RAFAEL STEMPNIAK IASCZCZAKI

INTERACTIONS BETWEEN AN EXTRAFLORAL NECTAR-FEEDING PREDATOR AND ANTS AND THEIR EFFECTS ON PEST CONTROL

Dissertation submitted to the Post Graduate Program of Entomology of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Angelo Pallini

Co-advisers: Arne Janssen
Madelaine Venzon

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“O que temer? Nada. A quem temer? Ninguém. Por quê? Por que aqueles que se unem a Deus obtêm três grandes privilégios: onipotência sem poder; embriaguez, sem vinho; e vida sem morte”.

(São Francisco de Assis)

ABSTRACT

IASCZCZAKI, Stempniak, Rafael, M.Sc., Universidade Federal de Viçosa, July, 2024. **Interactions between an extrafloral nectar-feeding predator and ants and their effects on pest control**. Adviser: Angelo Pallini. Co-advisers: Arne Janssen and Madelaine Venzon.

The strategical adding of plants species that provide alternative foods (nectar, pollen, alternative prey) can positively affect the diversity and densities of natural enemies of crop pests and consequently improve the biological control they offer in agricultural fields. Extrafloral nectar-producing *Inga* trees are often consorted to coffee agroforestry systems and, due to this nectar, they can attract and arrest a diversity on natural enemies that contribute to the control of coffee pests. Mites from the Phytoseiidae family are key natural enemies of coffee pest mites, but no study has investigated whether *Inga* extrafloral nectar increases the performance of predatory mites from coffee ecosystems. During the chapter I of my dissertation, I assessed whether the extrafloral nectaries of *Inga edulis* Mart. (Fabaceae) can be considered a suitable nutritional resource for the predatory mite *Amblyseius herbicolus* (Chant) (Acari: Phytoseiidae), one of the most frequent phytoseiids on Brazilian coffee crops. We found that feeding on extrafloral nectar allows for development and survival of *A. herbicolus,* but not reproduction. Furthermore, we speculated that the various natural enemies attracted to and feeding on the nectar of *Inga* are likely to interact with each other and some interactions within this community, e.g., hyperpradation and intraguild predation, may be detrimental for some species. Ants, for example, are known to interact strongly with plants through extrafloral nectar and they are important natural enemies of crop pests. They can aggressively defend nectar-producing plants towards other arthropods, including natural enemies and pollinators. Because the predatory mite that I investigated here also feeds on the Inga nectar, during the chapter II I provide the first study on the interaction between the most common ant species that visit extrafloral nectaries from Inga trees, i.e., *Camponotus rufipes* (Fabricius, 1775), *Camponotus crassus* Mayr, 1862 and *Wasmannia auropunctata* (Roger, 1863) and the mite *A. herbicolus*. My results showed no evidence that ants attacked and killed eggs, juveniles or adults of the predatory mite, either with or without *Inga* nectar present. Therefore, I suggest that an extrafloral nectar-producing plant can simultaneously benefit ants and predatory mites, which control different coffee pest species. In conclusion, the presence of Inga and other plants producing extrafloral nectar may improve the performance and biological control by predatory mites and ants in coffee fields and perhaps in other agroecosystems.

Keywords: Alternative food items; Ant-mite interactions; Arthropod-plant interactions; Natural pest control; Nectar producing plants.

RESUMO

IASCZCZAKI, Stempniak, Rafael, M.Sc., Universidade Federal de Viçosa, julho, 2024. **Interações entre um predador que se alimenta de néctar extrafloral e formigas e seus efeitos no controle de pragas**. Orientador: Angelo Pallini. Co-orientadores: Arne Janssen e Madelaine Venzon.

A adição estratégica de espécies vegetais que fornecem alimentos alternativos (néctar, pólen, alternativo) pode afetar positivamente a diversidade e densidades de inimigos naturais de pragas agrícolas e, consequentemente, melhorar o controle biológico que elas oferecem em campos agrícolas. Árvores de *Inga* produtoras de néctar extrafloral são frequentemente associadas a sistemas agroflorestais de café e, devido a esse néctar, podem atrair e deter uma diversidade de inimigos naturais que contribuem para o controle de pragas do café. Os ácaros da família Phytoseiidae são os principais inimigos naturais dos ácaros da praga do café, mas nenhum estudo investigou se o néctar extrafloral de *Inga* aumenta o desempenho de ácaros predadores dos ecossistemas cafeeiros. Durante o capítulo I da minha dissertação, avaliei se os nectários extraflorais de *Inga edulis* Mart. (Fabaceae) podem ser considerados um recurso nutricional adequado para o ácaro predador *Amblyseius herbicolus* (Chant) (Acari: Phytoseiidae), um dos fitoseídeos mais frequentes na cafeicultura brasileira. Descobrimos que a alimentação de néctar extrafloral permite o desenvolvimento e a sobrevivência de *A. herbicolus*, mas não a reprodução. Além disso, especulamos que os vários inimigos naturais atraídos e alimentados do néctar de *Inga* provavelmente interagem uns com os outros e algumas interações dentro dessa comunidade, por exemplo, hiperpredação e predação intraguilda, podem ser prejudiciais para algumas espécies. As formigas, por exemplo, são conhecidas por interagir fortemente com as plantas por meio do néctar extrafloral e são importantes inimigos naturais das pragas agrícolas. Elas podem defender agressivamente as plantas produtoras de néctar contra outros artrópodes, incluindo inimigos naturais e polinizadores. Como o ácaro predador que investiguei aqui também se alimenta do néctar de *Inga*, durante o capítulo II apresento o primeiro estudo sobre a interação entre as espécies de formigas mais comuns que visitam nectários extraflorais de árvores de *Inga*, ou seja, *Camponotus rufipes* (Fabricius, 1775), *Camponotus crassus* Mayr, 1862 e *Wasmannia auropunctata* (Roger, 1863) e o ácaro *A. herbicolus*. Meus resultados não mostraram evidências de que as formigas atacam e matam ovos, juvenis ou adultos do ácaro predador, com ou sem a presença de néctar de *Inga*. Portanto, sugiro que uma planta produtora de néctar extrafloral pode beneficiar simultaneamente formigas e ácaros predadores, que controlam diferentes espécies de pragas do café. Em conclusão, a presença de *Inga* e outras plantas produtoras de néctar extrafloral pode melhorar a performance e o controle biológico por ácaros predadores e formigas em lavouras de café, e talvez em outros agroecossistemas.

Palavras-chave: Alimentos alternativos; Controle natural de pragas; Interações artrópode-planta; Interações formiga-ácaro; Plantas produtoras de néctar.

Summary

General Introduction

The ecological simplicity of modern agricultural fields, cultivated under monocultures and receiving frequent pesticide applications, contribute to the typical arthropod pest outbreaks in such systems (Emden & Williams, 1974; Pimentel, 1961). Crop monoculture ecosystems have no conditions and resources regularly available to support rich-species communities of predators, parasitoids and pathogens of crop pests (Landis et al., 2000). Further, pesticide applications aiming at controlling pest infestations also impacts natural enemies. Pesticides can act directly, causing mortality, or indirectly, reducing or eliminating prey availability, on natural enemies, what contributes to decrease their diversity and density in crops (Desneux et al., 2007; Janssen & van Rijn, 2021). Consequently, such declines in natural enemy populations, impacts the biological control services they offer.

Indeed, lower natural enemy diversity and density associated to high levels of pest resistance to many active principles of pesticides benefit pests even more, making pesticides an ineffective pest management strategy in the longer term (Janssen & van Rijn, 2021). Furthermore, there are many other concerns related to pesticide applications, such as water and air pollution, soil degradation, wild life and human health, and climate change (Geiger et al., 2010; Köhler & Triebskorn, 2013; Yamamuro et al., 2019). Therefore, there is an emergent need to develop and apply more sustainable practices in agriculture aiming at controlling pest infestations and reduce the negative effects of these harmful practices adopted in modern agriculture.

A key alternative strategy to pesticide applications, used to control pest populations in crops, is biological control (Bouvet et al., 2019; Eilenberg et al., 2001; Hajek & Eilenberg, 2018). This is defined as the use of living organisms (natural enemies) as pest control agents (Waage et al., 1997). These natural enemies include predators, parasitoids, competitors, parasites and even phytophages for weed control. The targets include weeds, plant diseases, viruses vectors and herbivorous arthropod pests (Waage et al., 1997). This practice is not new, and its first records of applications are longer before its definition. All early efforts began with men attempting to control several types of pests using general predators such as cats, mongooses, owls, toads, ants and so on (Driesche & Jr, 2012).

Defined as an environmental-friendly pest management strategy, nowadays biological control generally results in higher-quality and safer food (Altieri, 1999; Bale et al., 2007; Tixier, 2018). Moreover, in contrast to pesticides, biological control does not cause environmental pollution, development of resistance in pests, secondary pest outbreaks and negative impacts on beneficial arthropods (Calvo et al., 2015; Geiger et al., 2010).

Biological control can generally be applied in three major ways: 1) classical, when an invasive pest is controlled by the introduction of a natural enemy from the same area of origin; 2) augmentative, rearing high amounts of a natural enemy species (mass-reared) in order to release it and control a pest; or 3) conservation biological control, where the landscape of the agricultural fields are managed to enhance the occurrence and control provided by indigenous natural enemies (Bale et al., 2007; Calvo et al., 2015; Tixier, 2018; Van Lenteren & Bueno, 2003).

With respect to the practices adopted in conservation biological control, the strategical increase of plant diversity in crops stands out (Venzon, 2021). Plant diversity can promote and enhance biological control in agricultural fields by increasing resources (e.g., structure, prey diversity, alternative food) and beneficial conditions (e.g., microclimatic conditions such as temperature and humidity,) to natural enemies (Altieri, 1999; Landis et al., 2000; Pfiffner & Wyss, 2004). These systems will support higher densities and diversity on natural enemies, consequently leading to increased predation and lower densities of the target pests (Rezende et al., 2014). That is because natural enemy species differ in pest species preference, strategies of attack, time and places, so that biological control is maximized by this sort of complementarity their feeding niches (Ives et al., 2005; Macarthur & Levins, 1967).

In this respect, plants providing alternative foods (pollen, nectar, alternative prey) can be especially important for generalist natural enemies (Altieri, 1999; Landis et al., 2000; Venzon, 2021). These enemies can or have to feed from a variety of food types, including plant derived food items such as pollen or nectar (Venzon, 2021). The populations of the natural enemies feeding on these alternative foods will increase in density leading to the pest populations to be reduced (Janssen & Sabelis, 2015; Nomikou et al., 2010; van Rijn et al., 2002). This is a phenomenon similar to apparent competition (Holt, 1977) but, instead of only being caused by an alternative prey, it is also induced by plant derived food items. Therefore, plants providing alternative foods can interact indirectly with herbivores in a crop, cause their reduction and thus improve the biological control services provided by natural enemies.

However, to reach such improvements in biological control through plant diversification on agricultural fields, detailed knowledge on the biology and requirements for survival, development and reproduction of the natural enemies of pests are needed (van Rijn & Wäckers, 2016). Based on this information it is possible to select plant species which attract and maintain the natural enemies of pests in the crop. But at the same time, the selected companion plants ought not to benefit pests (Venzon, 2021).

Plants species possessing extrafloral nectar glands have gained attention in the conservation biological control practices (Rezende et al., 2014, 2021; Venzon, 2021). This nectar is not directly related to the plant reproduction but instead, to the attraction and arrestment of natural enemies of its herbivores (Bentley, 1977). These natural enemies go for the nectar produced by such plants to gain energy but, they may extend their foraging activity to nearby plants (Rezende et al., 2014). Thus, predators and parasitoids can attack, kill and reduce the damages that herbivorous pests cause to the nearby crop plants, whenever nectar producing trees are present in the system (Rezende et al., 2014, 2021). In contrast to the extensive knowledge on the benefits of extrafloral nectar to insect natural enemies (e.g., predators, parasitoids), however, fewer is known on its effects on the performance of predatory mites (Bakker & Klein, 1992; van Rijn & Tanigoshi, 1999; Rojas & Morales-Ramos, 2008). Predatory mites, especially those from Phytoseiidae family, are among the most studied and most commercialized biological control agents in the world (McMurtry et al., 2015; Knapp et al., 2018). For these reasons, this was the subject of study in the chapter 1 of the present dissertation: I bring novel results on the effects of extrafloral nectar produced by *Inga* trees associated to coffee fields on the performance of an important predatory mite of coffee pest mites, *Amblyseius herbicolus* (Chant).

Another subject of importance to biological control, are the interactions between natural enemies that share the same source of food (Aebi et al., 2011; Amarasekare, 2008; Gagnon et al., 2011). Nectar producing plants are known for attracting and benefiting many natural enemy species of importance for biocontrol (Venzon, 2021; Rezende et al., 2014, 2021). However, interactions within the community of natural enemies feeding on this nectar can potentially be detrimental for some species, for example, through competition for nectar or through hyperpredation and intraguild predation (Polis *et al.*, 1989; Rosenheim et al., 1993, 1995; Janssen et al., 2006). Ants, for example, are one of the first and best-studied organisms described to interact with plants through extrafloral nectar (Janzen, 1966). Despite they are broadly recognized as natural enemies of plant pests (Drummond & Choate, 2011), ants can act aggressively towards other beneficial arthropods (e.g., natural enemies, pollinators), especially when in presence of extrafloral nectar or other sugar sources (Anjos et al., 2021; Calixto et al., 2024; Rosenheim et al., 1995). However, to the best of our knowledge, no effort has been dedicated to study interactions of predatory ants and predatory mites in a system containing extrafloral nectar. In this respect, during the chapter 2 of this dissertation I explored the interactions of the most common visiting ant species on *Inga* extrafloral nectaries and a predatory mite (*A. herbicolus*) that also feeds on *Inga* nectar. Practical implications of adding nectar producing plants in agricultural fields and future directions for the research area are discussed on the light of the presented results.

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Chapter I

Extrafloral nectar from associated trees is alternative food for a predatory mite in coffee ecosystems

Abstract

Plant diversity can enhance natural pest control in agriculture by providing natural enemies of crop pests with resources and conditions that are not regularly available in conventional crops. Extrafloral nectar-producing plants, for example, might cause reduction of pest densities on neighboring plants because the nectar can increase the performance of natural enemies. Coffee agroforestry systems often contain extrafloral-nectar-producing *Inga* trees, that serve several purposes. Recent studies suggest that they attract and arrest a diversity on natural enemies that contribute to the control of coffee pests. Mites from the Phytoseiid family are key natural enemies of coffee pest mites, but no study has investigated whether *Inga* extrafloral nectar increases survival, developmental rate and reproduction of predatory mites from coffee ecosystems. Thus, in the present paper, we assessed whether the extrafloral nectaries of *Inga edulis* Mart. (Fabaceae) can be considered a suitable nutritional resource for the predatory mite *Amblyseius herbicolus* (Chant), one of the most abundant phytoseiids on coffee crops. We found that feeding on extrafloral nectar allows for development and survival of *A. herbicolus,* but not reproduction. Our findings suggest that interplanting *Inga* trees is a strategy that can maintain populations of predatory mites in crop ecosystems through provision of nectar and may boost biological control services. Future research should investigate the effects of extrafloral nectar-producing trees on coffee pest control by these predatory mites.

Key-words: Food type; Generalist predator; Natural pest control; Nutritious value; Phytoseiidae; Plant-derived-food items.

Introduction

Current agriculture faces the challenge to manage the trade-off between food production and ecosystem services (Foley, 2005). The intensification of agriculture has impacts on atmospheric constituents, soil and water health as well as on wildlife (Matson *et al.*, 1997). With respect to the practices adopted in conventional monoculture agriculture aiming at controlling pest infestations, pesticide use stands out. This practice has harmful effects on human health and on the environment, such as pollution and direct impacts on biodiversity, which limits many ecosystem services (Geiger *et al.* 2010; Köhler & Triebskorn 2013; Yamamuro *et al.* 2019). Furthermore, pesticides sometimes cannot effectively control pests.

Although pesticide applications can reduce pest densities in the short term, target pest resurgence occurs some time after applications, with pest densities reaching higher levels than before (Hardin et al. 1995; Guedes *et al.* 2016). These are likely due to negative effects on natural enemies, even when the pest is more sensitive to the pesticide than natural enemies are, making this practice inefficient in pest control in the longer term (Janssen & van Rijn 2021). Indeed, arthropod pests remain among the major causes of yield loss despite the efforts to control them with pesticides (Johnson *et al.* 2020; Janssen & van Rijn 2021). Thus, there is a need to develop alternative pest control practices that decrease the risks associated with conventional agricultural practices without jeopardizing yield production.

Conservation biological control is a strategy aiming at a more sustainable agriculture. To this end, landscape manipulations are carried out, primarily through increase in plant diversity inside or near cropped area (Altieri, 1999; Landis *et al.* 2000; Denys & Tscharntke 2002; Pfiffner & Wyss 2004; Frank, 2010), with the purpose to attract and conserve natural enemy populations, thus promoting natural pest control.

Using detailed knowledge of the biology of the natural enemies of target pests (van Rijn & Wäckers 2016), it is possible to select plant species which can provide shelter, suitable microclimatic conditions as well as alternative food (e.g., pollen, nectar, alternative prey) to increase natural enemy densities (Pemberton, 1993; Wäckers *et al.* 2005, Venzon 2021). Plant species possessing extrafloral nectaries are potentially important in this respect (Rezende *et al.* 2014, 2021). These nectaries are not directly related to plant reproduction, but are glands outside flowers that produce nectar, either continuously or inducible (Agrawal & Rutter 1998; Heil *et al.* 2000). Herbivore damage on leaf or floral tissues, root damage, exploitation by carnivores (nectar removal) and even volatile signals associated with herbivory may induce or increase nectar production (Agrawal & Rutter 1998; Heil *et al.* 2000; Sabelis *et al.* 2005; Choh et al. 2006). This nectar attracts predatory arthropods that can subsequently defend the plant against herbivores (Janzen, 1966; Bentley, 1977; Sabelis *et al.* 2005; Calixto *et al.* 2018, Rezende et al. 2021). Extrafloral nectar is a sugar-rich fluid, but also contains other compounds in low concentrations, such as amino acids. Thus, it can provide energy for foraging and increase survival and reproduction, thus improving performance of many natural enemies (Rezende *et al.* 2014).

Many natural enemies are generalists and can or have to feed on food sources provided by plants (Wäckers, 2005). The provision of non-prey food by plants to generalist predators induces a phenomenon similar to apparent competition (van Rijn *et al.* 2002; Nomikou *et al.* 2002, 2010; Janssen & Sabelis 2015), in which predator densities will be increased by feeding on a prey or other food sources, and the density of the target pest will be reduced (Holt, 1977). Therefore, plants providing alternative food (e.g., pollen or nectar) can interact indirectly with herbivores in a crop. Mites from the Phytoseiid mites are among the most studied and most used arthropod biological control agents in the world (Moraes & Flechtmann 2008; McMurtry *et al.* 2015; Knapp *et al.* 2018), but not much is known about the effects of extrafloral nectar on these predators and their control of pests (Bakker & Klein 1992; van Rijn & Tanigoshi, 1999; Gnanvossou *et al.* 2005; Pekas & Wäckers, 2017). Here, we investigate the effects of extrafloral nectar on the performance of a predatory mite occurring in coffee.

Coffee (*Coffee arabica* L. and *Coffee canephora* L.) (Rubiaceae) is an economically important plant species, covering more than 10 million ha, mainly in Latin America (Philpott *et al.* 2007; Venzon, 2021). Insects are the main arthropod pests jeopardizing coffee production (Reis et al. 2002; Vega *et al.* 2009; Oliveira *et al.* 2013; Milligan *et al.* 2016; Avelino *et al.* 2018; Cure *et al.* 2020), but herbivorous mites can also cause significant yield reduction (Reis *et al.* 2002). Coffee ecosystems naturally harbor a great diversity of natural enemies, including predatory mites, of which *Amblyseius herbicolus* (Chant, 1959) (Acari: Phytoseiidae) is one of the most abundant (Pallini *et al.* 1992; Reis, 2002). Under laboratory and semi-field conditions, this species can suppress coffee pest mites such as *Oligonychus ilicis* (McGregor, 1917) (Acari: Tetranychidae) and *Polyphagotarsonemus latus* (Banks, 1904) (Acari: Tarsonemidae) (Franco *et al.* 2007; Reis *et al.* 2007; Duarte *et al.* 2015; Toledo *et al.* 2018), but its effectiveness in the field has not been tested yet. *Amblyseius herbicolus* is a generalist predator; it can feed on small arthropod pests but also on non-prey food sources such as pollen (Rodríguez-Cruz et al. 2013; Duarte *et al.* 2015; Marcossi *et al.* 2020; Kalile *et al.* 2021; Kalile *et al.* 2023).

Although coffee is predominantly grown in monocultures, it is also cultivated in agroforestry systems in many countries, including Brazil (Vandermeer *et al.* 2010; Jha *et al.* 2014; Rezende *et al.* 2021). These diversified systems contribute to the conservation of biodiversity and provisioning of ecosystem services such as natural pest control (Perfecto *et al.* 2007; Jha *et al.* 2014; Rezende *et al.* 2014). For example, coffee plants intercropped with nectar-producing *Inga* trees experienced less foliar damage, lower pest densities, increases in weight of coffee fruits and increased abundance and a higher diversity of natural enemies that were attracted to the extrafloral nectar (Rezende *et al.* 2014, 2021). Another study carried out in the Brazilian Cerrado, revealed a high richness of predatory mite species on *I. edulis* trees intercropped with coffee (Ferla *et al.* 2023). Among the 14 predatory species found, the Phytoseiidae family and *Amblyseius* genus stood out. However, to our knowledge, no study has investigated whether extrafloral nectar of *Inga* trees increases survival, developmental rate and reproduction of predatory mites from the Phytoseiidae family. In the present paper, we assessed whether the extrafloral nectaries of *Inga edulis* Mart. (Fabaceae) can be considered an adequate nutritional resource for *A. herbicolus*.

Materials and methods

Pollen

The pollen used for the predatory mite rearing and experimentations was collected directly from *Typha* spp. plants from rural areas in Viçosa, state of Minas Gerais. This pollen was chosen due to its suitability for *A. herbicolus* in previous experiments (Duarte *et al.* 2015; Marcossi *et al.* 2020). After collecting, it was dried in an oven at 40ºC for a period of 12 h and stocked in containers in a refrigerator (-6ºC). Small amounts were removed periodically from the container, put in 1.5 ml microtubes (Eppendorf), dried at 60 ºC for 48 h and then used.

Predator rearing

The predatory mite strain used in the experiments was collected from tomato plants in the municipally of Prados (latitude: 21°03′00″ S; longitude: 44°04′47″ W), Minas Gerais, Brazil and has been reared in our laboratory since 2014. The rearing was maintained on arenas that consisted of a black plastic sheet (10 x 14 cm) surrounded by a barrier of moistened cotton wool, placed on a wet sponge inside a plastic tray containing water (McMurtry & Scriven 1965).

This rearing was fed with pollen of *Typha* spp. twice per week. Cotton threads covered with pieces of black plastic sheet (1 x 1 cm^2) were provided to serve as oviposition sites and refuge, respectively. To obtain cohorts of similar-aged individuals, threads with eggs were collected from the rearing arenas and placed on a new arena. For experiments that compared developmental times, larvae of similar age were collected immediately after egg hatching. For other experiments, young adult females were collected 10-12 days after egg hatching. The rearing arenas were maintained in a room with controlled conditions (25 ± 2 °C, 70 ± 10% RH e L:D 12:12).

Extrafloral nectar

Extrafloral nectar was collected daily from *I. edulis* trees in the surroundings of the Federal University of Viçosa by cutting young branches with completely expanded composite leaves bearing producing nectaries (Pireda *et al.* 2018). To remove arthropods from the leaves, they were submersed in water for about 30 minutes. Subsequently, the leaves were dried and their stems were inserted into water for 24 h to allow for nectar secretion. Subsequently, nectaries containing similar amounts of nectar were selected. The nectaries of *I. edulis* consist of a raised concave surface with a central depression in which nectar accumulates (Fig. 1, *Pireda et al.* 2018), and we carefully cut them out from the leaves using a surgical knife and scissors. The nectaries were checked once again for remaining arthropods and symptoms of fungal infestation because predatory mites may feed on these (Zemek & Prenerov 1997; Momen & Abdelkhader 2010; Wu *et al.* 2016). Only uninfected nectaries were used to provide extrafloral nectar in the arenas for the experiments.

Survival and juvenile development of *A. herbicolus* on extrafloral nectar

In the lab, developmental time and survival of *A. herbicolus* juveniles were compared among four diets: a) *I. edulis* nectar; b) *Typha* spp. pollen; c) nectar plus pollen; d) no food. The combination of *Typha* pollen plus nectar was offered because van Rijn & Tanigoshi (1999), observed that *Iphiseius degenerans* (Berlese) was not able to develop into adult or reproduce when feeding only on extrafloral nectar of castor bean (*Ricinus communis* L.), but when nectar was offered together with pollen, they observed higher egg production than on a pollen diet. Furthermore, several predatory mites are known to perform better on a mixed diet than on single diets, and predators may prefer feeding on more than one type of food (Messelink *et al*. 2008; Marques *et al*. 2015, 2022).

Small black plastic dishes (ø = 5.5 cm; 1.4 cm high) with transparent lids were used as experimental units (arenas) for providing the food sources. Pollen was supplied in abundance in the arenas using a fine brush. For the treatments with nectar, one nectary full of nectar was provided in each experimental unit. The amounts of food offered was higher than the quantity consumed by the mites as assessed in earlier experiments.

Newly emerged larvae from a cohort of eggs were placed each in one arena with the assigned diet. Every day, individuals were transferred to new arenas with fresh food and checked for survival and development until adulthood. Once adulthood was reached, females were observed daily until they produced their first egg.

We measured the developmental time and survival of 22 *A. herbicolus* individuals with extrafloral nectar; 23 with pollen plus nectar; 24 with pollen and 20 without food. This experiment was carried out at 23ºC, 80 – 85% RH, light:dark 16:8 h. Effects of diet on survival and development of *A. herbicolus* were analyzed with a Cox proportional hazards model (survival package in R, Therneau 2020). We used R software version 4.0.0 (R Project for Statistical Computing. http://www.r-project.org) for all statistical analyses.

Oviposition of *A. herbicolus* on extrafloral nectar

This experiment was designed to compare the oviposition rates of *A. herbicolus* among the diets described above, using the same experimental arenas. Because *A. herbicolus* reproduces thelytokously (de Moraes & Mesa 1988) young mite females (10 - 12 days old since egg) were each placed in a separate arena and their oviposition was recorded every 24 h for a period of four days (Duarte *et al*. 2015). The oviposition rate of predatory and phytophagous mite peaks soon after the pre-oviposition period and is a good proxy for the total fecundity and the intrinsic rate of increase (Janssen & Sabelis 1992; Sabelis & Janssen 1994). The experiment was carried under the same conditions as above with 15 replicates per diet. Oviposition was analyzed using linear mixed effects models (LME of the package NLME in R, Pinheiro *et al*. 2020) with individual as a random factor to correct for repeated measures and with diet, time and their interaction as fixed factors. Non-significant factors and interactions were removed until a minimal adequate model was obtained (Crawley, 2013). Significance of factors and interactions were determined with likelihood ratio (L.R.) tests after model simplification and comparison of models using the anova function of R (R Core Team, 2017). Models were checked for normality of errors, and contrasts among treatments were assessed with the package emmeans with a Tukey correction for multiple comparisons (Lenth, 2019). To avoid effects of the previous diet (Sabelis, 1990), oviposition from the first day was excluded from analysis.

Hatching rate of eggs produced on a diet of extrafloral nectar

We also analyzed the viability of eggs from the three diets (treatment without food excluded). We isolated the eggs from the third day of oviposition in the experiment described above (thus excluding effects from previous diet) on arenas and allowed them to hatch during 48 h. We then assessed the proportions of hatched eggs and compared them among diets using a generalized linear model with a binomial error distribution (log link).

Crossed-diet experiment with extrafloral nectar and pollen

The previous experiment showed that adults that developed on a diet of extrafloral nectar did not produce eggs. Based on this, the following questions were addressed: 1) Are mites that developed on nectar capable of reproducing when their diet is changed to pollen? 2) Do mites that developed on a pollen diet and were then changed to a nectar diet stop ovipositing through time?

To answer these questions, we set up a crossed-diet experiment where a group of adults was allowed to develop from egg until adult on extrafloral nectar (initial n = 37) or pollen (initial n = 33). Twenty-three out of 37 individuals reached adulthood on pollen and 15 out of 33 became adults on nectar. After becoming adults on pollen and ovipositing for two days, ca. of half of them (n = 11) were placed on a nectar-based diet, while the other 12 were maintained on pollen. Subsequently, their oviposition was monitored for nine days. The same was done with individuals that had developed to adulthood on nectar: seven of them were shifted to pollen immediately after becoming adults, and the other eight was kept on nectar. Food was refreshed either every other day or daily, when necessary.

Juvenile development on the two diets was analyzed with a Cox proportional hazards model (survival package in R, Therneau 2020). Oviposition rates after changing the diets were analyzed with a linear mixed effects model (LME) with treatment and time and their interaction as fixed factor and individual as a random factor to correct for repeated measures. Oviposition data were transformed to log(x+1). Significance of interactions and contrasts were assessed as above.

Results

Development and survival

We observed that various stages of *A. herbicolus* fed on nectar (Figure 1). There were significant differences in the survival of *A. herbicolus* among diets (Figure 2, Log rank test = 88.7, d.f. = 3, $p < 0.0001$). No predators survived without food, and survival on nectar, pollen or pollen plus nectar was significantly higher (Figure 2). The development from larva to adult differed significantly among diets (Log rank = 36.4, d.f. = 2, $p < 0.0001$) and was longer on a diet of nectar than on pollen or pollen plus nectar (Figure 2).

Figure 1. A protonymph (A) and an adult female (B) of *A. herbicolus* feeding on *I. edulis* extrafloral nectar.

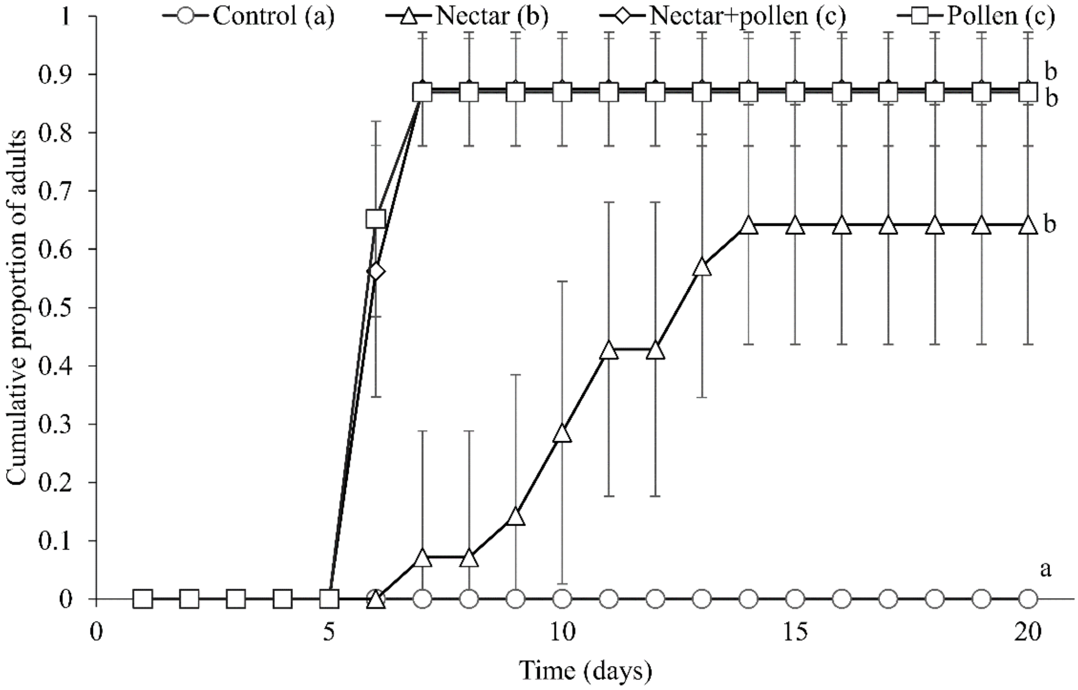

Figure 2. Juvenile development and survival (± 95% CI) of *A. herbicolus* fed only on *I. edulis* extrafloral nectar, *Typha* spp. pollen, pollen plus nectar or no food (control). Shown are the cumulative proportions of adults as a function of time. Total survival is the final cumulative proportion of individuals that reached adulthood (i.e., the proportions at day 20) Significance among diets is given by letters next to the lines (contrasts after coxph, $p < 0.05$). Significance of differences in development is indicated with letters in the legend.

Oviposition

Diet significantly affected oviposition of *A. herbicolus* through time (lme, interaction of diet with time: L.R. = 9.58, d.f. = 3, $p = 0.023$), with the highest oviposition rates with pollen (overall mean: 1.66 eggs per day) and pollen plus nectar (overall mean: 1.71 eggs per day). Oviposition on these two diets did not differ significantly. Oviposition by mites fed on nectar (overall mean: 1.0 eggs per day) was significantly higher than without food (overall mean: 0.33 eggs per day), but lower than on diets containing pollen. As expected, oviposition without food decreased between day 2 and 3 and ceased completely on day 4 (Figure 3).

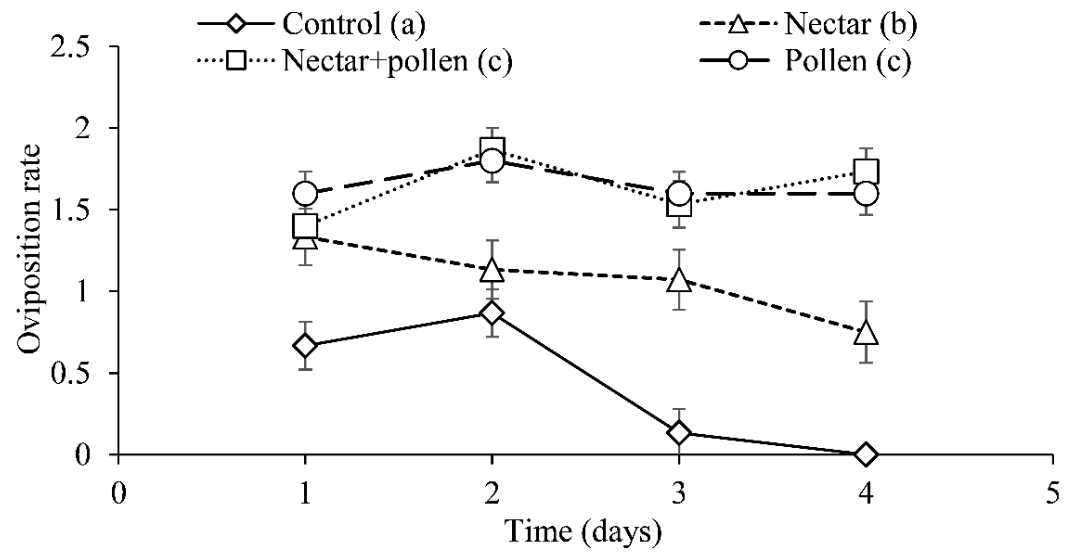

Figure 3. Oviposition rate (± SE) of *A. herbicolus* fed on various diets during four days. Significance of oviposition on different diets through time are indicated by the different letters in the legend (contrasts after lme, $p < 0.05$).

Viability of eggs produced on a nectar-based diet

The hatching rate of eggs produced on the third day of oviposition on a diet of extrafloral nectar, pollen, and pollen plus nectar did not differ significantly (GLM: Deviance = 0.20, d.f. = 2, $p = 0.91$; Figure 4).

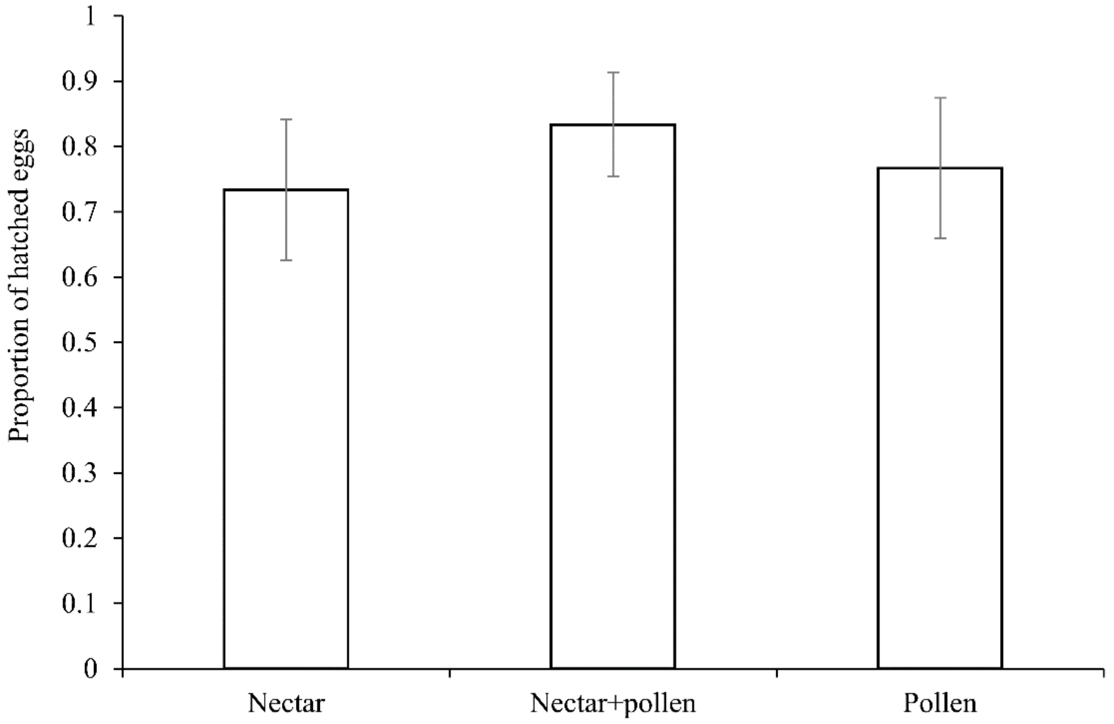

Figure 4. Proportion of hatched eggs (± SE) after 48h from the third day of oviposition by *A. herbicolus* feeding on extrafloral nectar; nectar plus pollen or pollen.

Crossed diet experiment

In contrast to the previous experiment, juvenile survival of *A. herbicolus* was significantly higher on a pollen diet than on nectar (Log rank test = 10.7, d.f. = 1, p = 0.001). Development from larva to adult took significantly longer on nectar than on pollen (Log rank test = 47.6; d.f. = 1; p < 0.0001; figure 5).

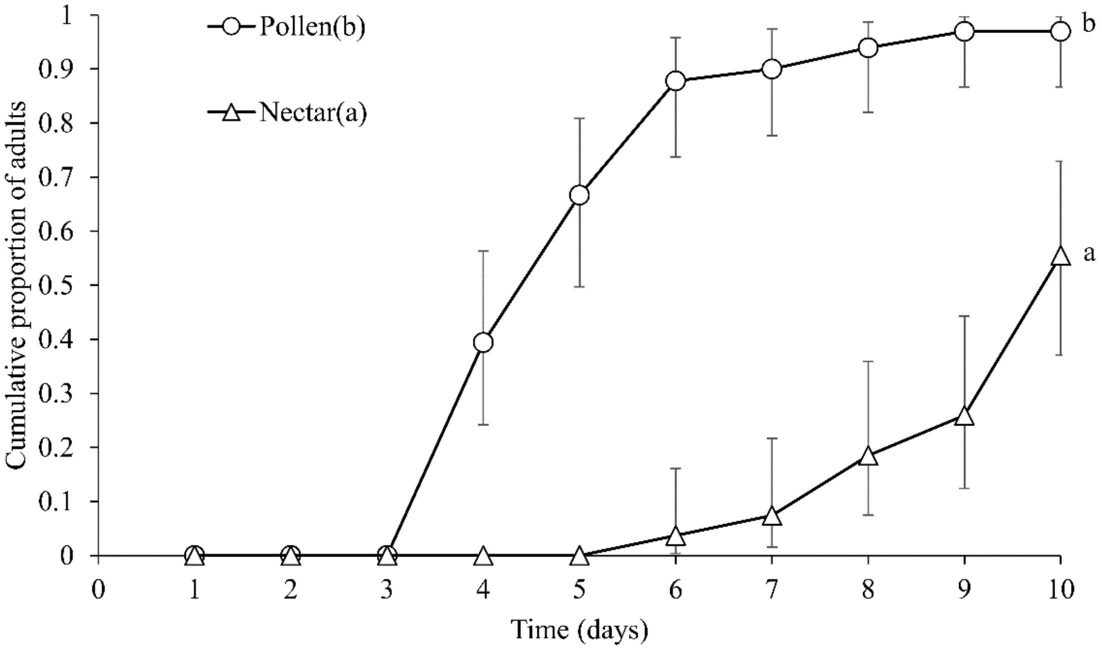

Figure 5. Juvenile development and survival (± 95% CI) of *A. herbicolus* fed on *I. edulis* extrafloral nectar or *Typha* spp. pollen during the crossed-diet experiment. Shown is the cumulative proportion of adults as a function of time. Total survival is the final cumulative proportion of individuals that reached adulthood (i.e., the proportions at day 10) Significance of survival between diets is given by letters next to the lines). Significance of differences in development is indicated with letters in the legend (contrasts after coxph, p < 0.05).

Individuals that developed on nectar became adult later than those on pollen and they were therefore shifted to the pollen diet one day later (day 10 from the start of the experiment) than the individuals that developed on pollen (day 9) (figure 6). Oviposition rates differed significantly among diets through time after changing the food type (L.R. = 23.7, d.f. = 3, $p < 0.0001$). Adults that had developed on nectar and were kept on a diet of nectar never oviposited, but those that were switched to a pollen diet did start reproducing (Figure 6). Individuals that developed on a diet of pollen and were then shifted to a diet of extrafloral nectar showed a gradual decrease in oviposition until it ceased completely on the 8th day, whereas individuals supplied with a diet of pollen continued ovipositing (Figure 6).

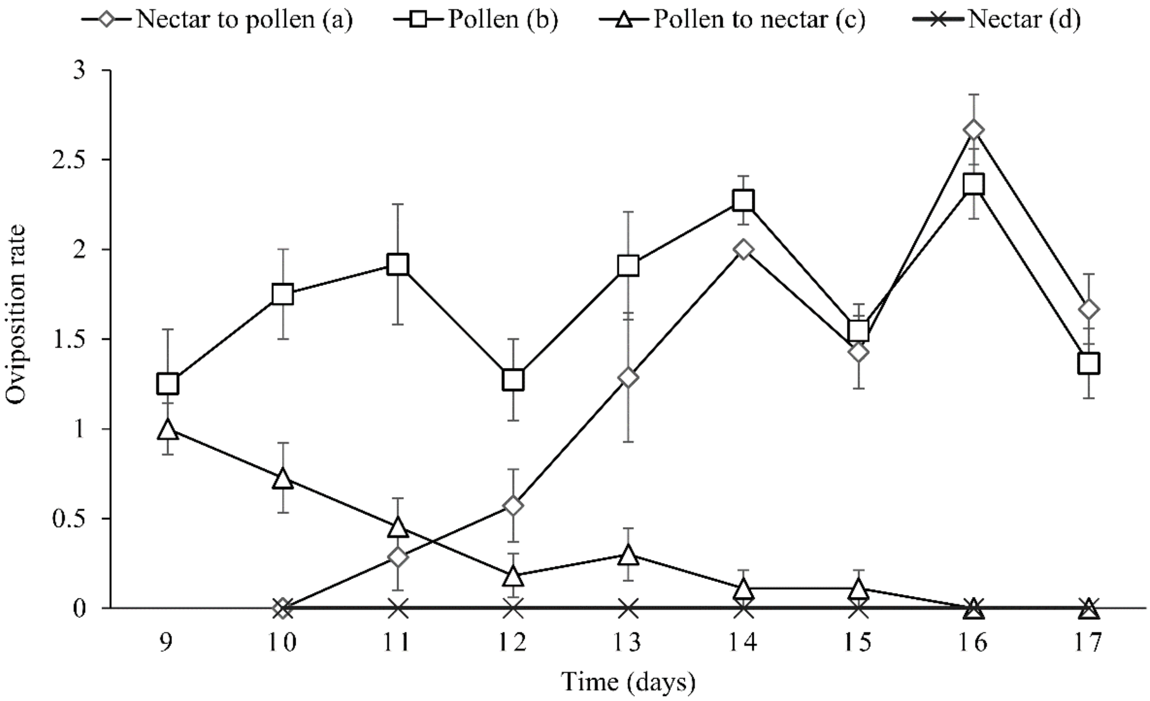

Figure 6. Oviposition rate (± SE) of *A. herbicolus* adults that developed either on pollen or extrafloral nectar over 9 - 10 days and were then changed from pollen to nectar (triangles), nectar to pollen (diamonds), or maintained on pollen (squares) or nectar (crosses). Overall significant effects of diets through time are indicated by the different letters in the legend (contrasts after lme, $p < 0.05$).

Discussion

It is well-known that extrafloral nectar can increase survival, development and reproduction of natural enemies (Lewis *et al.* 1998; Tylianakis *et al.* 2004; Jamont *et al.* 2013). Our study revealed that the extrafloral nectar produced by *I. edulis* trees allows for development and survival of *A. herbicolus*, but is probably not sufficient for reproduction. Thus, extrafloral nectar of *I. edulis* trees can serve as alternative food for juvenile development of *A. herbicolus*.

In our study, we offered entire nectary structures to supply nectar to the mites. In contrast, van Rijn & Tanigoshi (1999) scraped droplets from nectaries of castor bean (*Ricinus communis* L.) with a small plastic card and then provided them on arenas of *Iphiseius degenerans* (Berlese). Our study may have more realistic results in this respect because we could observe the predator individuals assessing the nectar directly from the nectaries (Figure 1). Because we cleaned and carefully selected nectaries without apparent infestations of fungi, we suggest that the observed results were all caused by nectar consumption. Some authors observed Phytoseiid species feeding on leaf tissue (Magalhães & Bakker 2002; Nomikou *et al*. 2003) and since the nectaries placed in the arenas in our study are part of the foliar tissue, the mites may have fed on it. Whether *A. herbicolus* indeed consumes leaf tissue remains a matter of further investigation.

To the best of our knowledge, this is the first time that development from egg to adult of a predatory mite was shown on a diet of extrafloral nectar. The presence of amino acids and other organic compounds beyond sugars in extrafloral nectar (Bentley, 1977) is probably not enough for oviposition, but is sufficient for development of this phytoseiid. Koptur (1994) revealed a large variation in composition and concentration of amino acids of eight *Inga* species: whereas the nectar of some species contained almost all essential amino acids, others simply did not contain any of them (Koptur, 1994). Pireda *et al*. (2018) found evidence for protein synthesis with a histochemical analysis in the nectar gland tissue of *I. edulis*. These authors, however, did not study the chemical composition of *I. edulis* nectar, the plant used in our study, which therefore deserves future investigation. Another possible explanation for the juvenile development of *A. herbicolus* is that the mites feed on other food sources such as yeasts and bacteria that frequently grow in the nectar (Canto & Herrera, 2012). Even so, some essential nutrients are missing in the nectar, either with or without these micro-organisms, at least for oviposition.

We observed variation in juvenile survival and in the developmental rate of *A. herbicolus* between the two development experiments we carried out (Figure 2 and 5). In contrast to the first experiment (Figure 2), we noticed a significantly lower survival rate of mites on nectar than on a pollen diet during the crossed-diet experiment (Figure 5). Furthermore, the surviving mites in the last experiment developed faster (the last individuals became adults on day 10, Figure 5) than those from the first experiment (around day 14, Figure 2). Two factors may have contributed to this: variation in time of refreshment of water and food, and the different seasons in which the experiments were carried out. Possibly, refreshing pollen and nectar every 48 h in the crossed-diet experiment compared to 24h in the first experiment resulted in a decrease of the quality of the foods and/or the availability of water, and this may have affected juvenile survival and developmental rates. Additionally, the first experiment was carried out in July, 2023, whereas the crossed-diet experiment was conducted in December of the same year, and the latter coincides with the period in which the *Inga* trees were flowering. Considering that extrafloral nectar quality varies largely through seasons (Koptur, 1994) it is possible that the quality of the extrafloral nectar was reduced due to the investment in flower/pollen production (Vilela *et al.* 2014; Heil, 2015; Calixto *et al.* 2021), causing the reduction in juvenile survival and developmental rate compared to pollen. However, this seasonal change in extrafloral nectar quality remains to be tested.

High amounts of protein are needed to sustain egg production in animals for longer periods (Barry & Wilder 2013). Pollen contains protein, nitrogen, amino acids, starch, sterols and lipids (Schmidt *et al.* 1989; Roulston & Cane 2000), reflected in the oviposition of *A. herbicolus* on this food source in our and other studies (Rodríguez-Cruz et al, 2013; Marcossi *et al.* 2020; Kalile *et al.* 2021). The differences in the nutritious value of pollen and nectar can also be inferred from the observation that adult females that received pollen after becoming adults on nectar did start ovipositing, and females that had developed on a diet of pollen and were then changed to nectar stopped reproducing after 8 days. Nevertheless, our results show that extrafloral nectar is a promising alternative food: when other food is scarce, nectar can be crucial for persistence of *A. herbicolus* populations in the field until prey or other higher quality food becomes available. In contrast to studies showing that predatory mites that feed on mixed diets have higher fitness (Marques *et al.* 2015, 2022), *A. herbicolus* did not perform better on a mixed diet of pollen and nectar.

An advantage of extrafloral nectar as food for enemies of plant pests is that it is produced throughout the year (Bentley, 1977), whereas pollen and floral nectar are only available during the flowering period (Cruz-Neto *et al.* 2011). In addition, extrafloral nectars are easier accessible to natural enemies because of their open structure compared to many flowers (Bentley, 1977; Jervis, 1998; Wäckers, 2005; van Rijn & Wäckers, 2016). Thus, despite our results showing that extrafloral nectar is less nutritious than pollen, it can be considered a good source of energy in terms of availability.

The high numbers of *Amblyseius* observed on *I. edulis* in coffee fields (Ferla *et al.* 2023) are possibly related to the availability of extrafloral nectar. Ferla *et al*. (2023) observed that the coffee pest mite *O. ilicis* was rare on *Inga*, suggesting that these mites did not benefit from the availability of nectar. Probably, these phytophagous mites do not need nectar for development and reproduction. In addition, they may avoid the nectaries because these are frequented by predatory mites and other natural enemies (Sabelis *et al*. 1999).

Ferla *et al*. (2023) observed that all predatory mite species found on *Inga* were also present on surrounding coffee plants, suggesting that they moved between *Inga* and coffee. In contrast to flying natural enemies (wasps, parasitoids, lacewings, ladybugs) and other, larger arthropods (ants, spiders), mites are quite small and disperse longer distances mainly by wind (Sabelis & Dicke 1985) and phoretic interactions with other animals (Moraes & Flechtmann, 2008), and cover shorter distances to neighboring plants mainly by walking. So, for predatory mites to move from associated nectar-producing trees into a crop, factors such as the positioning of intercropped plants in a crop may be of importance.

The other natural enemies found associated with *Inga* nectaries do not only attack the herbivores on *Inga* (Bentley, 1977; Begg *et al*. 2017; Rezende *et al*. 2021), but also on the associated coffee plants, resulting in decreased herbivory and increased yield by coffee plants associated with *Inga* (Rezende *et al*. 2014, 2021). It remains to be investigated whether the same applies to the phytoseiid mites studied here, which are key natural enemies of herbivorous pest mites of coffee plants (Franco *et al*. 2007; Duarte *et al*. 2015; Toledo *et al*. 2018).

The few studies on extrafloral nectar and mites (Bakker & Klein 1992; van Rijn & Tanigoshi 1999; Weber *et al*. 2016; Rojas & Morales-Ramos 2008) show that nectar and sugar solutions promoted survival and reproduction of predatory mites in different systems, at least when offered together with other food types. Moreover, Bakker & Klein (1992) noticed that the predatory mite *Typhlodromalus limonicus* (Garman & McGregor) persisted longer on cassava plants producing a sugar-rich exudate (similar to nectar) at the base of the petioles of the leaves. Furthermore, these authors showed that the predators reached higher densities when supplied with honey, even when prey densities were low. This shows that sugar-rich food such as extrafloral nectar and honey might confer advantages to predatory mites in various cultures and contexts and may improve biological control services.

When landscape management aims to support natural pest control, the plants used in or around agricultural fields should have suitable and accessible resources for the natural enemies (van Rijn & Wäckers 2016) such as extrafloral nectaries of *Inga* trees (Venzon 2021). Thus, our results suggest that interplanting *Inga* trees is a viable strategy to maintain populations of predatory mites in crop ecosystems through provision of alternative food. To the best of our knowledge, our study is the first to identify such results for a predatory mite from Phytoseiidae family.

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Chapter II

Ants do not prey upon an extrafloral-nectar feeding predatory mite

Abstract

Extrafloral nectar-producing plants associated with crops can increase the densities and diversity of natural enemies, which can improve the control of crop pests. However, the various natural enemies that feed on the nectar are likely to interact with each other, which can have negative effects on pest control. Ants, for example, are well known for their interactions with plants producing extrafloral nectar and they are important natural enemies of crop pests. They can aggressively defend the nectar-producing plants towards other arthropods, including natural enemies and pollinators. Predatory mites are important natural enemies of crop pests, and several generalist species are known to feed on nectar. Here, we investigated the interaction between the most common ant species that visit extrafloral nectaries of *Inga* trees and a nectar-feeding predatory mite, *Amblyseius herbicolus* (Chant, 1959) (Acari: Phytoseiidae). *Inga* is used to intercrop with coffee plants and its nectar is known to attract many natural enemies of coffee pests, including several ant species. The predatory mite is a natural enemy of the main coffee pest mites and was recently found to feed on *Inga* nectar. We therefore studied the interaction among *A. herbicolus* and three ant species that frequent *Inga* nectaries, i.e., *Camponotus rufipes* (Fabricius, 1775), *Camponotus crassus* Mayr, 1862 and *Wasmannia auropunctata* (Roger, 1863). We found no evidence that the ants attacked and killed eggs, juveniles or adult predatory mites, either with or without nectar present. In contrast, they did attack termites when offered together with nectar, showing that the ants were motivated to attack. This suggests that an extrafloral nectar-producing plant can simultaneously benefit ants and predatory mites, which control different coffee pest species, hence, the presence of *Inga* and other plants producing extrafloral nectar may improve natural pest control in coffee fields.

Key-words: Ant-mite interactions; Conservation biological control; Generalist predators; Natural pest control; Arthropod plant interactions.

Introduction

Plant diversification can potentially improve the densities and diversity of natural enemies and the service of pest control that these offer in agricultural fields, by the provision of resources and conditions not regularly available in conventional crops (Altieri, 1999; Landis *et al.*, 2000; Denys & Tscharntke, 2002; Sabelis *et al.*, 2005; Venzon, 2021). However, just increasing biodiversity does not necessarily translate into improved pest control (Albrecht *et al.*, 2020; Rinehart & Long, 2022; Crowther *et al.*, 2023). The resources provided by the plants used for this strategy must be tailored to the requirements of the target natural enemy taxa (Tschumi *et al.*, 2015, 2016). Thus, using knowledge of the biology and ecology of natural enemies of crop pests, it is possible to select plant species which promote survival, development and reproduction of these natural enemies (van Rijn & Wäckers, 2016).

Plant-derived food items (i.e., pollen, nectar, exudates) can be especially important for increasing natural enemy densities, mainly those with generalist feeding habits (van Rijn et al., 2002; Venzon, 2021). Generalist predators can colonize and reach increased densities in a crop by feeding on nectar and pollen provided by plants associated with the crop (Tylianakis *et al.*, 2004; Géneau *et al.*, 2012). Once attracted and established in the system, the predators can attack and kill crop pests, leading to lower pest densities in comparison to crops without such resources. This is similar to a phenomenon known as apparent competition (Holt, 1977; van Rijn *et al.*, 2002; Nomikou *et al.*, 2010; Janssen & Sabelis, 2015) but, instead of being caused by the increased densities of an alternative prey species, it is mediated by non-prey food sources supplied by companion plants.

Plants producing extrafloral nectar have shown potential at promoting natural pest control in crops (Tylianakis *et al.*, 2004; Brown *et al.*, 2010; Géneau *et al.*, 2012; Rezende *et al.*, 2014, 2021). This nectar is not directly related to plant reproduction but results in the recruitment of natural enemies of its herbivores (Bentley, 1977a; Koptur, 1994). Many predator and parasitoid species are attracted to this nectar to gain energy (Bentley, 1977a; Sabelis *et al.*, 2005; Frank, 2010; Calderón-Arroyo *et al.*, 2023), and defend the plant against herbivores. This defense extends to plants nearby the nectar-producing plants, including crops, resulting in a kind of associational defense (Brown *et al.*, 2010; Staab *et al.*, 2023). For example, experiments in coffee fields in Minas Gerais - Brazil showed that *Inga* trees that produce extrafloral nectar attract many natural enemies of coffee pests, resulting in increased natural control in nearby coffee plants (Rezende *et al.*, 2014, 2021).

However, since many natural enemies are attracted to and share the same source of nectar, a range of interactions is expected to occur among them. Ants, for example, can be important natural enemies of crop pests (Van Mele, 2008; Drummond & Choate, 2011; Jones *et al.*, 2017), but they show aggressive and territorial behavior towards other arthropods, especially when extrafloral nectar is present (Bentley, 1977b; Beattie, 1985; Jones *et al.*, 2017; Calixto *et al.*, 2024). Indeed, ants are the best-studied organisms that interact strongly with plants through extrafloral nectar (Janzen, 1966; Beattie, 1985; Agrawal & Rutter, 1998). This interaction can be beneficial or detrimental to the plant, depending on the species involved and ecological conditions (Bronstein, 1994; Assunção *et al.*, 2014; Calixto *et al.*, 2024; Way, 1963). The ants collect and feed on nectar and protect the extrafloral nectaries against other arthropod competitors that may reduce nectar availability. They consume a variety of arthropods but also physically remove or cause avoidance responses (e.g., dropping) of arthropods from plants (Way 1963; Sudd 1965). The plants can benefit from the ants because these attack and reduce herbivore densities (Atsatt & O'Dowd, 1976; do Nascimento & Del-Claro, 2010; Leal *et al.*, 2023). However, when ants attack other natural enemies and/or pollinators (Eubanks, 2001; Mathews *et al.*, 2011; Anjos *et al.*, 2017; Calixto *et al.*, 2024), their presence may well be detrimental to the plant. For example, ants associated with extrafloral-nectar-producing peach trees were found to disrupt biological control of lepidopteran pests by parasitoids (Mathews *et al.*, 2011). Therefore, the effects of extrafloral nectar on pest densities may be complex, and understanding these can help us to manage the environment of crops and increase densities of beneficial arthropod groups and the ecosystem services they offer (e.g., biocontrol and pollination).

Phytoseiid mites and ants are natural enemies of various pests of coffee plants (Franco *et al.*, 2007; Lomeli-Flores *et al.*, 2009; Toledo *et al.*, 2018; Perfecto & Vandermeer, 2020) and many species of both taxa are generalists (Hölldobler & Wilson, 1990; McMurtry & Croft, 1997; Calixto *et al.*, 2021). They co-occur in coffee crops and are both found on the extrafloral nectar-producing *Inga* trees associated with coffee (Rezende *et al.*, 2014; Ferla *et al.*, 2023). Recently, we observed that nectar feeding allowed for juvenile development and increased adult survival of *Amblyseius herbicolus* (Chant, 1959) (Acari: Phytoseiidae) (Iasczczaki *et al.*, in prep.), one of the most abundant phytoseiids found in Brazilian coffee fields and a natural enemy of coffee pest mites, such as *Oligonychus ilicis* (McGregor, 1917) (Acari: Tetranychidae), *Polyphagotarsonemus latus* (Banks, 1904) (Acari: Tarsonemidae) and *Brevipalpus phoenicis* (Geijskes, 1939) (Reis *et al.,* 2001; Franco *et al*. 2007; Reis *et al*. 2007; Duarte *et al*. 2015; Toledo *et al*. 2018). This suggests that control of these pests in coffee could be increased when the predatory mite has access to nectar of *Inga*. However, it is unclear whether the ant species frequenting *Inga* nectaries are also aggressive to these nectar-feeding predatory mites. In general, the effects of the presence of ants on the performance of predatory mites and their biological control services are understudied, especially in the presence of extrafloral nectar (Haney *et al*., 1987; Osborne *et al*., 1995; Otsuki & Yano, 2014; Adachi & Yano, 2017). Indeed, basic questions such as whether ants prey on eggs, juveniles or adults of predatory mites are still unexplored.

There is some evidence of ants preying on mites in soil and leaf litter ecosystems. Some authors (Masuko, 1994; Wilson, 2005) demonstrated that ants from the *Myrmecina* and *Pheidole* genera can readily collect and eat slow-moving, armored members of mites of the order Oribatida, and carry them to their nests to feed larvae (Wilson, 2005). Other studies suggest that ants are predators of spider mites (Haney *et al*., 1987; Osborne *et al*., 1995; Otsuki & Yano, 2014), but no study has focused on interactions of ants with Phytoseiidae, whereas they will encounter each other when feeding on extrafloral nectar. Given that both can be important natural enemies of crop pests, there is a need to understand whether ants are harmful to predatory mites that feed on extrafloral nectar (Franco *et al*., 2007; Lomeli-Flores *et al*., 2009; Toledo *et al*., 2018; Perfecto & Vandermeer, 2020).

Interactions between mites and ants are quite difficult to study. First, mites are small (< 0.5 mm) and are difficult to observe without a microscope. Second, ants can carry the mites off the plants to their nests (Wilson, 2005; Otsuki & Yano, 2014), making it impossible to distinguish between predation and mite dispersal. We therefore used a simplified system of arenas in the laboratory, to which we could add ants and predatory mites in the presence of *Inga edulis* Mart. (Fabaceae) extrafloral nectaries. In this way, we assessed whether ants of various species prey upon the predatory mite *A. herbicolus*.

Material and methods

Ant species

The ants *Wasmannia auropunctata* (Roger, 1863), *Camponotus rufipes* (Fabricius, 1775), *Camponotus crassus* Mayr, 1862 and *Crematogaster* spp. are among the most frequent nectary visitors of *I. edulis* plants (Rezende *et al.*, 2014; Sinisterra *et al.*, 2016). *Camponotus* spp. are predators of coffee leaf miners (Lomeli-Flores *et al.*, 2009), while *Wasmannia* spp. and *Crematogaster* spp. prey on coffee berry borers (Lomeli-Flores *et al.*, 2009; Perfecto & Vandermeer, 2020). For this reason, we selected these ants for our study.

Individuals of all ant species used in our experiments were collected directly from branches of *Inga* trees on the campus of the Federal University of Viçosa (Figure 1). This allowed us to obtain ant workers foraging outside the nest (Hölldobler & Wilson, 1990). The ants were collected in a plastic container using a pooter and taken to the lab. Because the ants were agitated due to the sampling, we incubated them inside a refrigerator (2 to 5ºC) for a period of 7 to 10 minutes to facilitate their handling before using them in an experiment. After the experiments, all ant individuals were stored in alcohol (70%) and the species identity was confirmed by Júlio Cézar Mário Chaul (Federal University of Viçosa), a taxonomist specialized in the Formicidae family. The ant individuals were then deposited in the insect collection of the Department of Entomology at the Federal University of Viçosa.

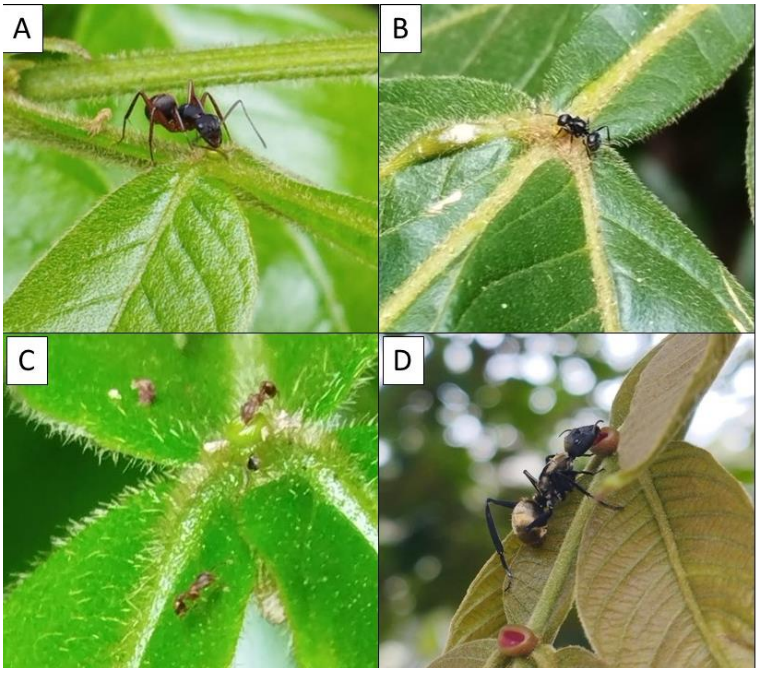


Figure 1. Ant workers from several species foraging on extrafloral nectaries from *Inga* plants on the campus of the Federal University of Viçosa. A) *Ca. rufipes*; B) *Ca. crassus*; C) *W. auropunctata*; D) *Ca. sericeiventris*. Pictures by the author.

Predatory mite rearing

The predatory mites used in the experiments were from a strain of *A. herbicolus* collected from tomato plants in the municipally of Prados (latitude: 21°03′00″ S; longitude: 44°04′47″ W), Minas Gerais, Brazil, in 2014 (Cardoso *et al*., 2024; Kalile *et al*., 2021). The rearing was maintained on arenas consisting of a black plastic sheet (10 x 14 cm) surrounded by a barrier of moistened cotton wool, put on a wet sponge inside a plastic tray containing water (McMurtry & Scriven, 1965) at 25 ± 2 °C, 70 ± 10% RH and L:D 12:12.

The predators were fed with *Typha* spp. pollen, twice per week. We provided cotton threads under pieces of black plastic sheet (1 x 1 cm²) to serve as ovipositing sites and refuge, respectively. *Amblyseius herbicolus* reproduces thelytokously (De Moraes & Mesa 1988) and to obtain cohorts of similar-aged individuals, threads with eggs were collected from the rearing units and were placed on a new arena. Ten - 12 days after hatching, young adult females were available for experiments. When larvae were needed, they were obtained immediately after the collected eggs had hatched.

Extrafloral nectaries

Extrafloral nectaries were collected by cutting young branches with completely expanded composite leaves from an *I. edulis* tree located around the Federal University of Viçosa, because nectaries on young branches produce more nectar (Pireda *et al*., 2018). To remove arthropods from the leaves, they were submersed in water for about 30 minutes. Subsequently, the leaves were dried and placed in a jar (ø = 10.5 cm; 6 cm high) with their stem inserted into water for 24 h to allow for nectar secretion. Then, nectaries containing similar amounts of nectar were selected and carefully cut out, using a surgical knife and scissors. Finally, the selected nectaries were used for experiments.

Validation of the experimental method

Because we removed the ant workers from their natural environment near their colony and placed them in a simplified system, these experiments aimed to validate whether the ants showed predation behaviour in our experimental set-up. Termites of *Cornitermes* spp. (Termitidae: Syntermitinae) were gently collected with the help of tweezers from a nest in the surroundings of the Federal University of Viçosa. We used them as prey model in our experiment and we used ants from *Ca. rufipes* as predator, because termites have been successfully used as prey in testing ant predation (Saks & Carroll, 1980; Oliveira, 1997; Apple & Feener, 2001; Calixto *et al.*, 2021). To test predation for *Ca. crassus* and *W. auropunctata* ants, we used coffee berry borers *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae) collected from fruits obtained from abandoned coffee plants found in the surroundings of the Federal University of Viçosa. In lab we gently opened the coffee fruits and collected either, coffee berry borer adults, or first-second instar larvae, to use them as prey in two experiments.

The experimental units comprised artificial arenas consisting of black plastic dishes (ø = 5.5 cm; 1.4 cm high) covered with transparent lids. One termite worker and one *Ca. rufipes* ant individual were released per arena, either in the presence of extrafloral nectar (N = 29) or not (N = 25). A third treatment consisted of one termite without an ant to check for natural mortality (N = 24). We made the same for coffee borers, but always in presence of a producing *Inga* nectary in the arenas. We placed one borer adult per replicate, in presence of *W. auropuntata* (N=26), *Ca. crassus* (N=24) or no ants (N=22). Finally, in the second experiment involving this species, we placed one larvae of coffee borer in each arena in presence of *W. auropunctata* ants (N=) or not (N=). We also used borer larvae for *W. auropunctata* ants, because other studies noticed this pest stage is frequently preyed by this ant (Morris & Perfecto, 2016). The availability of nectar at the end of the experiments was considered as a factor in the analysis, because it could influence the ant predation activity.

Twenty-four h later, the proportion of alive termites, coffee berry borer adults or larvae was recorded and compared among treatments with a generalized linear model (GLM) with a binomial error distribution (link = logit) and contrasts among treatments were assessed with the package emmeans with a Tukey correction for multiple comparisons (Lenth et al., 2019).

Ant predation on eggs, juveniles and adult mites

This series of experiments was designed to identify whether workers from various ant species prey upon different life stages of predatory mites in the presence of *Inga* extrafloral nectar. The experimental units were the same as above. Adults, larvae and eggs of *A. herbicolus* were tested individually with each ant species, together with a control without ants to assess natural mortality, always in the presence of a producing nectary. The availability of nectar at the end of the experiments varied because of consumption by the ant workers and some extent of evaporation in the 24h period. Because the ants may attack other food sources (i.e., mites) in the absence of nectar, the presence/absence of nectar at the end of the experiment was used as factor in our analysis. All experiments and their respective numbers of repetitions are summarized in Table 1 and 2. *Crematogaster* spp. ants were harder to find in the field, resulting in too few replicates with this species. For this reason, we decided not to analyze data from these experiments (Table 1 and 2; but see also the attachment 1).

We released one young adult female mite (10-12 days) per experimental unit with a nectary full of nectar and subsequently one ant worker of one of the species. Subsequently, we checked the arenas for accidental ant and/or mite deaths or escapes during the manipulation. When this occurred, we added a new mite and/or ant individual. Twenty-four hours later, we searched for evidence of predation. Because we could not collect all ant species at the same day, two experimental blocks with different ant species combinations (as treatments) were carried out for predation on adult mites: A) *Ca. rufipes*, *W. auropunctata, Ca. crassus* and no ants; B) *Ca. crassus*, *Crematogaster* spp., and no ants (Table 1, predation on adults A and B). In all cases, the proportions of alive adult mites were compared using a GLM with a binomial error distribution, considering nectar availability (yes/no) at the end of the experiment. Replicates in which ants died during the experiment or where mites drowned in the nectar were excluded from the analysis (Table 1).

The same protocol was applied with *A. herbicolus* larvae. We collected the larvae directly from the rearing units and released them each on a separate arena with nectar, either in the presence of ants or not. Two experimental blocks testing different ant species (as treatments) were done. During the first block, we tested *W. auropunctata*, *Ca. rufipes* and *Ca. crassus* ants, comparing them to a control without ants. In the second block, mite larvae were exposed to *Ca. crassus*, *Crematogaster* spp., *W. auropunctata* and no ants for control (Table 1, predation on juveniles A and B). The proportion of alive mite larvae was compared among treatments as above.

Table 1. Ant predation experiments on juveniles and adults of *Amblyseius herbicolus*. Shown are the initial numbers of replicates per treatment, including those excluded

followed by the reason for exclusion (either the mite drowned, or the ant died) and the final number of replicates considered for analysis.

Experiment	Treatment	Initial N	Ant death	Drowned mite	Final N
Predation on adults (A)	*Ca. rufipes*	25	2	1	22
	W. auropunctata	26	1	2	23
	Ca. crassus	27	1	2	24
	No ants	23	-	0	23
Predation on adults (B)*	*Ca. crassus*	32	1	10	21
	Crematogaster spp.	9	0	0	9
	No ants	17	-	0	17
Predation on juveniles (A)	*W. auropunctata*	28	2	2	24
	Ca. rufipes	25	0	1	24
	Ca. crassus	26	3	3	20
	No ants	24	-	1	23
Predation on juveniles (B)*	*Ca. crassus*	17	1	1	15
	Crematogaster spp.	9	0	0	9
	W. auropunctata	15	1	2	12
	No ants	15	-	1	14

*Data not analysed

To measure predation of predatory mite eggs by ants, we collected eggs from the mite rearing and placed them directly on the nectary structures inside the arenas. Subsequently, ant workers were released as above. Because we could not collect all ant species at the same day, three blocks with different ant species were carried out on different days. In the first block (A) we tested only *Ca. rufipes* with a control without ants. The mite egg numbers varied from 1 up to 3 per arena in this test. During the second block (B) we exposed exactly 2 mite eggs per arena to *Ca. crassus*, *Crematogaster* spp. and *W. auropunctata* and to a control without ants. The same was done in the third block (C), where 2 mite eggs were exposed to *W. auropunctata* and *Ca crassus* ants and to a control without ants (Table 2). In all cases, we assessed the proportions of intact eggs after 24 h and compared them among treatments using a generalized linear model (GLM) with a binomial error distribution (log link). Because we failed to score the availability of nectar at the end of blocks A and B, its effect was only analyzed for block C.

Table 2. Ant predation experiments on eggs of *Amblyseius herbicolus*. Shown are the initial numbers of replicates per treatment, including those excluded followed by the reason for exclusion (ant died) and the final number of replicates considered for analysis.

Experiment	Treatment	Initial N	Ant death	Final N
Predation on eggs (A)	*Ca. rufipes*	30	0	30
	No ants	21	-	21
Predation on eggs (B)*	*Ca. crassus*	15	0	15
	Crematogaster spp.	15	3	11
	W. auropunctata	15	3	10
	No ants	15	-	12
Predation on eggs (C)	*W. Auropunctata*	28	0	28
	Ca. crassus	25	0	25
	No ants	22	0	22

*Data not analysed

Results

Validation of the experimental method

The proportion of alive termites after 24 h varied significantly among treatments (GLM: Deviance = 41.3, d.f. = 2, $p < 0.001$; Figure 2) and was significantly lower in the presence of ants. The *Ca. rufipes* workers preyed readily upon the termites and the extrafloral nectar did not influence the proportion of surviving termites.

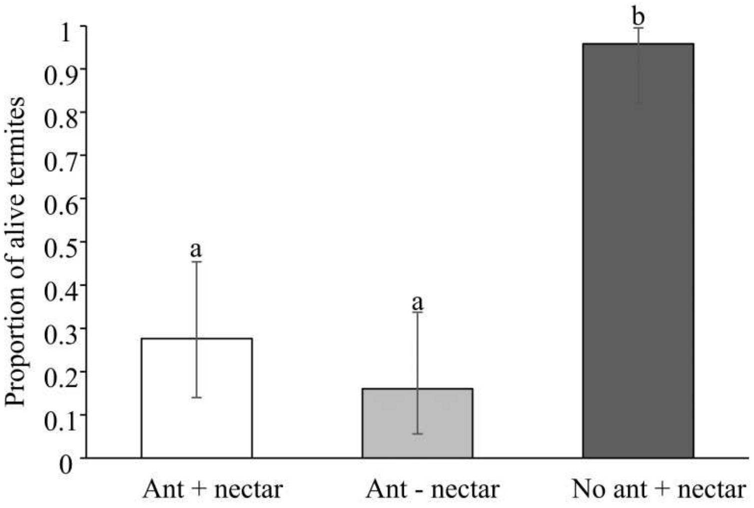


Figure 2. Proportion of alive termites (± 95% CI) after 24 h of exposure to ants, either with extrafloral nectar or not, and no ants but with nectar (control). Significant differences among treatments are indicated with letters above the bars with $p < 0.05$ (contrasts after GLM).

Similar results were observed when we offered coffee berry borers to *Ca. crassus* and *W. auropunctata* ants. The proportion of alive adult borers varied significantly among treatments (GLM: Deviance = 7.6, d.f. = 2, p = 0.002; Figure 3A) but there was no effect of nectar availability in such results. The number of adult borers found alive in the arenas was significantly higher without ants but similar between the two ant species (Figure 3A). We also noticed that borer larvae were preyed upon by *W. auropunctata* ants, with the number of alive individuals being significantly lower in presence of ants (GLM: Deviance = 4.96, d.f. = 1, p = 0.02; Figure 3B), and no effects of nectar availability were detected. Therefore, our simplified system of individual arenas was adequate for studying predation behaviour of the tested ant species, also when isolated from their nests.

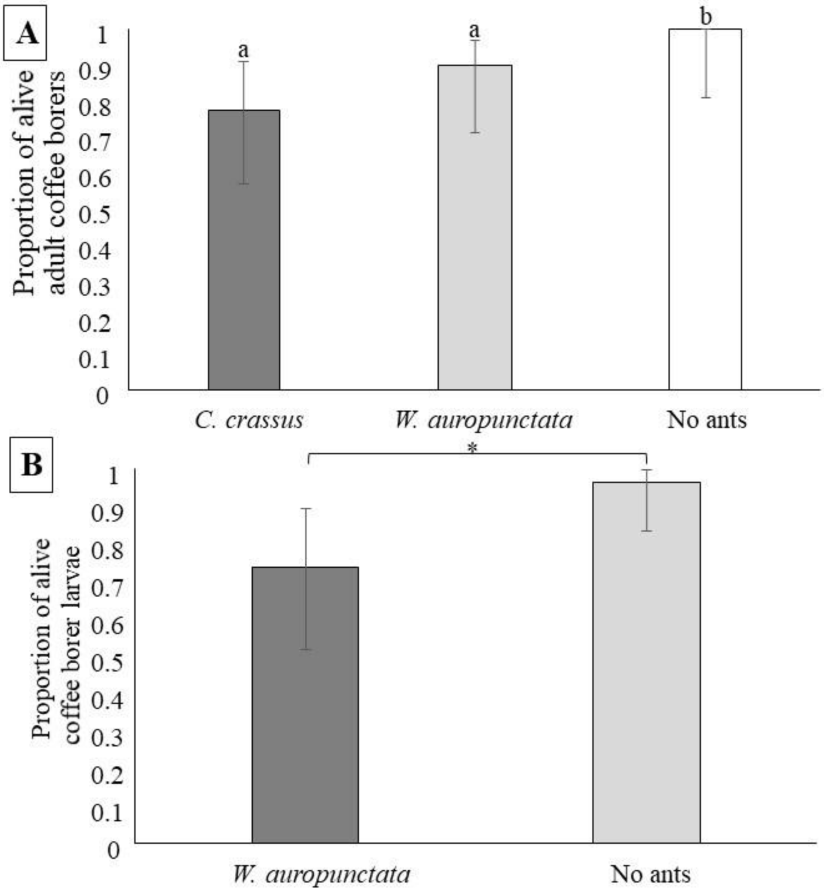


Figure 2. Proportion of alive adults (A) and larvae (B) of coffee berry borers (± 95% CI) after 24 h of exposure to ants or no ants, but always in presence of nectar. Significant differences among treatments are indicated with letters or asterisks above the bars with $p < 0.05$ (contrasts after GLM).

Ant predation on adult mites

The presence of *Ca. rufipes*, *W. auropunctata* and *Ca. crassus* ants did not affect the proportion of alive adult mites after 24 h in the presence of *Inga* extrafloral nectar (GLM: Deviance = 4.53, d.f. = 3, p = 0.20; Figure 3) and it did not differ with nectar availability at the end of the experiment (GLM: Deviance = 0.20, d.f. = 1, p = 0.65)

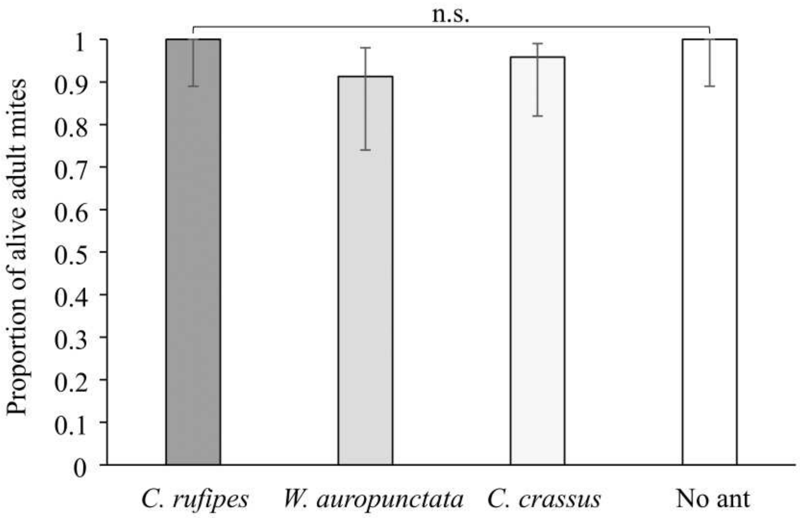


Figure 3. Proportion (± CI) of alive adult mites in the presence of extrafloral nectar and either ant workers from *Ca. rufipes*, *W. auropuntata* and *Ca. crassus* or no workers (control).

Ant predation on juvenile mites

Neither of the three species of ants affected the proportion of alive *A. herbicolus* larvae in the presence of *Inga* extrafloral nectar compared to a treatment without ants (GLM: Deviance = 1.24, d.f. = 3, p = 0.69; Figure 4). The nectar availability at the end of the experiment also did not affect the survival of predatory mite larvae (GLM: Deviance = 0.97, d.f. = 1, p = 0.32).

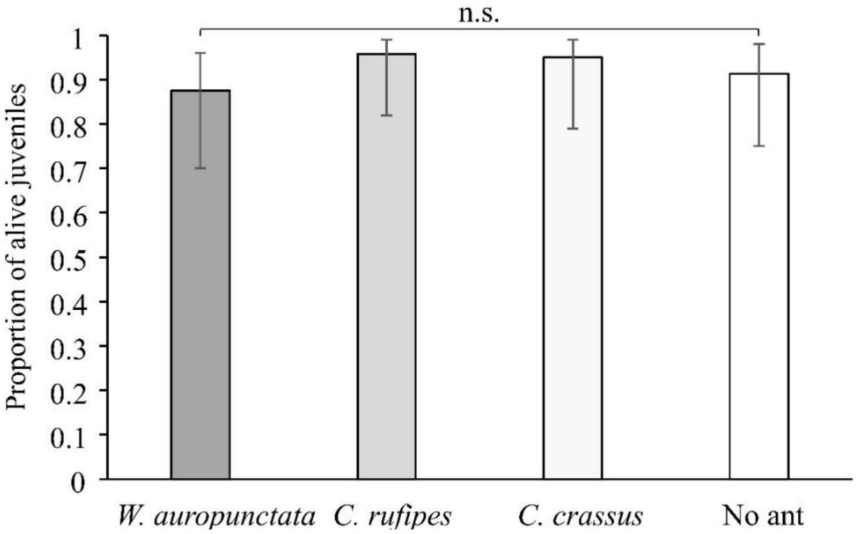
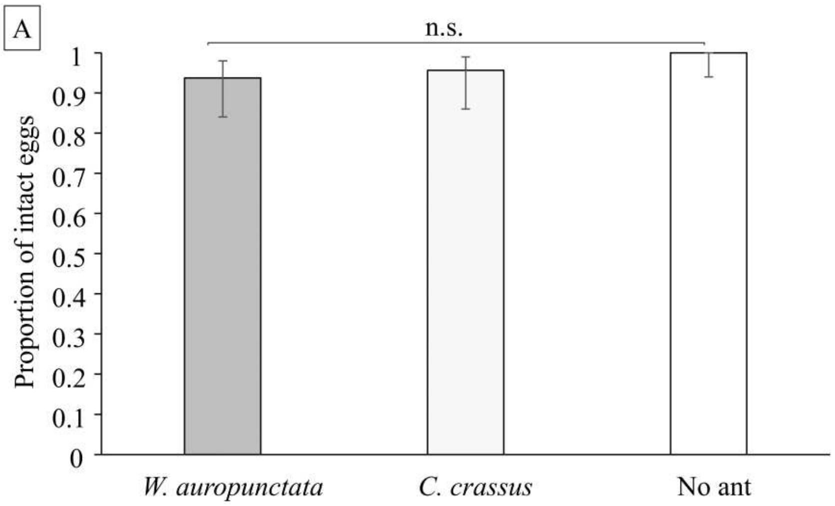


Figure 4. Proportion of alive larvae (juveniles) (± CI) of *A. herbicolus* in the presence of extrafloral nectar and either *Ca. rufipes, W. auropunctata*, *Ca. crassus* ant workers or without ants (control).

Ant predation on mite eggs

We did not find evidence for *W. auropunctata* or *Ca. crassus* ants preying upon mite eggs (GLM: Deviance = 3.65, d.f. = 2, p = 0.16; Figure 5A). Predation was also not influenced by the presence of nectar at the end of this experiment (GLM: Deviance = 0.004, d.f. = 1, p = 0.94). Similar results were found during the second experiment with *Ca. rufipes*, where the proportion of intact eggs after ant exposure was similar to that without ants (GLM: Deviance = 0.20, d.f. = 1, p = 0.65; Figure 5B).



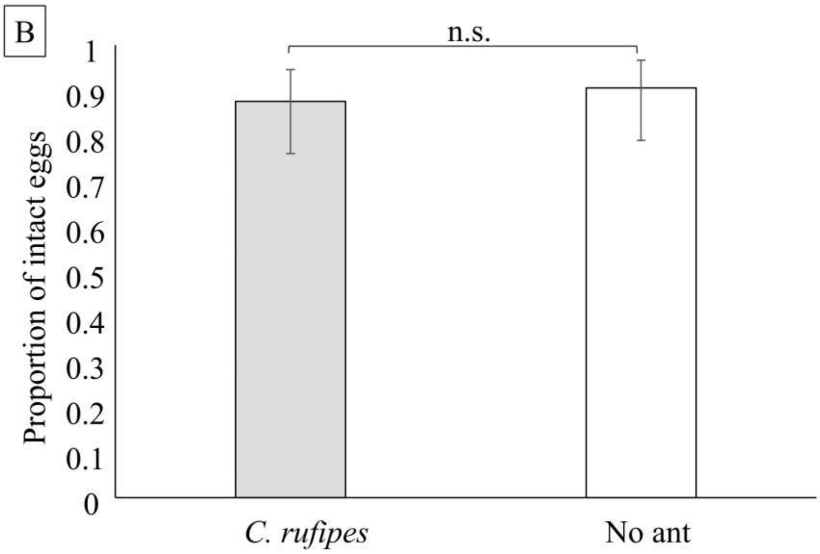


Figure 5. Proportion of intact mite eggs (± CI) in the presence of extrafloral nectar and, either A) with ants of the species *W. auropunctata* and *Ca. crassus* or no ants (control); B) likewise with *Ca. rufipes* ants or no ants (control).

In contrast to *W. auropunctata*, we observed that *Ca. rufipes* and *Ca. crassus* ants drank all nectar present in the arenas during the experiments with mites and termites (Figure 6).

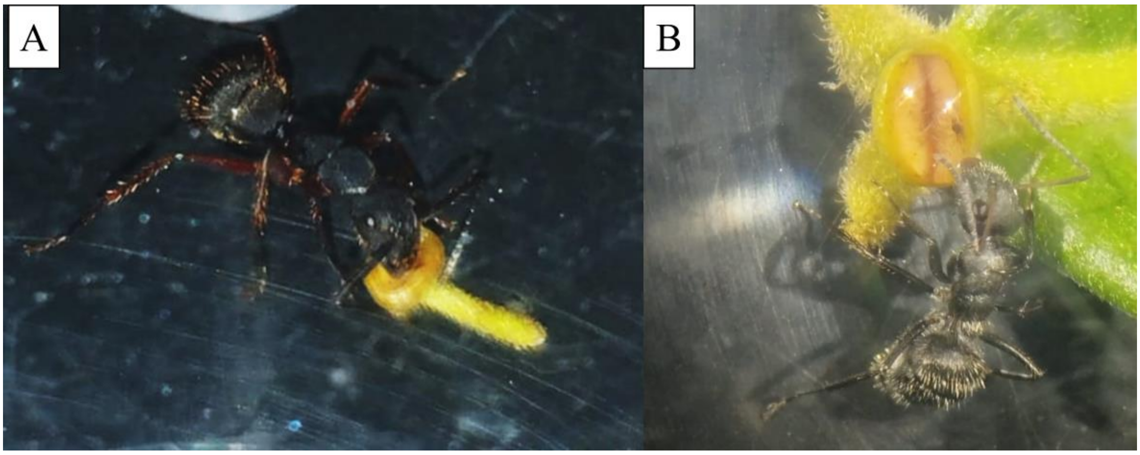


Figure 6. Ant workers from A) *Ca. rufipes* and B) *Ca. crassus* drinking the nectar from nectaries in the experimental units.

Discussion

Our study is the first to identify that various species of ants, which are important natural enemies of coffee pests (Lomeli-Flores *et al.*, 2009; Perfecto & Vandermeer, 2020), do not prey upon any life stage of a predatory mite in the presence of extrafloral nectar.

Our results suggest that the ants are not directly harmful to predatory mites, suggesting that these can persist with ants.

Many plants produce extrafloral nectar continuously, but nectar secretion can also be induced by attacks by herbivores (Wäckers & Bonifay, 2004; Heil, 2015). Ants are attracted to plants with extrafloral nectar and are known to act aggressively towards other potential competitors for the nectar, but also towards herbivores attacking the plant (do Nascimento & Del-Claro, 2010; Leal *et al.*, 2023) and other arthropods (Mathews *et al.*, 2011; Calixto *et al.*, 2024). In our study, the ants and the *Inga* producing nectaries were placed in a small arena in the presence of predatory mites. This would promote predation events and other aggressive behaviours by ants, if they recognize the mites as potential prey or nectar competitors. Because the ants were collected and almost immediately used in our experiments, we do not know whether they were satiated or starved or whether this affected the outcome of our experiments. However, we observed the ant workers feeding on the nectar inside the arenas (Figure 6), revealing some level of hunger. Moreover the *Ca. rufipes* workers preyed on almost all termite individuals, and the other ant species were also found to prey on the coffee berry borers, both on adults and larvae, suggesting that these ants do prey on other species under our experimental conditions. However, they did not attack the predatory mites, suggesting that the tested ant species do not prey on *A. herbicolus* in the presence of extrafloral nectar. In several replicates, however, the extrafloral nectar was completely consumed, and the ants would then perhaps search for other food sources, such as the mites. We therefore tested whether the predation of mites was higher in replicates where the nectar was finished, but found no such effect for any of the ant species.

Wilson (2005) offered a range of arthropod groups in soil-litter samples as prey to *Pheidole* ants and showed that the ants did not feed on fast-moving, predatory mite species of the suborder Mesostigmata and Prostigmata. Possibly, *A. herbicolus* adults were fast enough to escape from the ants in the arenas used in our study. However, eggs and relatively slow-moving juveniles, were also not preyed by any of the ant species, further confirming that these ants are no predators of *A. herbicolus*. Furthermore, mites may simply not be recognized as potential competitors or prey by ants due to their small size and the small amount of nectar they consume. We noticed that one full nectary of *Inga* is enough to feed more than 20 starved adult *A. herbicolus* mites during 24 h (RS Iasczczaki, pers. obs.). van Rijn & Tonigoshi (1999) offered three droplets of castor bean (*Ricinus communis* L.) extrafloral nectar per arena, which was enough for three days of feeding by ca. 30 adults of *Iphiseius degenerans* (Berlese). This suggest that mites need quite small amounts of nectar in comparison to ants and other larger arthropods, and depending on the mite densities ants may simply ignorethem as competitors.

In contrast, the fast consumption of nectar by the ants suggest that they are strong competitors for nectar, as also observed by other authors (Janzen, 1966; Beattie, 1985; Agrawal & Rutter, 1998). The tested ant species differed in the amount of nectar consumed, with the *Camponotus* species consuming all available nectar in less than 24 h in almost all replicates. Therefore, testing whether this competition for nectar, but also the sole presence of ants, affects long-term performance and biological control by predatory mites is of importance. Hence, many questions remain on the interactions of ants, extrafloral nectar and *A. herbicolus*, with possible practical implications.

Predatory mites do not only feed on nectar, but mainly on herbivorous mites, which are quite abundant on *Inga* (Ferla et al., 2023). Generalist phytoseiids such as *A. herbicolus* also feed on pollen (Duarte et al., 2015), which is also present during the limited period that *Inga* flowers, but whether this pollen is edible to *A. herbicolus* needs further investigation. *Inga edulis* leaves are densely covered with trichomes (hairs) which may capture pollen from the air (Addison et al., 2000; Schmidt, 2014; Montserrat et al., 2013), and also function as substrate for oviposition and refuge for the predatory mites (Schmidt, 2014). In contrast to predatory mites, ants are social organisms, and may visit extrafloral nectar plants for limited periods (Belchior et al., 2016), where they also hunt and kill herbivorous arthropods. Hence, whereas ants go to the *Inga* plants for nectar and consume it fast, predatory mites have other types of food (e.g., pollen) that may not be shared with ants, as well as nectar to use during periods where ants are not present.

Pests may suffer increased mortality in agricultural systems harboring a higher diversity of natural enemies (Tylianakis *et al.*, 2004; Brown *et al.*, 2010; Géneau *et al.*, 2012; Jones *et al.*, 2017; Rezende *et al.*, 2014, 2021). Richer communities of predators consist of natural enemies that attack different pest species, use different hunting strategies at different times and places (Ives *et al.*, 2005). This may cause complementarity in the feeding niches of the natural enemies (MacArthur & Levins, 1967). Our preliminary results suggest that an extrafloral-nectar-producing plant can simultaneously benefit ants and predatory mites, which control different pest species in an associated crop, hence, the presence of *Inga* and other plants producing extrafloral nectar may improve the ecosystem service of biocontrol.

Author contributions

Rafael Stempniak Iasczczaki, Arne Janssen, Angelo Pallini and Madelaine Venzon conceived the ideas and designed methodology; Rafael Stempniak Iasczczaki, Gabriel Modesto Beguelli, Caio Binda de Assis and Gabriel Henrique Pio collected the data; Rafael Stempniak Iasczczaki and Arne Janssen analyzed the data; Rafael Stempniak Iasczczaki and Arne Janssen led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Conflict of interest

The authors declare no conflicts of interest. This research requires no ethical approval for the use of animals.

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ATTACHMENT 1. Data of experiments not considered in the analysis.

Table 3. Ant predation experiments on adults and larvae of *Amblyseius herbicolus* that were not considered in the analysis. Shown are the summarized results of adults and larvae before (total of individuals) and after 24 h of releasing ants from various species in the arenas. The proportions of mites (adults and larvae) and lower/higher 95% confidence intervals are also presented

Experiment	Treat	N of mites after 24h	N total of mites	Prop.	LwrCI	UprCI
Predation on juveniles (B)	*W. auropunctata*	8	12	0.67	0.28	0.20
	C. crassus	9	14	0.64	0.26	0.20
	No ants	11	14	0.79	0.26	0.15
	Crematogaster spp.	9	9	1.00	0.24	0.00
Predation on adults (B)	*Crematogaster spp.*	9	9	1.00	0.24	0.00
	C. crassus	17	21	0.81	0.20	0.12
	No ants	16	17	0.94	0.19	0.05

Table 4. Ant predation experiment on mite eggs not used in the analysis. Shown are the summarized results of eggs before (total of eggs) and after (egg n after 24h) releasing ants from various species in the arenas. The proportions of eggs and lower/higher confidence intervals are also presented

Experiment	Treat	Egg n after 24 h	Total of eggs	Prop.	LwrCI	UprCI
Predation on eggs (B)	No ants	24	24	1	0.09	0
	W. auropuctata	20	20	1	0.11	0
	C. crassus	27	30	0.9	0.14	0.07
	Crematogaster spp.	22	22	1	0.10	0

General conclusion

My dissertation showed for the first time that the extrafloral nectar produced by coffee-intercropped *Inga* trees allows for development and survival of an important predatory mite which is natural enemy of various crop pests. This suggests that interplanting *Inga* and other extrafloral nectar-producing plants can maintain populations of predatory mites and possibly boost their biological control of pests in agricultural crops. Furthermore, I offered the first study on the interactions of various ant species and a predatory mite in the presence of *Inga* nectaries, a food source shared by both natural enemy taxa. We revealed that ants are not directly harmful to predatory mites and thus, nectar producing plants may attract and provide alternative food for these two natural enemy groups of importance in agricultural fields. Therefore, this dissertation contributes to the fields of ecology and biological control, and I argue to the importance of this knowledge to the development of more sustainable practices of pest control in coffee and other agroecosystems.