sampled and an additional four organic and four conventional orchards were added for a total of 20 orchards. All orchards consisted of mature trees with harvestable crop, ranging in age from 10 to 30 years and in tree height from 5 to 12 m. Each orchard was divided into four blocks and, within each block, 20 trees were sampled. Employing the standardized sampling protocol developed by Pickel *et al.* (2014), aphid density was estimated by counting all walnut aphids on five randomly selected leaves from the lower and mid canopy of each tree using a telescoping pole pruner. Walnut aphids are not highly mobile and do not detach from the leaves. The total number of aphids sampled from the 20 trees was divided by the total number of leaves sampled to provide a single measure of aphid density per leaf for each orchard block. Foliar nitrogen status was monitored by taking CCI readings from the three terminal leaflets of each of the five leaves sampled on each tree. Orchards were sampled in early June and early September to coincide with the seasonal peaks of aphid activity. All sampling in each season was completed within 5 days.

Aphid density and CCI were measured as block means nested within orchard for each season ($n = 4$ blocks $\times$ 12 orchards in 2012; $n = 4$ blocks $\times$ 20 orchards in 2013). As in the walnut seedling experiment, only a subset of the sampled trees was analyzed to verify the relationship between CCI and nitrogen content of the foliage in mature commercial trees. Two leaves were collected in both June and September from each of the 12 orchards sampled in 2012 ($n = 48$). These leaves were analyzed for soluble nitrate content only using the same methods described above for the foliage collected from walnut seedlings (Sechtig, 1992; Miller, 1998).

Statistical analysis

Data were analyzed using R (R Core Team, 2013) and LME4 (Bates, 2011). In all analyses, the significance of the fixed effects was estimated by model comparison between the full model with the fixed effect included and a reduced model lacking the fixed effect using a chi-square test (Bolker *et al.*, 2009). Models were fitted either as mixed models using the LMER function of the LME4 package or as linear models; parameters were estimated using maximum likelihood so that comparisons would be valid between models with different fixed effects (Bolker *et al.*, 2009). Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality. The variables used are described below.

For the data from the walnut seedling experiment, linear mixed effect models were fitted to test the effects of the nitrogen

Figure 1 Influence of nitrogen treatment on (a) percentage soluble nitrogen ($n=57$), (b) chlorophyll content index ($n=108$), (c) percentage soluble phosphorus ($n=57$) and (d) soluble nitrogen : phosphorus ratio ($n=57$) in dried foliage of walnut seedlings. Means with different lowercase letters are significantly different ($P < 0.05$, Holm pairwise comparison test). The box represents the median and interquartile range. The whiskers show the furthest data points within 1.5 times the interquartile range; any individual points are outliers.

treatments on foliar nutrient content and CCI and, subsequently, to test the effect of treatment, nitrogen content, phosphorus content, nitrogen : phosphorus ratio and CCI on the instantaneous rate of growth of the walnut aphid populations. Percentage foliar nitrogen and phosphorus as measurement variables were first transformed using log odds ratios (Crawley, 2013) and CCI and percentage foliar nitrogen were $\log_{10}$ transformed for normality. Pairwise comparisons of means between nitrogen treatments were based on the Holm method with pooled SD (Crawley, 2013). Block was included in all models as a random effect. A linear model was fit to estimate the effect of treatment on aphid weight; there were no random factors because they were all taken from the same block. Generalized linear models with a binomial error distribution were used to estimate the effect of nitrogen treatment on the proportion of aphids in different stages of development (instars).

For the data collected from mature walnut trees in the field, linear mixed effects models were fitted to analyze the relationships between soluble nitrogen content and CCI of the foliage, and between aphid density and CCI with type of orchard management (conventional versus organic) added as a second fixed factor. Percentage foliar nitrogen and CCI were $\log_{10}$ transformed for normality. In addition to the fixed factors, the mixed models also included the influence of random effects associated with the hierarchical structure of the sampling design. Blocks were nested within orchard and orchards were nested within type of management. Orchard within type of management was included as a scalar random effect for intercept, whereas year and season were included as nested random effects for slope indexed by orchard (Bates, 2011; Crawley, 2013). Accounting for the other variables in the sampling design as hierarchical random effects allowed us to focus directly on the influence of the fixed factors on the variance in the measurement variable for both models. Indexing season within year by orchard was necessary to account for

the temporal pseudoreplication arising from sampling the same orchards in both seasons and both years.

When appropriate, we used the conditional r^2 , the variance explained by both fixed and random factors in the mixed models, and the marginal r^2 , the variance explained by just the fixed factors (Nakagawa & Schielzeth, 2013). The change in marginal or conditional r^2 values between full and reduced models was also used to estimate the amount of variation explained by individual factors in the models (Nakagawa & Schielzeth, 2013).

Results

Aphid population growth on walnut seedlings

Nitrogen treatment had a significant positive influence on the soluble nitrogen content of the foliage of the walnut seedlings ($\chi^2 = 13.03$, d.f. = 2, $n=57$, $P=0.002$) (Fig. 1a). Pairwise comparisons of treatments indicated that dried foliage from the high nitrogen treatment had a significantly higher nitrogen content than dried foliage from the low ($P=0.002$) and standard treatments ($P=0.04$). There was no difference between the low and standard treatments ($P=0.26$). Similarly, the foliar CCI was positively influenced by nitrogen treatment ($\chi^2 = 7.98$, d.f. = 2, $n=108$, $P=0.02$) (Fig. 1b) and pairwise comparisons of treatments showed a significant difference between low and high treatments ($P=0.02$) but not between low and standard ($P=0.30$) or standard and high ($P=0.30$) treatments. As expected, nitrogen treatment did not have a significant influence on the mean percentage soluble phosphorus in dried foliage of the walnut seedlings ($\chi^2 = 4.32$, d.f. = 2, $n=57$, $P=0.12$) (Fig. 1c) but, more unexpectedly, there was no significant variation in the mean soluble nitrogen : phosphorus ratio between nitrogen treatments ($\chi^2 = 4.70$, d.f. = 2, $n=57$, $P=0.10$) (Fig. 1d). There

Figure 2 The relationship between percent nitrogen content and chlorophyll content index of leaves from (a) walnut seedlings in the nitrogen treatment experiment ($n=57$) and (b) mature trees in commercial walnut orchards in 2012 ($n=48$).

was no effect of nitrogen treatment on aphid weight ($F=0.04$, d.f. = 2, $n=20$, $P=0.96$).

There was a significant positive relationship between foliar soluble nitrogen content and foliar CCI of the walnut seedlings after the experiment ($\chi^2=10.49$, d.f. = 1, $n=57$, $P=0.001$) (Fig. 2a). There was also a significant positive relationship between the nitrogen : phosphorous ratio in the dried foliage and foliar CCI ($\chi^2=5.71$, d.f. = 1, $n=57$, $P=0.02$). This was a result of the positive relationship of nitrogen content with foliar CCI because CCI did not vary with soluble phosphorus content ($\chi^2=0.79$, d.f. = 1, $n=57$, $P=0.38$, not shown).

There was no relationship between the instantaneous rate of increase (r) of the experimental walnut aphid populations and nitrogen treatment ($\chi^2=0.97$, d.f. = 2, $n=108$, $P=0.62$) (Fig. 3a). There was substantial variation in final numbers of aphids and, therefore, in r , although only 0.7% of variance was explained by nitrogen treatment (change in marginal $r^2=0.007$). Similarly, there was no relationship between the instantaneous rate of increase of the aphid populations and foliar chlorophyll content after the experiment CCI ($\chi^2=0.04$, d.f. = 1, $n=108$, $P=0.85$) (Fig. 3b) with only 0.03% of the variance explained by CCI (change in marginal $r^2=0.0003$). Moreover, there was no relationship between the instantaneous rate of increase (r) and foliar chlorophyll content at the beginning of the experiment ($\chi^2=0.50$, d.f. = 1, $n=108$, $P=0.48$) with only 0.4% of variance explained by CCI at the start of the experiment. There was also no effect of nitrogen ($\chi^2=0.02$, d.f. = 1, $n=57$, $P=0.96$) or of the nitrogen : phosphorous ratio ($\chi^2=0.84$, d.f. = 1, $n=57$,

$P=0.36$) on the instantaneous rate of increase of the walnut aphids. Furthermore, the nitrogen treatments did not change the age structure of the populations because the proportion of first and second instars (deviance = 0.21, d.f. = 2, $n=108$, $P=0.90$), third and fourth instars (deviance = 0.31, d.f. = 2, $n=108$, $P=0.85$) and adult aphids (deviance = 0.06, d.f. = 2, $n=108$, $P=0.97$) was the same between all nitrogen treatments.

Aphid density on mature trees in walnut orchards

Regarding walnut seedlings, there was a significant positive relationship between foliar nitrogen content and foliar CCI of the mature orchard trees in the field ($\chi^2=24.61$, d.f. = 1, $n=48$, $P>0.001$) (Fig. 2b). The range of foliar nitrogen content and CCI measurements was similar between the orchard trees in the field and the walnut seedlings used in the greenhouse experiment (Fig. 2). There was also no relationship between aphid density and foliar CCI in the commercial walnut orchards sampled ($\chi^2=1.78$, d.f. = 1, $n=128$, $P=0.13$) (Fig. 4), with only 3.1% of the variance explained by CCI (change in marginal $r^2=0.03$). In addition, there was neither a significant difference in aphid density between organic and conventional orchards ($\chi^2=0.14$, d.f. = 1, $n=128$, $P=0.71$, change in marginal $r^2=0.02$), nor a significant difference in foliar CCI between organic and conventional orchards ($\chi^2=0.18$, d.f. = 1, $n=128$, $P=0.67$, change in marginal $r^2=0.06$). For the full model, 47% of the variance in aphid density was explained by the random factors (conditional $r^2=0.87$ and marginal $r^2=0.40$).

Discussion

Insect herbivores are generally considered to be constrained in their population growth by the nutritional quality of their host plant (Kytö *et al.*, 1996; Sauge *et al.*, 2010). In the present study, however, the nitrogen treatments did not appear to have a strong bottom-up influence on the population growth of *C. juglandicola* on potted seedlings, even though the nitrogen fertilizer treatments had a significant effect on both the soluble nitrogen content and foliar CCI of the walnut seedlings. Not only was there no difference in population growth, but also there was no difference in aphid weights or the age structure of the populations between treatments. This finding was further corroborated by the lack of a relationship between aphid density and foliar CCI of mature walnut trees in commercial orchards, despite a clear relationship between foliar nitrogen content and CCI. This is an unusual finding because aphids, including some tree aphids, generally show a positive or dome-shaped response (measured either as increased life-history performance in laboratory studies or as greater abundance in field studies) to nitrogen fertilizer and to CCI (Kytö *et al.*, 1996; Zehnder & Hunter, 2009; Couture *et al.*, 2010; Noma *et al.*, 2010; Sauge *et al.*, 2010; Schüepp *et al.*, 2014). There are several potential explanations for the lack of walnut aphid response: (i) walnut seedlings and mature trees did not respond to increased foliar nitrogen in a way that improved food quality for the aphids; (ii) nitrogen is not the limiting nutrient for walnut aphids; and (iii) factors other than nutritional quality limit aphid population growth on walnut seedlings and mature trees.

Figure 3 Effect of (a) nitrogen treatment ($n=108$) and (b) chlorophyll content index on the instantaneous population growth rate of aphid cohorts on walnut seedlings ($n=108$). The boxes in (a) represent the median and interquartile range. The whiskers show the furthest data points within 1.5 times the interquartile range; any individual points are outliers.

It is clear from the present study that the walnut seedlings did respond to the increased nitrogen through an increase in both soluble nitrogen content and CCI of the foliage. Higher foliar nitrogen content and CCI did not lead, however, to a corresponding increase in aphid population growth on seedlings in the greenhouse or aphid densities on mature trees in the field. Fertilization and nitrogen addition do lead to increases in many other aphids (Kytö *et al.*, 1996) and phloem feeding insects, as would be expected in the present study if walnut aphids were responding to nitrogen present in the leaves. It is possible, however, that the aphids may be more responsive to the presence of particular amino acids rather than to total soluble nitrogen (Douglas & van Emden, 2007). Although nitrogen fertilization has been shown to increase free amino acid concentration in phloem sap, as well as increase the soluble nitrogen content of foliage in at least some plants (Nowak & Komor, 2010; Ryan *et al.*, 2014), a key source of essential amino acids for aphids comprises their obligate symbionts (Douglas & van Emden, 2007; Shigenobu & Wilson, 2011; Hansen & Moran, 2014). Thus, the population growth rate of *C. juglandicola* may still have been limited by the availability of symbiont-produced amino acids even when levels of soluble nitrogen and perhaps free amino acids increased in the phloem sap. Because amino acids in the phloem were not measured directly in the present study, further research will be needed to determine whether

Figure 4 The absence of a relationship between average aphid density per leaf and average chlorophyll content index of leaves from mature trees in organic and conventional commercial walnut orchards. Each point represents a block within an orchard ($n=128$).

specific amino acids could limit walnut aphid population growth and thus densities in the field.

The effect of nitrogen fertilization of trees on aphid population growth has been variable, although generally positive (Kytö *et al.*, 1996). More recent work on peaches, however, has shown that, at very high nitrogen levels, aphid populations declined (Sauge *et al.*, 2010). Aphids appear to be able to tolerate a range of nitrogen content in their host trees (Dixon, 1998), although they suffer when nitrogen levels are either below or above the normal range, similar to aphids on non-woody plants (Zehnder & Hunter, 2009; Hosseini *et al.*, 2010). Higher levels of nitrogen than those used in the present study might eventually cause negative effects on the population growth of *C. juglandicola*; however, they would not be relevant to walnut production because the ranges of CCI readings and foliar nitrogen content of our walnut seedlings were similar to those observed in the field (Fig. 2).

Nitrogen is not always a limiting nutrient or the only limiting nutrient for insect herbivores because phosphorus can also be limiting (Sterner & Elser, 2002). The nitrogen : phosphorus ratio, moreover, may play a regulating role for some insects, possibly causing populations to decline when there are nutrient imbalances (Zehnder & Hunter, 2009). The lack of any relationship between aphid population growth and the soluble nitrogen : phosphorus ratio of the foliage of the walnut seedlings used in the present study suggests that nutrient imbalance in the walnut seedlings did not affect the performance of *C. juglandicola*, although it does not exclude the possibility that phosphorus may have been a limiting nutrient for the aphid at all nitrogen levels used in the greenhouse study. A study that directly manipulated phosphorus, as well as nitrogen would be needed to determine whether phosphorus could be a limiting nutrient for *C. juglandicola*, and could further illuminate the importance of the nitrogen : phosphorus ratio for this aphid.

It is also possible that plant defence, rather than plant nutrition, was responsible for the apparent absence of bottom-up effects on walnut aphid populations in the present study because it has been shown that plant defence can vary with level of nitrogen fertilizer (Herms & Mattson, 1992; Koricheva *et al.*, 1998; Behmer, 2009; Sauge *et al.*, 2010). Nitrogen has been shown to alter both

the quantity and quality of defensive compounds (Olson *et al.*, 2009; Sauge *et al.*, 2010). Although chemical defence was not measured in the present study, walnut foliage is known to contain juglone, which is toxic to many insects (Daglish, 1950; Piskorski *et al.*, 2011). Because juglone does not contain nitrogen (Daglish, 1950), it would not be expected to be directly influenced by increased available nitrogen; we cannot, however, exclude the possibility of an indirect linkage between nitrogen and phenolic defences because higher levels of nitrogen could also increase carbon by allowing for extra photosynthetic tissue (Vannette & Hunter, 2011). Being specialists, walnut aphids may even benefit from juglone. Further study of defensive compounds and their relationship to fertilizer application and insect populations is needed to determine whether plant defences could drive the dynamics of walnut aphid populations.

The lack of difference in either aphid density or foliar CCI between organic and conventional walnut orchards was unexpected because Berry *et al.* (2002) found organic farms to be generally nitrogen limited. Conventional orchards use urea fertilizer, whereas organic orchards use poultry manure. Because the orchards showed no difference in foliar CCI or aphid densities despite any differences in management practices and nitrogen sources, we did not explore the differences further for the present study. As is the case with many field studies, there were many factors potentially affecting aphid density on mature, commercial trees. By including the hierarchical structure of the random effects from the sampling design into our mixed effects models, we were able to show that, collectively, they explained 47% of the variation in aphid density, which allowed us to more effectively address the minimal additional effects of foliar nutrient status and type of orchard management on aphid densities. These results are consistent with our finding that nitrogen does not appear to be the driving factor behind walnut aphid population growth.

The results from the present study suggest that bottom-up effects from fertilizer management did not affect walnut aphid populations as a result of changes in nitrogen either on seedlings in the greenhouse or on mature trees in the field. Thus, it appears unlikely that fertilizer management could contribute effectively to the integrated management of *C. juglandicola* in walnut orchards. In view of this, it is important to maximize the role of top-down control by natural enemies, such as *T. pallidus*, in the management of walnut aphid populations. The recent switch to reduced-risk pesticides for the control of primary pests, such as codling moth *Cydia pomonella* (L.) (Lep.: Tortricidae), may have destabilized the management of secondary pests, such as walnut aphid, by suppressing parasitoid activity but not killing aphids (Jones *et al.*, 2010). Thus selectivity, in the choice of pesticides used in walnuts, aiming to focus on materials that are less harmful to *T. pallidus*, may be more effective in restoring the top-down control of *C. juglandicola* by *T. pallidus* than management of nitrogen fertilizer input.

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