

# Functional Ecology

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**TITLE:** Excessive nutrient input induces an ecological cost for aphids by modifying their attractiveness towards mutualist ants.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Data will be permanently archived in the Institutional Digital Repository of Universidad Nacional del Comahue, <http://rdi.uncoma.edu.ar/handle/uncoma/16831> (Lescano et al., 2022)

#### **AUTHOR CONTRIBUTIONS**

All authors contributed to the conception and design of the study. MNL and CQ performed the experiment and collected the data. MNL and EB did the chemical analysis and interpretation of those results. The first draft of the manuscript was written by MNL and all authors commented on earlier versions of the manuscript. All authors read and approved the final manuscript.

**TITLE:** Excessive nutrient input induces an ecological cost for aphids by modifying their attractiveness towards mutualist ants.

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## ABSTRACT

1. Enhanced soil nutrient availability often favors herbivore performance by bringing the carbon:nutrient ratio of plants closer to herbivore requirements. However, a surplus of nutrients can promote a too low carbon:nutrient ratio in plants, making them of poor quality for herbivores. In addition, increased soil nutrients can trigger cascading effects altering higher trophic levels, resulting in indirect costs for herbivores.
2. Through experiments under three increasing fertilization levels (unfertilized, NPK-rich, and 2NPK-rich soils), we studied how the enhancement of soil nutrient availability, by modifying the C:N ratio of thistles, affects the performance and homeostatic response of the aphids, and the consequent ant attraction.
3. Fertilized soils increased the biomass and reduced the C:N ratio of thistles, also increasing aphid abundance. The stoichiometric homeostasis of aphids was modulated through changes in honeydew production and composition; fertilization treatments reduced by more than half the quantity of honeydew secreted and lead to 2.5 to 6.4 times higher honeydew N concentration compared with the unfertilized treatment. In addition, in the highest fertilization treatment, the aphids increased the content of uric acid (a waste toxic compound involved in amino acid deamination) excreted in their honeydew. Aphid-infested thistles had the highest number of aphid-tending ants when they grew on intermediate rich-substrates. Ants selected honeydew with a lower C:N ratio (compared to unfertilized plants), but fewer workers patrolled plants with the highest fertilization treatment likely due to increased uric acid in the honeydew.
4. We showed that enhanced soil nutrients brought plant C:N ratio closer to aphid requirements, enhancing their performance and promoting ant attendance. But a disproportionate increase in fertilization did not further improve aphid performance

while it decreases the attraction of protective ants, which would make aphid populations more vulnerable to attack by natural enemies, inducing an ecological cost.

5. This study highlights the complex role of bottom-up cascading effects triggered by increases in soil nutrient availability and the importance of evaluating not only the physiological and population cost and benefits of it but also the ecological ones; especially when it alters mutualistic interactions.

**Keywords**

ants; aphids; bottom-up cascades; ecological stoichiometry; fertilization; knife-edge hypothesis; trophic interactions

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## RESUMEN (Second Abstract)

1. Un aumento en la disponibilidad de nutrientes del suelo, al disminuir el desbalance elemental (carbono:nitrógeno:fosforo, C:N:P) entre plantas y herbívoros, puede favorecer el rendimiento de los herbívoros. Sin embargo, un excesivo incremento de nutrientes podría resultar desfavorable si promueve una relación C:N:P de las plantas demasiado baja respecto de la requerida por los herbívoros. Estos cambios podrían, además, desencadenar efectos en cascada ascendente que alteren los niveles tróficos superiores, lo que podría implicar un costo indirecto para los herbívoros.
2. En experimentos de invernadero, utilizando tres niveles crecientes de fertilización (sin fertilizar, 1 dosis de NPK, 2 dosis de NPK), estudiamos cómo el aumento de la disponibilidad de nutrientes en el suelo, al modificar el balance C:N de los cardos, afecta el rendimiento y la respuesta homeostática de los pulgones, así como la atracción de hormigas melívoras hacia plantas con pulgones.
3. Los cardos que crecieron en suelos fertilizados tuvieron mayor biomasa, menor relación C:N y mayor abundancia de pulgones que los cardos en suelos sin fertilizar. La homeostasis estequiométrica de los pulgones fue modulada a través de cambios en la producción y composición de su melaza: la adición de fertilizante redujo a más de la mitad la producción de melaza y produjo un aumento en la cantidad de N excretado en la melaza. El tratamiento 2NPK

generó un aumento en la cantidad de ácido úrico (un compuesto tóxico de desecho que interviene en la desaminación de los aminoácidos) presente en la melaza. Los cardos con pulgones que crecieron bajo fertilización intermedia tuvieron la mayor abundancia de hormigas melívoras. Esto podría ser explicado por las preferencias de las hormigas: melaza con menor C:N pero también menor cantidad de ácido úrico.

4. Demostramos que el aumento de los nutrientes del suelo disminuye la proporción C:N de las plantas, mejorando el rendimiento de los pulgones y promoviendo su atracción hacia hormigas melívoras. Dado que las hormigas protegen a los pulgones del ataque de sus enemigos naturales, el aumento desproporcionado de la fertilización, al generar una disminución de la atracción de las hormigas hacia plantas con pulgones, podría inducir un costo ecológico para los pulgones.
5. Este estudio pone de manifiesto el complejo papel de los efectos en cascada ascendentes desencadenados por el aumento de la disponibilidad de nutrientes en el suelo, y destaca la importancia de evaluar no sólo los costos/beneficios fisiológicos y poblacionales sino también ecológicos; especialmente cuando involucra interacciones mutualistas.

## INTRODUCTION

Constraints on herbivore growth and reproduction due to nutrient concentrations can occur at both ends of a resource gradient (Sterner & Elser, 2002; Boersma & Elser, 2006). On one hand, herbivores demand large amounts of nutrients other than carbon to sustain their vital functions (particularly nitrogen (N) and phosphorus (P) are the two most important and critical limiting nutrients, Fagan et al., 2002; Cross et al., 2003, Elser et al., 2007), but autotrophs are usually poor in these nutrients (Elser et al., 2003; Frost et al., 2005). Herbivores, therefore, need to use different mechanisms to compensate for the low quality (i.e., high C:N:P ratios) of their diet, to sustain their stoichiometric homeostasis (Sterner & Elser 2002; Sitters et al., 2017), which restrict herbivore performance due to their energy demand. On the other hand, when N and/or P are in excess, herbivores also need to expend energy associated with the energetic and toxicity costs of metabolizing them (Piper et al., 2017). The penalty associated with these mechanisms may explain the reduced growth and reproduction of herbivores in response to excess nutrients (Anderson et al., 2005; Boersma & Elser, 2006). This reduced performance towards the extremes of the food quality gradient (C:N:P ratios) has been termed the stoichiometric knife-edge hypothesis (Boersma & Elser, 2006) and is supported by some empirical results that showed a maximal growth rate of herbivores at intermediate food nutrient concentrations (e.g., Morehouse et al., 2013; Elser et al., 2016). Despite this concept having been tested not only for herbivores but also for animals of higher trophic levels (e.g., omnivores, Bullejos et al., 2014; predators, Laspoumaderes et al., 2015), most evidence comes from aquatic systems and is focused on P availability (Hessen et al., 2013). Thus, there is a need for additional evidence of the knife-edge hypothesis, in particular from terrestrial systems and focusing in N intake.

The stoichiometric knife-edge hypothesis likely plays a major role in terrestrial systems, although it has been historically assumed that soil N additions will linearly increase

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herbivore performance (Matson, 1980; Mayntz et al., 2005). Although mean carbon:nitrogen ratio (C:N ratio) of terrestrial plants is more than threefold higher than for freshwater autotrophs, herbivorous insects and zooplankton had similar mean C:N ratios (Elser et al., 2000; Sterner & Elser 2002). This suggests that terrestrial herbivores experience a higher degree of N limitations than zooplankton (Sardans et al., 2012; Lemoine et al., 2014); so that, excess of fertilization in terrestrial systems, by resulting in high-N plants, could be detrimental to herbivores as they are specially adapted to extremely poor-N diets. Recent evidence supports this idea; for example, the size and survival of a dominant locust from north Asian grasslands decreased strongly with N fertilization (Cease et al., 2012), and the population growth rate of *Aphis nerii* was highest at intermediate levels of N additions in growth chamber experiments (Zehnder & Hunter, 2009). However, behavioral and physiological mechanisms associated with herbivore performance under different levels of soil fertilization and how it affects higher trophic levels are relatively understudied.

Given the complexity of food webs, research on interactions involving three or more trophic levels is more realistic than the traditional bitrophic interactions (Tschamntke & Hawkins 2002) and has the benefit to shed light on the complex impact of changes in soil nutrients at the community level. Particularly, plant-aphid-ant interactions are an excellent terrestrial system to study whether different availability of soil N, by altering the C:N ratio of plants, directly modify herbivore performance and/or indirectly impact on higher trophic levels. First, aphids are herbivores particularly specialized in low nutrient foods (phloem feeders) and may be sensitive to enhanced plant N availability because they face a great asymmetry between the N content of their tissues and those of the phloem sap they eat (Sterner & Elser, 2002; Douglas, 2006). Second, one adaptation of aphids to their poor-quality diet is to ingest a large quantity of sap to obtain adequate amounts of limiting N and other nutrients (Douglas, 2006); this results in an excess of carbohydrates, certain amino



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acids, and water that are excreted in the form of honeydew. Thus, the aphid regulation in the face of imbalanced C:N ratios in their food can be easily assessed by analyzing honeydew quantity and composition (proven for other phloem feeders, e.g., Bi et al., 2001). Third, indirect impacts on higher trophic levels are manifested through the patrolling of ants on a plant, given that honeydew is a valuable food resource and its quantity and quality determine the degree of ant attraction to a host plant with aphids (Blüthgen & Fiedler, 2004). Finally, during their patrolling activities, ants protect the aphids and host plants from other arthropods (Floate & Whitham, 1994; Styrsky & Eubanks, 2010; Devegili et al., 2020). Thus, increased ant attraction leading to greater numbers of ants patrolling an aphid infested plant may benefit both aphid and plant performance.

Changes in honeydew excretion allow aphids to deal either with low or high plant C:N ratios (Sterner & Elser, 2002; Douglas, 2006). Under high plant C:N ratios, as mentioned before, aphids ingest large quantities of sap to obtain adequate amounts of limiting nutrients; and thus, produce large quantities of honeydew. On the other hand, when aphids face N-enriched plants, one possibility is that they excrete less quantity of honeydew because they need to process lower volumes of phloem to cover their elemental demand. Another possibility is that aphids diminish their effort to retain N because this nutrient is more available; so that, honeydew N concentration increases (i.e., lower C:N). These scenarios not only may directly increase aphid performance (due to lower physiological costs) but also can indirectly favor aphid population growth as they become more attractive to ants (Blanchard et al., 2019). However, it is also possible that if the soil nutrient enrichment causes plant C:N ratio to drop below the stoichiometric knife-edge maximum (i.e., excess of N), aphids will need to excrete the additional N through their honeydew, partly as free amino acids but also in the form of uric acid, a waste toxic compound involved in amino acid deamination (Jennings et al., 2018). Because aphids are specially adapted to process low-quality food

(Boersma & Elser, 2006), the metabolic cost of dealing with excess N can be extremely high (Weihrach & O'Donnell, 2021) and impact negatively on their fitness. Moreover, the excretion of excess N by aphids, by altering the honeydew quality, could negatively affect the capacity of ants to digest this honeydew and thereby decrease ant attraction and defense service. Thus, by modulating the honeydew quality and quantity, changes in soil nutrient availability may indirectly affect aphid population growth through their impact on the attraction of aphid-tending ants; evidencing that changes in soil nutrients have consequences that may scale up to higher trophic levels.

In Patagonian steppes, the exotic thistle *Carduus thoermeri* is a naturalized plant species with an important role for the local insect assemblage as is one of the main host plants for the mutualism between native ants and aphids (Lescano & Farji-Brener, 2011). Moreover, it naturally grows in substrates of varying quality: from nutrient-poor steppe soils to nutrient-rich refuse dumps, which are organic waste piles accumulated on the soil surface by the leaf-cutting ant *Acromyrmex lobicornis* (Farji-Brener & Ghermandi, 2008). Field studies showed that refuse dumps have a positive direct effect on thistle performance and an indirect and positive effect on aphid density and aphid-tending ant abundance (Farji-Brener et al., 2009; Lescano et al., 2012). However, the physiological mechanisms behind this pattern remain poorly explored. Here, we performed a common garden experiment to study how the enhancement of soil nutrient availability, by modifying the C:N ratio of thistles, affects the homeostatic response of the aphids that colonize them, and through this, modify aphid population growth as well as ant attraction and behavior. For this purpose, we carried out experiments with thistles, aphids, and aphid-tending ants, under three increasing fertilization levels to test whether linear or non-linear responses to soil nutrient enrichment could arise at the herbivore and/or the third trophic level. Based on the stoichiometric knife-edge hypothesis we predict a unimodal response at both trophic levels. In other words, we expect

that increased soil nutrients, by diminishing the C:N imbalance between aphids and plants, would increase aphid performance (i.e., higher growth rate) and honeydew quality (i.e., lower C:N ratio). However, further increase in soil nutrient availability, would decrease aphid performance and/or the quality of their honeydew, decreasing ant attraction. Thus, we expected that aphid-tending ants would be more attracted by aphid-infested plants from the intermediate level of nutrient enrichment.

## MATERIALS AND METHODS

### *Study system*

The Northwestern Patagonian steppes correspond to the Subandean district of the Patagonian phytogeographic province, which is characterized by grasslands or perennial grass steppes (Cabrera, 1971). The soils have little vertical development, and low organic matter and moisture content (Satti et al., 2003). However, the activity of the leaf-cutting ant *Acromyrmex lobicornis* generates nutrient-rich patches around their nest; these are considered islands of fertility because they contain 5-10 times higher nutrient levels, particularly N, and better water retention capacity than adjacent soils (Farji-Brener & Ghermandi, 2008).

*Carduus thoermeri* (Asteraceae) is one of the most common plants on the fertility islands of *A. lobicornis* (Farji-Brener & Ghermandi, 2008). This musk thistle is an exotic and biennial plant species very abundant in disturbed areas of Northwestern Patagonia. It responds very plastically to differences in soil nutrients, significantly increasing aboveground biomass, leaf area, number of inflorescences, and investing more in physical defenses (i.e.; leaf toughness and spines) as well as increasing leaf N content (Lescano et al., 2019).

Although it is considered a serious weed in the invaded habitats, *C. thoermeri* has currently an important role in the community since it facilitates the abundance of native insect fauna

(Lescano & Farji-Brener, 2011). Among the aphid species reported colonizing this thistle in our area of interest, *Brachycaudus cardui* (Aphididae: Macrosiphini) is the most frequent one and its colonies are largely seen tended by ants (Lescano & Farji-Brener, 2011). One less common aphid associated with this thistle is *Capitophorus* cf. *carduinus* (Aphididae: Macrosiphini, hereafter “*Capitophorus*”). This aphid lives on the undersides of the lower leaves of thistles and thrives quite well in the absence of ants (Addicott, 1979), so its association with tending ants is not so common (Lescano, pers obs). In this study, three ant species were observed patrolling thistles and tending aphids: *Dorymyrmer tener*, *Camponotus distiguendus*, and *C. chilensis*. Although all of these ant species are ubiquitous, *D. tener* is numerically dominant in the region and one of the most frequent aphid-tending ant species on thistles (Pirk 2014; Lescano & Farji-Brener, 2011; Devegili et al., 2020) and, particularly, the most abundant species in the study site. All three ant species are opportunists with a generalized diet that includes honeydew, other insects, and spiders. Moreover, previous field observational and experimental studies in this system showed a decrease in aphid predation pressure and parasitism rates with increased ant activity (Lescano et al., 2012; Devegili et al., 2021); evidencing the active protective role of these ants to aphids.

### *Experimental design*

To determine the direct impact of increased soil nutrient availability on plant tissue traits, as well as the indirect effects on aphid density, aphid homeostatic response, and ant attraction, we performed a common garden experiment (we did not need permission for fieldwork). Second-year rosettes of *C. thoermeri* that had ~20-25 cm in diameter, 5-10 fully developed basal leaves and around 5 leaf buds in the rosette center were collected from nutrient-poor steppes soils in October 2017, transplanted into individual pots (volume 7 liters), and placed in a greenhouse where they grew under natural daylight and were watered

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as needed (N=44). We used NPK fertilizer (Nitrophoska 13:9:16, whose previous quantitative analyses indicated that it has approximately 12% nitrogen and 6.4% phosphorous; hence, 4.15 atomic N:P ratio) to carry out the experiment with three increasing fertilization levels. Thus, one month after the transplant, each pot was randomly assigned to one of three soil fertilization treatments: Control treatment (i.e., unfertilized nutrient-poor soils from the Patagonian steppe, n=15), NPK-rich soil (i.e., soil enriched with 5 gr of NPK, to emulate the average N content from refuse dumps of *A. lobicornis*; Lescano et al., 2019, n=15), and 2NPK-rich soil (i.e., soil enriched with 10 gr of NPK, to test any possible saturation threshold in the cascading effects as in nature it is possible to find refuse dumps extraordinarily enriched in N, with up to 14.8 g/kg N; Tadey & Farji-Brener, 2007, n=14). Pots were fertilized at 2-week intervals to total levels of 5 and 10 g of NPK (i.e., the total amount of N and P added per pot were 0.65 g and 0.32 g, respectively, for pots from NPK-rich treatment, and 1.3 g and 0.64 g, respectively, for pots from 2NPK-rich treatment). Greenhouse ventilation allowed entry of two aphid species, *B. cardui* and *Capitophorus*, which began colonizing plants from naturally occurring local populations 3-weeks after fertilization.

#### *Plant traits and observational measurements of aphids in focal plants*

In mid-December (spring-summer transition), we recorded several plant traits, such as maximum height, number of leaves, and number of inflorescences per plant. In addition, in a subset of six plants per treatment, we collected fully developed new leaves to assess carbon and nitrogen content and their C:N ratio (see chemical analysis). We also estimated the abundance of each aphid species per plant and its honeydew production. Aphids have mainly parthenogenetic reproduction and, thus, colonies are form mainly by wingless asexual females, born on the same host plant where they feed and have rapid population growth (Sullivan 2004). Thus, aphid abundance within a plant is a good proxy for aphid population

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growth and performance. For aphid abundance, we estimated the percentage of plant surface covered by individuals of each species, according to the following visual categories: 0 (0%), 1 (<5%), 2 (5–10%), 3 (10–25%), 4 (25–50%), 5 (50–75%), and 6 (>75%). For statistical analysis (see below), this level of aphid abundance was expressed as the approximate abundance using the maximum percentage of individuals per category. To collect honeydew, we put pre-weighted 5 cm<sup>2</sup> round aluminum foil disks below a defined sub-group of each aphid species for 5 days; for a total of 2 to 4 disks per aphid species/plant.

On the fifth day, disks with honeydew were removed, reweighed, and freeze-dried for posterior analysis. For each aphid species, we calculated the quantity of honeydew secreted as the difference between the final and initial weights (mg) of each disk, averaging the values of those 2–4 disks per plant to obtain the mg of honeydew secreted per aphid species per plant. Then, to obtain a parameter of honeydew production independent of aphid colony size, we divided mg of honeydew secreted per aphid species and plant by their aphid abundance and the number of days the foiled disks were collected (mg of honeydew per individual per day = honeydew production per aphid species and plant). Because aphids can move within the plants, some disks collected a mix of honeydew from both aphid species; but, those were discarded for future analyses. We only used those disks that were placed under a sub-group of a particular species, and on each day of collection, it was confirmed that there was no mixture of aphid species above it. Hence, the number of replications depended on the number of plants with disks that collected honeydew of each aphid species separately (N= 26 for *B. cardui* and N= 36 for *Capitophorus*).

#### *Chemical analysis*

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To test whether soil fertilization treatment affected the nutritional composition of plant tissues, aphid's body, and honeydew, we collected and analyzed samples in a sub-set of thistles per treatment (plants: N= 4, aphids: N=5, disk: N=6). For plant carbon and nitrogen analysis, 2-3 fully expanded new leaves per plant were dried at 60° C for 48 h up to constant weight, then crushed with mortar and pestle into a fine powder, and approximately 2–3 mg of dry weight was put into tin capsules weighed and analyzed with a suitable analyzer (Flash EA 1112; Thermo Finnigan, Milan, Italy). For aphid's body carbon and nitrogen analysis, we collected between 10 and 15 adult individuals per species/plant, which were washed with distilled water, dried and weighed, placed in tin capsules, and analyzed in the same way. For aphid's honeydew carbon and nitrogen content, one disk per aphid species/plant was defrosted and put into a tin capsule to be analyzed in the same way.

Given that the primary N-containing metabolite excreted by insects is uric acid (O'Donnell & Donini, 2017) we tested whether the two fertilized treatments affected the content of uric acid in the honeydew of *B. cardui*. To do this, we used a Uric Acid Assay Kit (Uricostat enzymatic AA, from Wiener lab) for detecting uric acid in biological samples. Briefly, honeydew samples were resuspended in 100  $\mu$ L Milli-Q water, and the reaction mix and standards were added, incubated for 20 min at 20°C, and then measured the absorbance with a double beam spectrophotometer (Shimadzu UV2450) at 505 nm. We analyzed 4 sub-samples of this species for each treatment.

#### *Foraging activity of ants*

To evaluate whether soil fertilization treatments indirectly influence ant attraction to aphids, we moved some pots with aphid-infested thistles (i.e. those plants in good condition before they begin to senesce, N=32) into a 1.5 x 5 m open common garden where the aphid-

tending ants were allowed free access to the pots. All pots were exposed to similar environmental conditions and the treatments were distributed randomly in the experimental area. After two days, we estimated the ant attraction to plants with aphids by counting the number of aphid-tending ants per plant three times (early morning, noon, and afternoon), and then we averaged the three counts. Also, we divided the average number of ants by the total number of leaves per plant to assess the influence of soil nutrient treatment upon ant attraction independently of plant size.

To investigate whether the attraction of the ant *Dorymyrmex tener* to aphid honeydew depend on soil fertilization treatment, we set up a choice experiment. Defrosted *B. cardui*'s honeydew disks were offered under a paired comparison design to an experimental colony of *D. tener* (one pair at a time, in all possible combinations: Control vs. NPK-rich treatments, Control vs. 2NPK-rich treatments, and NPK-rich vs. 2NPK-rich treatments). After one minute of habituation, we count the number of ant workers in each one of the disks. In total, we used four *D. tener* experimental colonies and each combination was replicated 2-3 times per colony on different days.

#### *Data analysis*

We compared plant performance traits (height, number of leaves, and C:N ratio) among the three soil fertilization treatments using one-way analysis of variance (ANOVAs), with fertilization treatment included as a fixed factor with three levels (control, NPK-rich, and 2NPK-rich). We checked ANOVA assumptions before analyses, and response variables were transformed when necessary to meet ANOVA assumptions. The number of inflorescences failed to meet the normality assumptions so a Kruskal–Wallis test, followed by a post hoc Dunn's test was applied.



Differences in aphid abundance for each aphid species among soil fertilization treatments were tested using Kruskal-Wallis tests followed by post-hoc Dunn's test. Variation in additional aphid variables (honeydew production, body C:N ratio and C:N ratio of honeydew) among the soil nutrient treatments, were assessed using one-way ANOVAs; one for each aphid species. In all cases, the soil fertilization treatment was considered as a fixed factor, and data were transformed when necessary to meet ANOVA assumptions. To compare the amount of uric acid in *B. cardui* honeydew between NPK-rich and 2NPK-rich treatments we used Mann-Whitney test.

Finally, to assess whether ant attraction to plants with aphids depended on the different soil substrates in which plants grew, we used one-way ANOVA, using soil fertilization treatment as a fixed factor. The response variables were total ant abundance per plant and average ant abundance/ total number of leaves per plant, a logarithmic transformation was performed to normalize the data for analysis. For the choice experiment, paired *t*-tests were used to compare the ant preferences between the aphid honeydew from different soil substrates (paired disk offered to four *D. tener* colonies), being the response variable the proportion of ant feeding on each disk.

## RESULTS

### *Plant traits and observational measurements of aphids in focal plants*

Plants height was similar among the three soil fertilization treatments ( $F_{2,41}=0.259$ ,  $P=0.773$  Figure 1A), but thistles grown in NPK-rich and 2NPK-rich treatments showed approximately 1.8 times more leaves and 2.5 times more inflorescences than plants grown on unfertilized pots (Figure 1 B-C; leaves:  $F_{2,41}=14,3338$ ,  $P<0.0001$ ; inflorescences:  $H=26.85$   $df: 2$ ,  $P<0.0001$ ). In the same way, both enriched substrates led to a lower plant leaf C:N

ratio compared with leaves of plants grown on unfertilized soils (C:N ratio,  $F_{2,9}=41.6$ ,  $P < 0.0001$ ). Specifically, we observed a 70 % and 78 % decrease in plant C:N ratio in NPK-rich and 2NPK-rich, respectively, compared with control plants (Figure 1 D). This reduction in leaf C:N ratios of fertilized plants is explained by the increase in leaf N content by 3.27 and 4.1 times higher in plants from NPK-rich and 2NPK-rich pots, respectively, than in plants from unfertilized pots ( $F_{2,9}=37.45$ ,  $P < 0.0001$ , Appendix S1: Table S1). In turn, no significant difference between NPK-rich and 2NPK-rich treatments was observed for any of these plant traits (Figure 1).

All experimental plants were colonized by both aphid species, *Brachycaudus cardui* and *Capitophorus*. However, the substrate on which thistles grew influenced the abundance of both aphid species (*B. cardui*:  $H=12.58$ ,  $df:2$ ,  $P < 0.05$ ; *Capitophorus*:  $H=32.55$ ;  $df:2$ ,  $P < 0.0001$ , Figure 2 A, B). Particularly, for *B. cardui*, plants growing on enriched soils supported 3.8-fold higher abundance than plants in unfertilized soils (with no significant difference between the two enriched soil treatments) (Figure 2A). In turn, for *Capitophorus* the response was stronger, with thistles growing on NPK-rich and 2NPK-rich soils supporting 6.7- and 6-fold higher abundances, respectively, than those in unfertilized soils (Figure 2B).

Honeydew production ( $\text{mg of honeydew ind}^{-1} \text{ day}^{-1}$ ) in both aphid species depended on the soil fertilization treatment (*B. cardui*:  $F_{2,23}=3.61$ ,  $P < 0.05$ , Figure 2C; *Capitophorus*:  $F_{2,33}=20.35$ ,  $P < 0.00001$ , Figure 2D), but the effect was stronger for *Capitophorus*. Specifically, individuals of *B. cardui* reared on NPK-rich plants secreted ~65% lower volume of honeydew than those reared on unfertilized plants. In 2NPK-rich plants, honeydew secreted by *B. cardui* was 55% less than in control plants, but with no significant difference from neither unfertilized nor NPK-rich plants (Figure 2C). In turn, *Capitophorus* reduced by 80 % the amount of honeydew when fed on plants from both fertilization treatments as compared to the quantity produced on unfertilized plants (Figure 2D).

### *Aphid-chemical analysis*

The body C content of *B. cardui* depended on the soil fertilization treatment ( $F_{2,12} = 7.86, P < 0.01$ ; Appendix S1: Table S1); individuals had lower body C content in both NPK and 2NPK treatments compared with individuals from unfertilized treatment. Instead, its body N content was similar among the three soil fertilization treatments ( $F_{2,12} = 0.02, P = 0.97$ ; Appendix S1: Table S1). Thus, this differential change in C resulted in *B. cardui* having a significantly lower body C:N ratio in fertilized than unfertilized treatments ( $F_{2,12} = 18.20, P < 0.001$ ; Figure 3 A). The N content of honeydew from *B. cardui* reared on NPK-rich and 2NPK-rich plants was 3.3 and 6.4 times higher, respectively, than honeydew from unfertilized plants ( $F_{2,15} = 16.48, P < 0.001$ ; Appendix S1: Table S1). Thus, individuals reared on both NPK and 2NPK rich-plants secreted honeydew of lower C:N ratio than those on control plants (honeydew C:N ratio, unfertilized:  $351 \pm 28$ ; NPK:  $114 \pm 18, 70$ ; 2NPK:  $70 \pm 11$ ;  $F_{2,15} = 54.78, P < 0.0001$ ). In addition to the tendency to secrete honeydew with higher N content, the nitrogen compounds differed between both fertilization treatments. We found 2.4 times more uric acid in honeydew of *B. cardui* from the 2NPK-rich than those from the NPK-rich treatment ( $U = -2.31, P < 0.05, N=4$ , Figure. 4). Finally, honeydew of *B. cardui* on 2NPK-rich treatments contained a slightly higher amount of C compared with those from NPK and unfertilized plants but it did not differ significantly among treatments ( $F_{2,15} = 0.037, P = 0.96$ ; Appendix S1: Table S1).

The body C and N content of *Capitophorus* did not differ between soil fertilization treatments (for carbon:  $F_{2,12} = 2.19, P = 0.15$ ; for nitrogen:  $F_{2,12} = 1.44, P = 0.27$ , Appendix S1: Table S1). Thus, these similar values of elemental contents resulted in relatively constant body C:N ratios of individuals reared under different soil fertilization treatments ( $F_{2,12} = 0.89, P = 0.43$ , Figure 3 B). For this aphid species, the C content of honeydew was similar between

the fertilization treatments ( $F_{2,15} = 0.94$ ,  $P = 0.41$ , Appendix S1: Table S1); instead, we have found that the N content of honeydew depended on the soil fertilization treatment ( $F_{2,15} = 6.86$ ,  $P < 0.01$ , Appendix S1: Table S1). The honeydew of *Capitophorus* had 2-3 times higher N content when individuals were reared on fertilized plants compared with those in unfertilized plants. Moreover, individuals of *Capitophorus* reared on plants from NPK-rich and 2NPK-rich treatments have a reduction of 66 % and 76% in honeydew C:N ratios, respectively ( $F_{2,15} = 11.98$ ,  $P < 0.001$ , Figure. 3 D), compared with individuals from the unfertilized treatment.

#### *Foraging activity of ants*

Three native tending-ant species were observed patrolling the aphid-infested thistles in the common garden plot, being *Dorymyrmex tener* the most abundant on plants, followed by *Camponotus distinguendus* and *C. chilensis*. The substrate on which plants grew influenced the total abundance of ants in aphid-infested thistles ( $F_{2,29} = 9.79$ ,  $P < 0.001$  4.51,  $P < 0.05$ ; Figure 5 A); the number of ants patrolling a plant increase with fertilization but the highest number of aphid-tending ants per plant was recorded in plants growing on the intermediate soil fertilization treatment (i.e., NPK-rich pots) and there was no difference between control and 2NPK plants. Independently of plant size, aphid-infested plants from the intermediate soil fertilization had the highest number of patrolling ants (unfertilized:  $0.11 \pm 0.01$ ; NPK:  $0.18 \pm 0.03$ ; 2NPK:  $0.07 \pm 0.01$ ;  $F_{2,29} = 4.51$ ,  $P < 0.05$ ). Accordingly, in the experimental choice arena, *D. tener* showed a marked preference for *B. cardui*'s honeydew disks from the NPK-rich versus disks with honeydew from the unfertilized treatment (paired  $t = -3.99$ ,  $P < 0.01$ ,  $N = 8$ ; Figure 5 B) or from the 2NPK-rich treatment (paired  $t = 3.40$ ,  $P < 0.05$ ,  $N = 8$ ; Figure 5 B). Instead, when we simultaneously offered disks from unfertilized and 2NPK-rich

treatments to the ants, both were equally harvested (paired  $t = -1.28$ ,  $P = 0.23$ ,  $N = 12$ ; Figure 5 B).

## DISCUSSION

Our results demonstrate that changes in soil nutrient availability, by modifying the extreme imbalance in C:N ratios between plants and herbivores, trigger bottom-up cascading effects as predicted by ecological stoichiometry theory (Sterner & Elser, 2002). But, the originality of our work lies in the fact that an excessive N input, although may not further benefit herbivore performance, decreases their attractiveness to protective mutualists. This would make herbivores more vulnerable to attack by natural enemies, suggesting an ecological cost for herbivores. In particular, we found that growing up in fertilized soils increases the biomass and the leaf quality of *C. thoermeri*, which leads to a greater abundance of aphids that colonize and feed on them. We also showed that aphids have a relatively strict stoichiometric homeostasis (with slight differences between species), and that it was achieved by significantly adjusting their honeydew production and composition. Specifically, fertilization treatments reduced by more than half the quantity of honeydew secreted and led to 2.5 to 6.4 times higher honeydew N concentration. Finally, these changes in honeydew quality and quantity also led to an indirect effect of soil nutrient enrichment on aphid-tending ant attraction. Aphid-infested thistles had the highest number of aphid-tending ants when they grew on intermediate fertilization substrates. Hence, ants preferred to collect honeydew with lower C:N ratios, like those from NPK and 2NPK rich plants; but honeydew from 2NPK-rich plants was less attractive than those from NPK-rich plants, likely due to the increase in uric acid. As a consequence, fewer workers patrolled 2NPK-rich plants which may increase the vulnerability of both aphids to natural enemies and plants to other, potentially more damaging, herbivores.

A more nutritious substrate is usually associated with enhanced plant performance, represented by either large biomass and more N content which usually improves herbivores' development rate, survival and/or fecundity, increasing their abundance (e.g., Rhainds & English Loeb, 2003; Perkinns et al., 2004; Huberty & Denno, 2006). However, the addition of nutrients like K or Na through fertilization and their consequent increase in leaf tissues may also enhance plant resistance to herbivores (Singh & Sood, 2007; Kaspari 2020). In this study, despite the use of a multinutrient fertilizer like NPK, we found no evidence of a negative effect on herbivores. In fact, our results support previous works in this system, showing that greater soil nutrient availability improves the quantity and quality of *C. thoermeri* tissues favoring herbivores that feed on them (caterpillars: Lescano et al., 2019; aphids: Farji-Brener et al., 2009; Lescano et al., 2012). Additionally, our results show the key role of stoichiometric theory in elucidating the mechanism behind this result.

According to ecological stoichiometry theory, we found that fertilized thistles supported 3.8 to 6.7 times higher abundance of aphids, *B. cardui* and *Capitophorus*, respectively— than thistles in unfertilized soils. However, we did not find significant difference in aphid abundance between the two enriched soil treatments, as we may expect if the double doses of soil fertilization result in an excessively high concentration of N in the plants. When elemental ratios of plants are really low (i.e., very high N and/or P content) herbivore performance or growth can be limited by carbon and/or affected by the deleterious effect of excessively high concentration of nutrients (Boersma & Elser, 2006; Zehnder & Hunter, 2009). This theory has been examined and proven mainly concerning P, as it can be explicitly linked to growth via the biosynthetic machinery (Hessen, 2013). But some evidence from terrestrial systems indicates that this stoichiometric knife-edge hypothesis would apply to other nutrients beyond P (e.g., Cease et al., 2012; Chesnais et al., 2016, Zhu et al., 2019). Based on that, we expected to find that excess N decreased aphid performance.

The lack of evidence of a decrease in aphid performance as plant fertilization increased beyond a certain limit, can be explained by three not mutually exclusive reasons. First, the imbalances in our extra fertilization treatment may have not been large enough to substantially affect aphid populations (indeed, we did not observe a proportional decrease in plant C:N ratio as fertilization increased, Figure 1 D). Second, aphids may have efficient physiological responses aimed at saving or disposing of elements in deficit or in excess, respectively, which was evidenced by their homeostatic responses to changes in plant C:N ratios (i.e., slightly variations in their body's nutrient balance despite the huge variations in the C:N of thistles). Finally, we may have not detected differences in the impact of NPK and 2NPK treatments on the aphid performance due to the usually unimodal responses of consumers to changes in food C:N ratios (Boersma & Elser, 2006). So, aphid performance for NPK and 2NPK treatments could be on either side of the maxima in their unimodal response to soil enrichment; which could be evidenced through changes in their post-digestive homeostatic regulation strategies (i.e., through changes in the volume and quality of honeydew secreted).

Honeydew production (mg of honeydew ind<sup>-1</sup> dia<sup>-1</sup>) by aphids reared on fertilized plants strongly diminished compared with those from unfertilized ones, and this was greater in *Capitophorus* (80%) than in *B. cardui* (65%). Also, we found that N content of honeydew increased as the C:N ratio of thistles decreased. These changes in the composition of honeydew may imply a reduction in the cost associated with both the excretion of excessive elements and/or the retention of scarce ones to maintain the aphid stoichiometric homeostasis (Sturner & Elser, 2002). Particularly, for each ml of honeydew secreted by *Capitophorus* in fertilized plants, individuals release 2 to 3 times more N than those individuals feeding on unfertilized plants; but for *B. cardui* such difference raises up to 3.4 to 6.6 times more N excreted when they feed on NPK and 2NPK-rich plants, respectively. When the carbohydrate

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content of food is relatively low, herbivores need to transform and/or excrete the extra nitrogen, by deamination of amino acids and the prevention of toxicity (Reed et al., 2017; Jennings et al., 2018), which produces uric acid as the main catabolite of this process. We found that *B. cardui*' honeydew from 2NK-rich plants had 2.4 times more uric acid than those from NPK-rich treatment, suggesting they are dealing with an excess of N in 2NPK-rich plants. Moreover, the changes that we observed in honeydew composition are consistent with some previous studies that showed that the nutritional status of plants affects the sugar and the amino-acid composition of aphid honeydew (e.g., Fischer & Shingleton, 2001, Pringle et al., 2014, but see Pringle et al., 2017). Therefore, it is expected that changes in honeydew be an important mechanism that allows aphids to deal with the nutritional imbalance of their food, regulating the excretion of elements in excess.

*Capitophorus* resulted more homeostatic than *B. cardui*, since the somatic C:N ratio remained more constant despite the changes in thistles elemental ratios due to fertilization. It could be expected that being more homeostatic, *Capitophorus* would produce more honeydew with a higher C:N ratio in the unfertilized treatment. Contrariwise to this expectation, *Capitophorus* excreted less amount of honeydew with a lower N content when thistles presented a higher C:N ratio. *Capitophorus* has a smaller body size (~ one-half that of *B. cardui*), so a less amount of honeydew ( $\text{mg ind.}^{-1} \text{ day}^{-1}$ ) should be expected. However, this does not explain the lower C:N ratio of the secreted honeydew. Respiration is one of the mechanisms used for the excretion of the excess carbon in the food, and it has been shown that respiration increases when the C:nutrient ratio of the food increases (Darchambeau et al., 2003). Although we did not measure respiration rates in our experiments, we can suggest that respiration had an important role in this elemental balance. Since smaller insects have higher mass-specific respiration rate (Ballesteros et al., 2018), we can expect that *Capitophorus* has



higher mass specific respiration rates (and thus more C lost through respiration) than *B. cardui*.

The highest fertilization used here (i.e., 2NPK-rich treatment) was neither deleterious for aphids, nor represented a benefit to them in terms of performance. Nevertheless, changes in honeydew quantity and quality at the highest fertilization level could imply an ecological cost. While collecting honeydew, ants protect aphids from predators and parasitoids and clean the colony of accumulated honeydew (Stadler & Dixon, 2008; Lescano et al., 2012; Devegili et al., 2020). Although some studies reported costs of ant attendance to aphids (e.g., Stadler & Dixon, 1999; Yao et al., 2000), most ant-aphid interactions are assumed to be mutualistic and it has been suggested that the strength of these interactions may be mediated by host plant quality (Breton & Addicott, 1992; Pringle et al., 2014). Our results showed that aphid-tending ants have a marked preference for honeydew of *B. cardui* reared on NPK-rich plants, which is consistent with the highest number of aphid-tending ants patrolling these plants. On the one hand, honeydew with a lower C:N ratio (i.e.; higher N concentrations) may be an attractive resource because ants are also N limited, as is usual for predatory arthropods (Fagan et al., 2002; Blüthgen & Fiedler, 2004). On the other hand, when the double dose of fertilizer was applied, the honeydew composition of *B. cardui* tended to be N enriched (with an increased proportion of uric acid, a waste toxic compound) compared to honeydew from NPK-rich plants; which could be a cause of the lower attraction of aphid-tending ants to plants in the 2NPK treatment. Moreover, we cannot exclude the role of other nutrients that can be in excess in the phloem sap of 2NPK-treated plants, that could have been ingested by aphids and then secreted as part of the honeydew (e.g., Rashid et al., 2016). Considering that we used a multi-nutrient fertilizer, future work should examine the effect of other macronutrients - besides C and N - (e.g.; K, Mg and S) in host plant quality, aphid honeydew composition, and the behavior of aphid-tending ants. Nonetheless, whether the effect is

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mostly driven by N solely or in combination with other macronutrients, fewer workers patrolling extra-fertilized plants may represent a higher vulnerability of aphids to natural enemies because the chances of ants to kill or expel aphid enemies is often positively correlated with the number of patrolling ants (e.g., Lescano et al., 2012). Besides ant abundance, the identity of the aphid-attending ant is also critical as species vary in aggressiveness (Clark & Singer, 2018; Devegili et al., 2020). In the studied system, *D. tener* is the species most frequently observed on aphid-infested thistles, both in this work and in the study area (Lescano et al 2012; 2014; Devegili et al., 2020). In addition, previous laboratory and field studies have shown that *D. tener* is one of the most aggressive species that effectively expels aphid natural enemies from thistles, and the abundance of their workers on the thistle is tightly related to the quality of its defense service (Devegili et al., 2020). Hence, this unimodal response in aphid-tending ant behavior highlights the ecological benefit for aphids feeding on N-enriched plants, but also suggests that, by decreasing ant attraction, there would be an ecological cost when the soil nutrient enrichment becomes in excess.

In sum, the increase of soil nutrient availability brings the plant nutritional quality closer to aphid requirements, enhancing aphid performance and promoting ant attendance. However, we also found evidence of a shift in the positive upward cascading effect, as a disproportionate increase in fertilization indirectly lead to diminished ant attraction to aphid-infested plants via physiological adjustments in honeydew composition (i.e., the aphids excreted the additional N by increasing the uric acid content of their honeydew). Lower abundance of patrolling ants would decrease the likelihood of driving out aphid enemies, which would make aphid populations more vulnerable to attack by their enemies. In addition, because the presence of tending-ants often decreases the abundance of other herbivorous insects on the host plant (Rosumek et al. 2009, Styrsky and Eubanks 2010), the ecological cost of excessive soil nutrients could also be costly for the host plant itself.

Understanding the bottom-up effects triggered by soil enrichment is particularly important because soil fertilization occurs naturally in several ecosystems, for example where nutrient-poor soils are enriched by the presence of “fertility islands” generated by the activity of ants (Lescano et al., 2012; Farji-Brener & Werenkraut, 2017) or other ecosystem-engineer animals (e.g., Jouquet et al., 2016; Le Bayron et al., 2017) or due to natural spatial variability in soil composition. Furthermore, human activities have strongly altered the biogeochemical cycles of key elements, such as C, N, and P via nutrient enrichment (Smith & Schindler, 2009; Peñuelas et al., 2012), so documenting the existence of a stoichiometric knife-edge at terrestrial ecosystems and with other nutrients besides P implies a strong potential impact of anthropogenic soil nutrient addition on plant-herbivore interactions and higher trophic levels (Elser et al., 2006; Zhou & Declerck, 2019). This study provides key insights on the complex role of bottom-up cascading effects triggered by increases in soil nutrient availability and highlights the importance of evaluating not only the physiological and population cost and benefits of it but also the ecological ones; especially when it alters mutualistic interactions.

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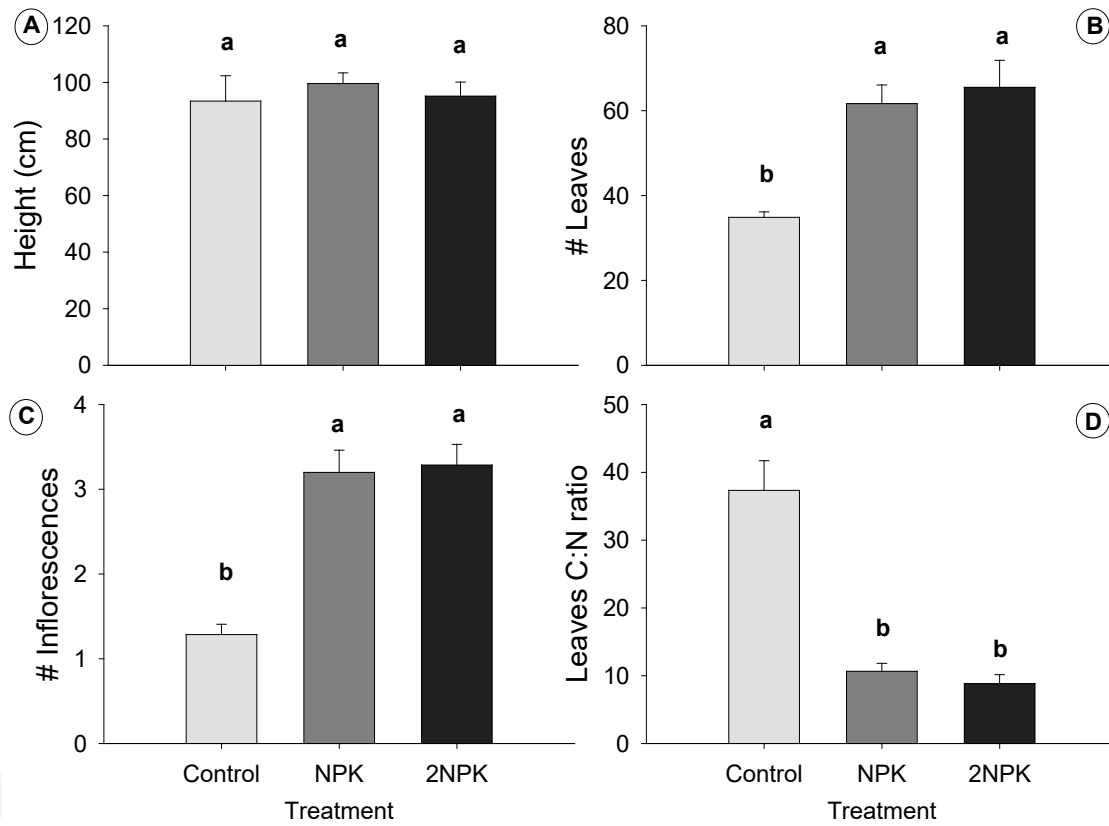
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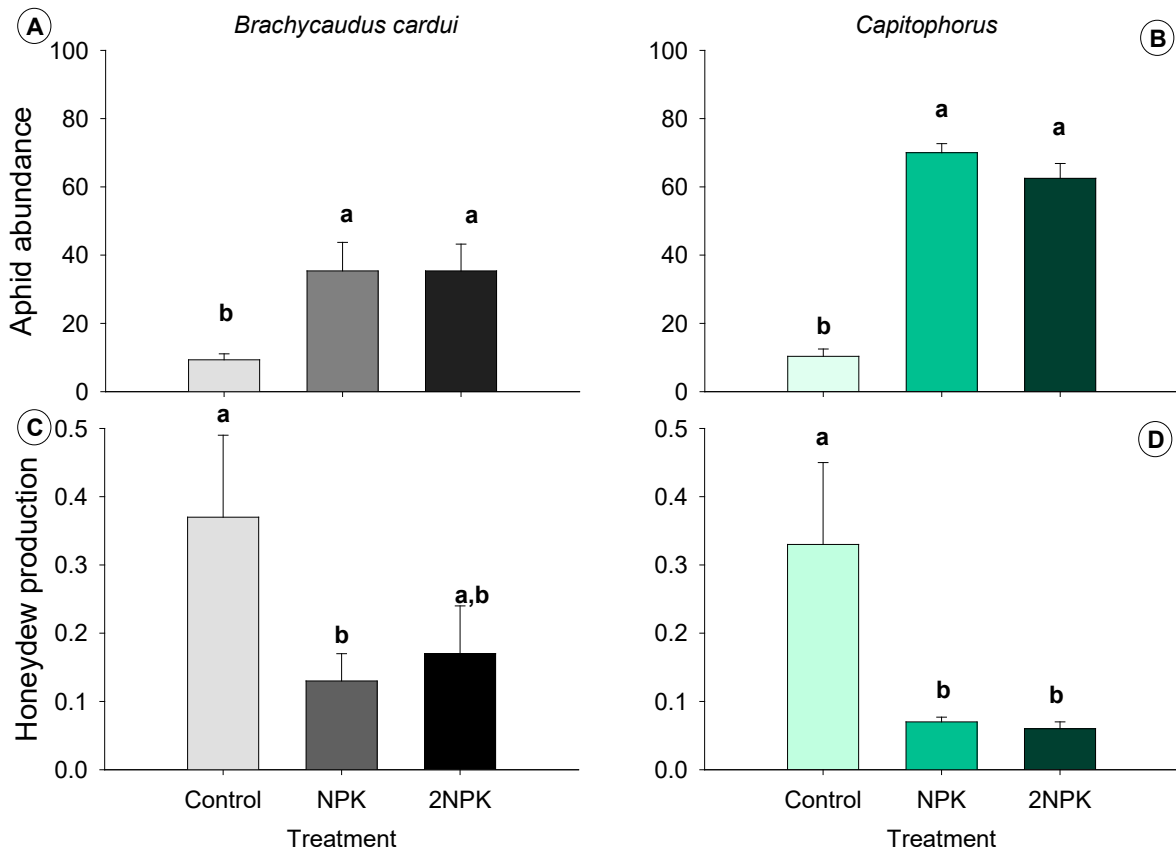
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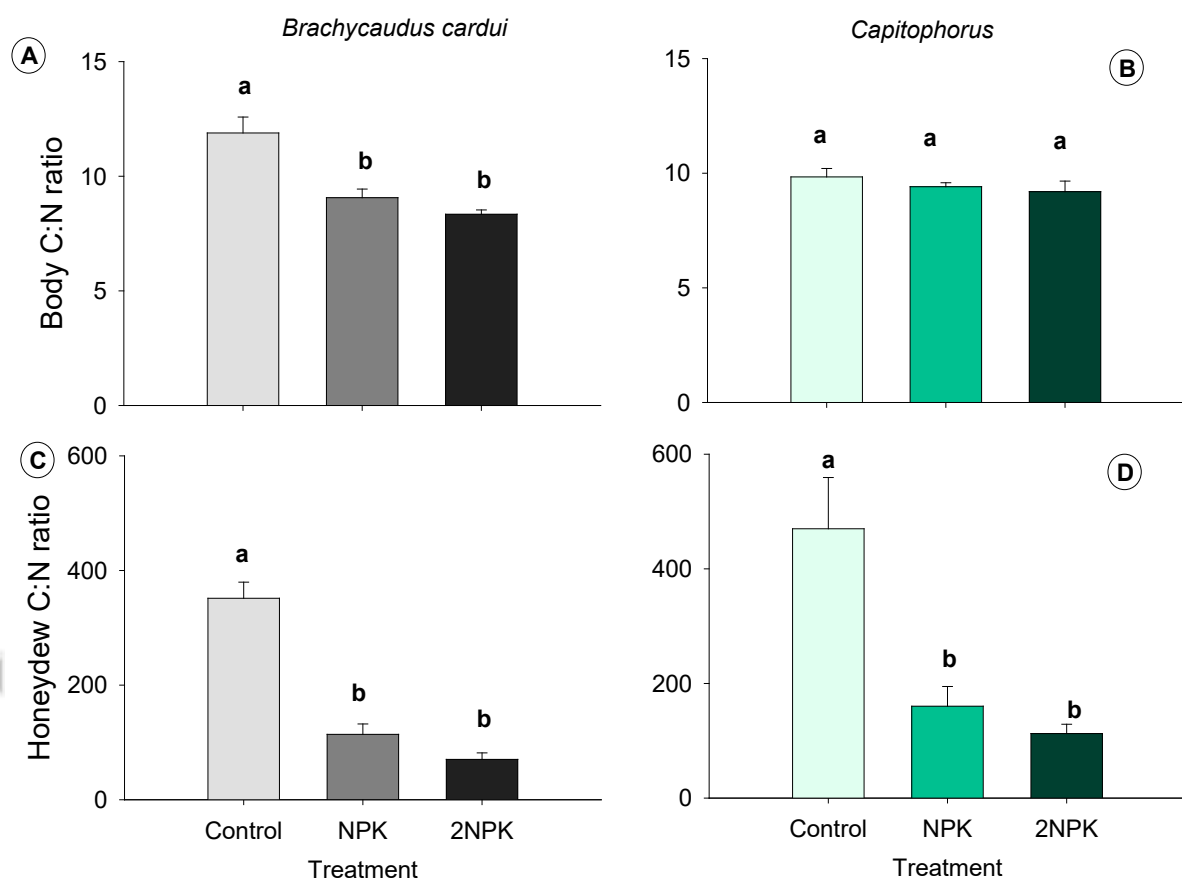
**Figure 1** *Carduus thoermeri* plant traits (mean  $\pm$  1 SE) growing on different soil fertilization treatments: control (unfertilized soil), NPK (soil fertilized with 1 dose of NPK), and 2NPK (soil fertilized with double dose of NPK). (A) Height, (B) Number of leaves, (C) Number of inflorescences, (D) C:N ratio. Different lower case letters indicate statistically significant differences between substrates ( $P < 0.05$ )



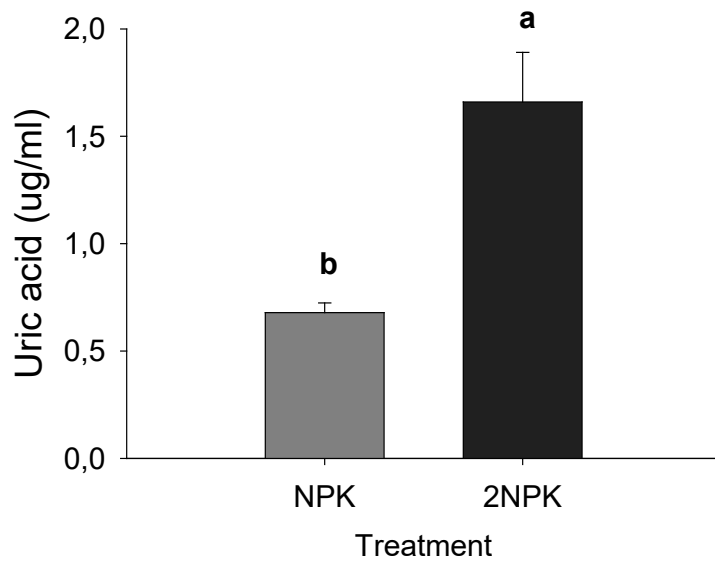
**Figure 2.** Mean ( $\pm 1$  SE) aphid abundance and honeydew production by each aphid species in plants growing on different soil fertilization treatments: control (unfertilized soil), NPK (soil fertilized with 1 dose of NPK), and 2NPK (soil fertilized with double dose of NPK). (A) and (B) Percentage of plants covered by individuals of *B. cardui* and *Capitophorus*, respectively. (C) and (D) mg of honeydew ind<sup>-1</sup> day<sup>-1</sup> produced by *B. cardui* and *Capitophorus*, respectively. Different lower case letters indicate statistically significant differences between substrates ( $P < 0.05$ )



**Figure 3.** Chemical analysis of aphids reared on plants under different soil fertilization treatments: control (unfertilized soil), NPK (soil fertilized with 1 dose of NPK), and 2NPK (soil fertilized with double dose of NPK) soils. (A) and (B) C:N ratio of the body tissue of *B. cardui* and *Capitophorus*, respectively. (C) and (D) C:N ratio of the honeydew secreted by *B. cardui* and *Capitophorus*, respectively. Different lower case letters indicate statistically significant differences between substrates ( $P < 0.05$ ).



**Figure 4.** Amount of uric acid in *B. cardui* honeydew from plants growing on different soil substrates: NPK (soil fertilized with 1 dose of NPK) and 2NPK (soil fertilized with double dose of NPK). Different lower case letters indicate statistically significant differences between substrates ( $P < 0.05$ ).



**Figure 5.** Mean ( $\pm 1$  SE) of number of ants foraging an aphid-infested plants, or *B. cardui* honeydew disks from plants reared on different soil substrates: control (unfertilized soil), NPK (soil fertilized with 1 dose of NPK), and 2NPK (soil fertilized with double dose of NPK). (A) Number of aphid-tending ants per plant. (B) Relative abundance of ants in each disk with honeydew, offered paired. Significant differences between the treatments are indicated with one asterisk ( $P < 0.05$ ) and two asterisks ( $P < 0.01$ ).

