MILENA OLIVEIRA KALILE

BIOLOGICAL CONTROL OF *Diaphorina citri* WITH THE PREDATORY MITE *Amblyseius herbicolus*

Dissertation presented at the Universidade Federal de Viçosa as part of the requirements of the Entomology Graduate Program, to obtain the title of *Magister Scientiae*.

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“A senhorita não compreende. Para a senhorita, é insuportável ferir alguém. Mas, para algumas mentes, mais insuportável ainda é não saber (...) para a mente científica, a verdade vem em primeiro lugar. A verdade, por mais amarga que seja, pode ser aceita e tecida num padrão de vida.”

Agatha Christie

ABSTRACT

KALILE, Milena Oliveira, M.Sc., Universidade Federal de Viçosa, February, 2020. **Biological control of *Diaphorina citri* with the predatory mite *Amblyseius herbicolus*.** Advisor: Arnoldus Rudolf Maria Janssen. Co-advisors: Angelo Pallini Filho, Morgana Maria Fonseca Porto, Simon Luke Elliot and Verônica Saraiva Fialho.

Pesticide use is becoming increasingly limited worldwide because of problems with the environment, food safety and their negative effects on beneficial animals such as pollinators and natural enemies. In addition, it can cause pest resistance, starting a vicious cycle of increases of doses that do not solve the pest problem but increase it. Biological control is a key alternative to pesticide use to control crop pests, because it is safe, often effective and environmental-friendly. Generalist predators are promising biological control agents because their populations can be established in the crop with alternative food earlier in the season, which makes the system more resilient to pest invasions. Predatory mites, many of which are generalists, are among the best-selling natural enemies to control crop pests. Nevertheless, much remains to be investigated about natural enemies controlling insect vectors and the possible reduction of pathogen spread. For example, there is still no natural enemy commercialized to control the Asian citrus psyllid *Diaphorina citri*, vector of the bacteria that cause Huanglongbing or Greening, the most devastating citrus disease in the world, also causing considerable economic losses in Brazil. Biological control with parasitoids of *D. citri* is not effective enough and mass rearing of this natural enemy is laborious. I studied *Amblyseius herbicolus*, a generalist predatory mite, which can potentially control *Bemisia tabaci* and *Polyphagotarsonemus latus*, and occurs on citrus and orange jasmine plants in Minas Gerais, Brazil. My aim was to evaluate the potential of this predator to control *D. citri*. Additionally, I explored methods to enable mass rearing of this predator for future field releases and commercialization. I show that *A. herbicolus* is potentially effective to control *D. citri* in laboratory tests and on orange jasmine plants. Besides, *A. herbicolus* can develop and reproduce on the storage mite *Thyreophagus cracentiseta*, which will facilitate mass rearing and releases of this predator in the field.

Keywords: Greening. Citrus. Asian citrus psyllid. Generalist predators. Mass rearing.

RESUMO

KALILE, Milena Oliveira, M.Sc., Universidade Federal de Viçosa, fevereiro de 2020. **Controle biológico de *Diaphorina citri* com o ácaro predador *Amblyseius herbicolus*.** Orientador: Arnoldus Rudolf Maria Janssen. Coorientadores: Angelo Pallini Filho, Morgana Maria Fonseca Porto, Simon Luke Elliot e Verônica Saraiva Fialho.

O uso de pesticidas é cada vez mais limitado por causa de problemas com o meio ambiente, segurança alimentar e efeitos negativos em animais benéficos como polinizadores e inimigos naturais. Além disso, pode causar resistência da praga, iniciando um ciclo vicioso de aumento das doses que não apenas não resolvem como agravam o problema. O controle biológico é uma alternativa chave ao uso de pesticidas para controlar pragas agrícolas, porque é seguro, frequentemente efetivo e favorável ao meio ambiente. Predadores generalistas são agentes promissores de controle biológico porque suas populações podem ser estabelecidas na cultura com alimento alternativo no início do cultivo, o que faz o sistema mais resiliente contra invasões de pragas. Ácaros predadores, a maioria dos quais é generalista, estão entre os inimigos naturais mais vendidos para controlar pragas agrícolas. Porém, muito precisa ser investigado sobre inimigos naturais controlando insetos vetores e a possível redução do espalhamento de patógenos. Por exemplo, ainda não há inimigo natural comercializado para controlar o psilídeo asiático dos citros, *Diaphorina citri*, vetor das bactérias que causam Huanglongbing or Greening, a doença mais devastadora dos citros no mundo, também causando consideráveis perdas econômicas no Brasil. O Controle biológico com parasitóides de *D. citri* não é efetivo o suficiente e a criação em massa é trabalhosa. Eu investiguei *Amblyseius herbicolus*, um ácaro predador generalista que pode potencialmente controlar *Bemisia tabaci* e *Polyphagotarsonemus latus* e ocorre em citros e plantas de murta em Minas Gerais, Brasil. O objetivo deste trabalho foi avaliar o potencial deste ácaro para controlar *D. citri*. Meu objetivo foi avaliar o potencial deste predador para controlar *D. citri.* Adicionalmente, eu explorei métodos para permitir criação em massa deste predador para futuras liberações em campo e comercialização. Eu mostrei que *A. herbicolus* é potencialmente efetivo para controlar *D. citri* em testes de laboratório e em plantas de murta. Além disso, *A. herbicolus* pode se desenvolver e reproduzir no ácaro de armazenamento *Thyreophagus cracentiseta* o que irá facilitar a criação em massa e liberações deste predador em campo.

Palavras-chave: Greening. Citros. Psilídeo asiático dos citros. Predadores generalistas. Criação em massa.

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GENERAL INTRODUCTION

Fossil evidence indicates that much of the planet is covered by plants since the beginning of multicellular life (Stebbins and Hill 1980; Karban and Baldwin 1997). The maintenance of this condition is related to the evolution of a sophisticated system of plant defence against consumption by herbivores (Murdoch 1966; Fox et al. 1981), which includes the actions of the third trophic level, which can be seen as part of the plant's set of defences (Price et al. 1980). The interactions between plants and the natural enemies of the herbivores result in reductions of herbivore densities (Holt 1977; Sabelis and Jong 1988; Sabelis et al. 1999; Price et al. 1980; Bouvet et al. 2019).

Biological control uses this phenomenon of natural enemies that control pest populations in crops (Elienberg et al. 2001). Nowadays, pesticide use is increasingly limited by law in several countries (Handford et al. 2015) and biological control is a key alternative (Hajek and Elienberg 2018; Bouvet et al. 2019). Biological control is an environmental-friendly pest management strategy that meets growing demand for higher-quality and safer food (Altieri 1999; Bale et al. 2008; Tixier et al. 2018). Moreover, biological control does not cause several problems arising from pesticide use, such as environmental pollution, development of resistance in pests, secondary pest outbreaks and negative effects on beneficial arthropods (Geiger et al. 2010; Calvo et al. 2015).

Natural enemies can be used in three biological control strategies: classical, when a natural enemy, from the same area of origin as the invasive pest, is introduced to control it; conservation biological control, where agricultural systems are managed to enhance the occurrence and control provided by natural enemies; or augmentative biocontrol, in which mass-reared natural enemies are released (van Lenteren and Bueno 2003; Bale et al. 2008; Calvo et al. 2014; Tixier et al. 2018).

Natural enemies can be specialists or generalists with respect to the range of prey species and other food types that they consume, including resources of plant origin (McMurtry 1992; Snyder and Ives 2001). In the past, generalist predators were considered poor candidates for effective biological control because, differently from specialists, they are polyphagous and their population dynamics are uncoupled from that of a specific prey. (Huffaker et al. 1971; Murdoch et al. 1985; Hassel and May 1986; Symondson et al. 2002). However, populations of specialist natural enemies will be reduced when pest populations decrease and cannot be introduced into a crop before the pest is present (Janssen and Sabelis 2015). This does not apply to generalists because they can feed on alternative food when pest densities are low (Murdoch and Oaten 1975; Hassel and May 1986). The increase of generalist predator densities, even in the absence of a specific pest, may allow the prevention of pest invasions and outbreaks (Murdoch et al. 1985) and they are therefore nowadays considered good biological control agents (Symondson et al. 2002).

In general, generalists show less need to disperse to new areas when densities of a particular prey are low because they can feed, reproduce and survive on other food sources in the area, such as pollen, nectar, fungi, and also on alternative prey (McMurtry 1992; van Rijin et al. 2002; Wäckers et al. 2008; Pilkington et al. 2010; Rao et al. 2018). As a consequence of the increased density of generalist predators feeding on alternative food, pest densities will be kept at a low level, a phenomenon similar to apparent competition (van Rijn et al. 2002; Nomikou et al. 2010; Janssen and Sabelis 2015). With apparent competition, predator densities will be increased by feeding on a prey or other food sources, and as a consequence the density of the target pest will be reduced (Holt 1977, Messelink et al. 2008).

Interactions between pests and natural enemies become more complex when the pest is the vector of a plant pathogen because the action of natural enemies of the herbivore can also affect disease spread (Queiroz et al. 2016; Tholt et al. 2018). In the case of pathogen vectors, every herbivore is not only a direct threat, but also acts as a potential Trojan horse, carrying an army of microscopic invaders to the plants (Karban and Baldwin, 1997). In order to limit the transmission of vector-borne plant pathogens, it is necessary to understand their effects on hosts, vectors and natural enemies in a multi-trophic perspective, because these interactions may affect the population dynamics of vector and disease (Wang et al. 2017; Zhang et al. 2019). Predation may be efficient to control vector of diseases and to reduce disease spread (Anderson and May 1991; Moore et al. 2010) and the use of predators in these systems is progressively increasing (Okamoto and Amarasekare 2012).

The control of vector-borne crop diseases and studies on biological vector control are still scarce, although this method has the potential to be safer and more cost-effective than others (Zhou and Yao 2014). The goal of biological control of vectors is not necessarily to exclude the pest, but to reduce the incidence of the disease, or even eradicating it, by limiting or interrupting pathogen spread (Murdoch et al. 1985; Moore et al. 2009; Okamoto and Amarasekare 2012; Zhou and Yao 2014). For this, it is necessary to identify the density threshold of the vector below which pathogen establishment is prevented, and which natural enemy density is sufficient to maintain the vector below this threshold (Moore et al. 2009).

Sufficient control of vector population to reduce pathogen spread depends on characteristics of vector and pathogen, such as transmission efficiency and infectiousness. In vector-borne diseases, transmission is closely linked to the population dynamics of the pest and natural enemies (Howard 2013). Even if predation does not remove the pathogen, this may still reduce the prevalence of the disease in the host plant population, because the densities of the vector and infected plants can be reduced (Moore et al. 2009; Zhou and Yao 2014). Predation of a vector may reduce disease transmission, especially in the initial phase of pathogen spread (Moore et al. 2009; Long and Finke 2015).

Predators are used to control vectors that transmit diseases to humans and animals. such as the mosquito species that transmit malaria and dengue fever (Legner 1995; Nelson and Jackson 2006; Chandra et al. 2008; Bowatte et al. 2013; Moore et al. 2010). There are also studies on the control of herbivorous pests that are vectors of crop plants, such as aphids that are vectors of pathogens that cause cereal yellow dwarf virus in wheat and beet yellow virus in sugar beet (Landis and Van der Werf 1997; Long and Finke 2015; de Oliveira et al. 2014). Control with predators of thrips and whiteflies that are also transmitters of several viral diseases such as tospoviruses, tomato leaf curl and cassava mosaic virus are also investigated (Castañé et al. 1999; Shun-xiang et al. 2008; Legg et al. 2014). However, biological control of vectors is still scarce.

Among the pathogens affecting agricultural crops, extensive damage is caused by diseases associated with *Candidatus* Liberibacter species, especially on citrus and potato, and the affected areas are increasing due to territorial expansion of the vectors worldwide. In all cases, the vectors of these pathogens are specific psyllids (Wang et al. 2017). In this context, huanglongbing (HLB) or greening is the most devastating incurable vector-borne disease that threatens the citrilaboratory worldwide. The pathogens are phloem-restricted bacteria (*Candidatus* Liberibacter spp.), widespread in citrus production areas and mainly transmitted by *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) ((McClean, 1970; Bové, 2006; Damsteeg et al. 2010; Wang et al. 2017). All citrus varieties are susceptible and symptomatic plants must be eradicated because the disease is incurable and lethal. However, symptoms may be expressed only several years after infection, and the main approach to limit the disease spread is therefore vector control (Grafton-Cardwell et al. 2013; Monzó and Stansly 2019).

Biological control of pests in citrus crops has been successfully used in several countries (Caltaginore and Doutt 1989; Tanigosh 1991; Murdoch et al. 1995; Jacas and Urbaneja 2010). The genus *Citrus* of the Rutaceae family is one of the most cultivated fruit crops in the world

(Wu et al. 2018) and the long cropping period favors the maintenance of ecosystem services such as biotic mortality of key pests provided by natural enemies (Bouvet at al. 2019).

Attempts have been made to control *D. citri* with its most important parasitoid, *Tamarixia radiata* (Hymenoptera: Eulophidae) (Chien and Chu, 1996), but with ambiguous results at different locations (Michaud 2004; Grafton-Cardwell et al. 2013; Diniz et al. 2020). Moreover, rearing the parasitoid is laborious because there is no artificial diet to replace the haemolymph of the nymphs, so it is necessary to rear *D. citri* to maintain populations of the parasitoid (Chen and Stansly 2014). Another alternative is the use of entomopathogenic fungi such as *Isaria fumesorosea* which is now available commercially, especially against adults of *D. citri* (Averi et al. 2009; Postali Parra et al. 2019). Although predators have been reported as an important mortality factor of *D. citri*, none of them are commercially available to control *D. citri* in citrus crops at the moment (Michaud 2002; Michaud 2004; Qureshi and Stansly 2009), hence, there is still a need for research on biological control of this pest.

After parasitoid wasps, predatory mites are the most commercialized biocontrol agents (van Lenteren 2012; Rao et al. 2018; Knapp et al. 2018). Predatory mites are cheaper and easier to rear than predatory bugs, especially when they can be mass-reared on storage mites or other alternative food (Ramakers and Lieburg 1982; Pilkington et al. 2010; Knapp et al. 2018), which allows them to be produced relatively cheaply and to be released in large numbers to control pests (van Lenteren et al. 2018). The family Phytoseiidae encompasses several predatory mites that are efficient biocontrol agents (Rao et al. 2018). Today, there are about 350 species of control agents used for biological control. Of these, 59 are predatory mites (van Lenteren 2012; van Lenteren et al. 2017), which are among the most sold for augmentative biological control, especially for the control of small phytophagous arthropods (McMurtry and Croft 1997; Knapp et al. 2018; Tixier et al. 2018).

Generalists are the most numerous Phytoseiids in citrus, and are crucial for biological control in this crop (Beltrà et al. 2017). Besides, studies showed that, if properly managed, citrus orchards can be stable ecosystems (Maoz et al. 2014) and they are specially promising to implement conservation biological control (Rusch et al. 2017; Bouvet et al. 2019). Until now, three predatory mites were tested to control *D. citri*, *i.e. Neoseiulus cucumeris* Oudemans, *Neoseiulus barkeri* Hughes and *Amblyseius swirskii* Athias-Henriot (Acari: Phytoseiidae). Of these species, *A. swirskii* showed the best results (Juan-Blasco et al. 2012; Fang et al. 2013). This predator is used to successfully control thrips and whiteflies that are virus transmitters (Knapp et al. 2018). However, it does not occur in Brazil (Cavalcante et al. 2017) and importation will be difficult, if not, impossible. The World Health Organization recommended the use of native species to control vector pests (Who 2002; Howard 2013) and the risks involved in the use of exotic natural enemies to control vector of diseases to animals and humans are also applicable to agricultural crops.

The generalist predatory mite *Amblyseius herbicolus* Chanti (Acari: Phytoseiidae) is not commercialized yet, but has been shown to be a potential biological control agent of whiteflies, *B. tabaci* on tomato plants (Cavalcante et al. 2015; Cardoso 2019) and broad mites, *Polyphagotarsonemus latus* Banks (Acari: Tarsonemidae) on chili pepper (Rodríguez-Cruz et al. 2016; Duarte et al. 2015). Besides, it can develop and reproduce on alternative food (Rodriguez-Cruz et. 2013) such as cattail pollen and it is naturally present on citrus and orange jasmine plants in Minas Gerais, Brazil (personal observations; Reis et al. 2000), so there is a possibility of developing conservation and augmentative biological control of *D. citri* with this predator. Therefore, my aim here was to evaluate the potential of this predator as a biological control agent of *D. citri*.

The commercialization of potential predatory mites is only possible if, besides being effective in controlling pest populations, mass rearing of the predator is easy and cheap (Calvo et al. 2015). The use of astigmatid mites as factitious prey is important to enable mass production and commercialization of predatory mites. It is not known yet if *A. herbicolus* can reproduce and develop on astigmatid mites. I therefore also investigated the potential of *Thyreophagus cracentiseta* (Barbosa, OConnor & Moraes) (Acari: Acaridae) as prey for future mass rearing of *A. herbicolus*.

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Chapter 1:

Suitability of the Asian citrus psyllid and storage mites as prey for the predatory mite *Amblyseius herbicolus* Chant (Acari: Phytoseiidae)

ABSTRACT

Diaphorina citri Kuwayama (Hemiptera Psyllidae) is vector of the bacteria that cause Huanglongbing or greening, the incurable and fatal disease threatening citri culture worldwide. Brazil is the largest producer of citrus and orange juice of the world. Since 2004, when the disease was discovered in the state of São Paulo, more than 35 million infected plants were destroyed to avoid spread of the pathogen. One of the most important management methods is the control of *D. citri* with pesticides, but their intensive use causes development of resistance and pollution. Currently, the main natural enemy used in biological control is the parasitoid *Tamarixia radiata* Waterston (Hymenoptera: Eulophydae). However, the control exerted is still not sufficient and a holistic approach is necessary to manage this vector-pathogen system. The generalist predatory mite *Amblyseius herbicolus* Chant (Acari: Phytoseiidae) was found on citrus and orange jasmine plants, sometimes co-occurring with *D. citri*. Our objective was to evaluate if *A. herbicolus* can reproduce and develop on *D. citri* eggs as well as on the storage mite *Thyreophagus cracentiseta*, which could facilitate mass rearing of the predator. Our results show that this predator can feed and develop on *D. citri* eggs. However, the predation rate was higher on eggs that were collected from plants in the field than on eggs of *D. citri* from small plants from the laboratory. Moreover, when the number of eggs provided was limited (30 eggs per day), the oviposition of *A. herbicolus* feeding on eggs from plants from the field was reduced while that of predators feeding on eggs from the laboratory was not. In a further experiment with limited numbers of eggs (10 eggs per day) this effect was not observed, which suggests that the reduction of quantity of food of both origins diluted the effect of differences in the quality of the eggs. Additionally, we observed that the predator preferred eggs from the laboratory, which may indicate that the higher predation rate of eggs from the field is a compensatory mechanism for lower quality of these eggs compared to eggs from the laboratory. Moreover, the predator was also able to develop and reproduce on *T. cracentiseta*, hence, this astigmatid can be used for mass rearing and future field releases for the control of *D. citri*.

INTRODUCTION

Huanglongbing (HLB) or Greening is the most devastating disease of citrus in the world and it is caused by three different phloem-limited gram-negative *Candidatus* bacteria species (Garnier et al. 1984; Halbert and Manjunath 2004; Grafton-Cardwell et al. 2013). Two insects are vectors of HLB: *Trioza erytreae*, the African psyllid (McClean and Oberholzer 1965) — whose occurrence was limited to Africa until its arrival on the Iberian Peninsula in 2014 (Pérez-Otero et al. 2015) — and *Diaphorina citri*, the Asian citrus psyllid, capable of transmitting the three species of bacteria, including *Ca*. Liberibacter americanus, known only from Brazil (Bové 2006). The disease transmitted by *D. citri* is characterized as being more severe than that transmitted by *T. erytreae* (Aubert 1987) because of higher heat tolerance. This makes *D. citri* currently the most important citrus pest worldwide (Amorós et al. 2019).

Diaphorina citri is an oligophagous insect that feeds on plants of the Rutaceae family (Catling 1970; Patt and Sétamou 2010). Population growth depends on the presence of new growing tips on which females exclusively oviposit (Catling 1970). The insect has five nymphal instars following the egg phase (Husain and Nath 1927; Catling 1970; Hall 2008), which develop and feed on immature leaves. The nymphs and adults produce large amounts of honeydew that accumulates on the plant, favouring the growth of fungi that affect the photosynthetic capacity (Husain and Nath 1927; Yang et al. 2006). Without the pathogens, psyllids are usually considered a secondary pest (Halbert and Manjunath 2004) because their sucking feeding habit only causes loss of productivity when population densities are high.

Huanglongbing can affect citrus plants of all cultivars (Batool et al. 2008; Zou et al. 2019). The symptoms, mottling and chlorosis of the leaves (Batool et al. 2008), can easily be mistaken for zinc deficiency (Lee 1921). In Chinese, Huanglongbing means "yellow dragon disease" which refers to mottled leaves that cause the characteristic yellow shoots symptom (Halbert and Manjunath 2004; Gasparoto et al. 2018) and the English term "greening" refers to the fruits staying green instead of developing the color of ripe fruits (Gottwald et al. 2007; Batool et al. 2008). Other HLB symptoms include poor development of the root system (Batool et al. 2008), seed abortion, asymmetrical fruits (Bové 2006; Gottwald et al. 2007; Gasparoto et al. 2018), premature fruit drop and bitter juice without economic value (McCollum and Baldwin 2017). In a production unit, plants may show variable titers of the pathogen and incomplete systemic infection (Gottwald et al. 2007; Batool et al. 2008).

The visual symptoms can vary greatly, even among plants that were infected at the same time, and are dependent on environmental conditions and cultivar (Gottwald et al. 2007). However, they are always preceded by a prolonged asymptomatic period, which may delay detection by months to years (Gottwald et al. 2020). Yet, asymptomatic infected plants act as a source of the disease and contribute to its rapid spread (Salifu et al. 2012; Lee et al. 2015). Huanglongbing causes quantitative and qualitative yield losses and ultimately plant death (Gottwald et al. 2007; Alvarez et al. 2016), leading to dramatic economic losses worldwide (Manjunath et al. 2008; Bassanezi et al. 2011; Alvarez et al. 2016).

Pesticides have been used to control the vector (Chen and Stelinski 2017; Mascarin et al. 2019). However, even with new pesticides being formulated, their effectiveness is limited (Li et al. 2019) because *D. citri* populations have developed resistance (Tiwari et al. 2011; Chen and Stelinski 2017; Chen et al. 2020) due to the aggressive insecticidal spray programs. These sprayings concomitantly disrupt the naturally occurring biological control (Monzo et al. 2014) and may cause secondary pest problems (Luck et al. 1977). Besides not effectively controlling the vector, the frequent and long-term pesticide applications have various undesirable effects on beneficial arthropods, human health and environment, such as citrus orchard pollution (Pimentel and Burgess 2014; Chow et al. 2016; Li et al. 2019).

An alternative to chemical control is the use of natural mortality factors of *D. citri*. The main natural factors that affect the vector are the flushing rhythms of host plants (growing tips are necessary for oviposition and development, Catling 1970; Chien and Chu 1996), weather extremes and natural enemies (Catling 1970). Biological control with natural enemies is an important pest control strategy and does not cause resistance development on pests, in contrast to insecticides (Bale et al. 2008). Thus, one way of controlling *D. citri* would be the use of natural enemies (Qureshi and Stansly 2007).

Natural enemies reported for biological control of *D. citri* include parasitoids such as *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae) (Chien and Chu 1996), generalist predators such as coccinellid beetles, hoverflies, spiders and lacewing larvae (Michaud 2004; Pluke et al. 2005; Kistner et al. 2016), entomopathogens (Hall et al. 2012; Arnosti et al. 2019), predatory mites such as *Amblyseius swirskii* and *Neoseiulus cucumeris* (Juan-Blasco et al. 2012; Fang et al. 2013) and *Pyemotes* mites (Prostigmata: Pyemotidae) (Lu et al. 2019). *Tamarixia radiata*, the most important parasitoid of *D. citri* used for biocontrol, attacks nymphs from the third to fifth instar (Pluke et al. 2008; Chen and Stansly 2014). However, its effectiveness is controversial: *T. radiata* did not seem to reduce the populations of *D. citri* enough to limit the spread of the pathogen in some regions, whereas it is considered an effective biological control agent in other regions (Hall 2008a; Grafton-Cardwell et al. 2013). Moreover, Michaud (2004) reported that the main natural mortality factor of *D. citri* is predation by generalist predators, which may be more efficient at controlling citrus pests than parasitoids (DeBach 1946; Symondson et al. 2002; Michaud 2004).

The consumption of *D. citri* eggs is considered crucial (Juan-Blasco et al. 2012; Fang et al. 2013), especially because it is the only ontogenetic stage that does not transmit greening. However, eggs of *D. citri* are oviposited on developing leaves, where they are protected from the consumption of large predators, but not from predatory mites (Juan-Blasco et al. 2012). Besides, large predators can feed on the parasitized nymphs of the 4th and 5th instars, which can result in high mortality of the parasitoid inside these hosts (Michaud 2004). To date, the disease continues to advance and no single approach is effective enough to control *D. citri* and HLB (Juan-Blasco et al. 2012; Lu et al. 2019). Therefore, the combined use of the parasitoid in association with a generalist predator that feeds on *D. citri* eggs, but not on parasitized nymphs, may be the best alternative.

Diaphorina citri has been reported in Brazil since the 1940s (Da Costa Lima 1942). However, HLB was only registered for the first time in 2004 in Araraquara, São Paulo (Coletta-Filho et al. 2004). Brazil is the principal producer of oranges in the world (Gasparoto et al. 2018) and the incidence of HLB in the citrus belt of São Paulo, Triângulo and Southeast of Minas Gerais State was 0.61% in 2008 and reached 18.15% in 2018, which corresponds to 35.3 million plants (Fundecitrus 2018). The Brazilian government has established that all plants of a production unit should be destroyed if more than 28% shows symptom, (Ministério da Agricultura, Pecuária e Abastecimento 2008). At the moment, the major strategy is to control the population densities of the insect vector, which should be kept as low as possible (Chu and Chien 1991) to reduce disease spread (Boina and Bloomquist 2015). The use of biological control in Brazil is growing, but is still limited (Bueno et al. 2019). Studies of biological control of *D. citri* with predators are scarce, despite the large diversity of natural enemies (Demite et al. 2014). Thus, the aim of this paper is to study a generalist predator as potential biocontrol agent of *D. citri* in Brazil.

Several generalist phytoseiid mites are excellent biological control agents due to their capacity to feed on alternative food or prey, enabling the persistence of populations in the absence of the target pest (van Rijn et al. 2002; Nomikou et al. 2003b; Janssen and Sabelis 2015). We therefore investigated the potential of a native Brazilian predatory mite for control of *D. citri*. At the moment, there are no studies of predators controlling *D. citri* on citrus plants in Brazil, and there are also no predators to control *D. citri* available in the country. We therefore collected predatory mites from citrus and orange jasmine plants (*Murraya paniculata*) in the state of Minas Gerais, Brazil. The predatory mite *Amblyseius herbicolus* Chant (Acari:

Phytoseiidae) was found on both plants. It is a generalist, found in several crops and can be reared on pollen (Rodríguez-Cruz et al. 2013; Cavalcante et al. 2015; Duarte et al. 2015; Lam et al. 2019). Additionally, it reproduces by thelytoky, hence has only females (de Moraes and Mesa 1988). Thelytoky can be advantageous in biological control because it can result in high rates of population increase and in reduced costs of mass rearing (Siepel 1994; Heimpel and Lundgren 2000; Ramirez-Romero et al. 2012).

The preferred host plant of *D. citri* is orange jasmine, *Murraya paniculata* (L.) Jack (Halbert and Manjunath 2004; Gasparoto et al. 2018), which is also a potential reservoir of *Candidatus* Liberibacter bacteria associated with HLB (Damsteegt et al. 2010). Orange jasmine is a widespread ornamental plant in Brazil (Lopes et al. 2010), which favors the spread of the pathogen. This plant was therefore used for rearing of *D. citri* and in experiments.

Besides the efficiency as a biological control agent, the use of natural enemies depends on the possibility of mass rearing, especially on alternative food, to favor large scale production in an economic way. Moreover, the use of alternative food may enable persistence of a population of natural enemies in the crop after release (de Klerk and Ramakers 1986; Van Baalen et al. 2001; Nomikou et al. 2005; Sabelis and van Rijn 2005). Rearing the predatory mite on a factitious prey makes its use economically viable. However, the development and reproduction of *A. herbicolus* when feeding on potential factitious prey has not yet been investigated. Therefore, our aim here was to evaluate the potential of *A. herbicolus* to control *D. citri*. Experiments were carried out in the laboratory to test the capacity of *A. herbicolus* to develop and reproduce when feeding on *D. citri* eggs and on *Thyreophagus cracentiseta* (Acaridae) Barbosa, OConnor & Moraes as a potential factitious prey.

MATERIALS AND METHODS

Predator sampling

To verify the presence of predatory mites on citrus and orange jasmine, plants of these species were sampled on the campus of the Federal University of Viçosa (20º46’9’’ S, 42º87’02’’ W) in the state of Minas Gerais, Brazil. Two methods of collection were used; the first was beating the leaves above a black tray, which was subsequently covered with plastic film. For the second method, we removed the branches with *D. citri* eggs in the growing tips, which then were placed in paper bags. Tray and paper bags were taken to the laboratory, where slides of predatory mites were made for morphological identification using a phase contrast microscope and a dichotomous key (Chant and McMurtry 1994, 2007). The predatory mite *A. herbicolus* was found on both citrus and orange jasmine plants, in the last case together with *D. citri*.

Acquisition and maintenance of clean plants

Orange jasmine plants (4-6 months old) were obtained from Viveiro Antuérpia in the vicinity of Viçosa, Minas Gerais, Brazil. Subsequently, the plants were kept in an insect-proof greenhouse (25 ± 2 ºC), where they were fertilized every two months with a mixture of NPK (4/14/8). The plants were pruned regularly to induce the development of growing tips, which are oviposition sites of *D. citri*. We assessed the presence of the three *Candidatus* Liberibacter species in plant material as well as in *D. citri* (see Supplementary Material).

Diaphorina citri **rearing**

Adults of *D. citri* were collected from orange jasmine trees of about 6 years old on the campus of the Federal University of Viçosa. They were incubated in a BugDorm-4F insect cages (0.5 x 0.5 x 1.0 m) to oviposit on pesticide-free orange jasmine plants (see above) and formed the basis of what will be referred to as the lab population. These insects were kept under laboratory conditions. The plants were watered two times a week and adults were regularly transferred to new plants. Besides eggs from this lab strain, eggs were also obtained directly from trees on the campus; they are referred to as the field strain.

Pollen

The pollen used for the predatory mite rearing and experiments was collected from *Typha sp.* plants from rural areas around Viçosa, Minas Gerais. This pollen was chosen because previous studies showed high oviposition and population growth rates of *A. herbicolus* when feeding on it (Duarte et al. 2015). It was dried in an oven at 60º C for 48 h and was stored in a container in the freezer. Periodically, small amounts of pollen were removed from the container and placed in 1.5 ml microtubes (Eppendorf), dried at 60° C for an additional 48 h and then stored in the freezer.

Thyreophagus **cracentiseta rearing**

The culture of *T. cracentiseta* was initiated with individuals obtained from the Laboratory of Acarology of the Escola Superior de Agricultura "Luis de Queiroz" (ESALQ), in 2019. The mites were reared in plastic pots (ø = 10 cm; 15 cm high) covered with mesh (90 µm). The rearing was maintained by providing sterilized wheat bran once a week and was kept under controlled conditions (25 ± 2° C, 70 ± 10% RH and 12 h of photophase).

***Amblyseius herbicolus* rearing**

The predators used for experiments came from an existing rearing of *A. herbicolus*, collected in 2014 from tomato plants in Prados (latitude: 21° 03' S; longitude: 44° 04' 47" W) in the State of Minas Gerais, Brazil. The predators were reared on arenas consisting of a plastic sheet (10 x 15 cm²) placed on a wet sponge in a plastic tray containing water (McMurtry and Scriven 1965) and surrounded by a moistened cotton wool barrier to prevent predator escapes (Rodríguez-Cruz et al. 2013). They were fed with cattail pollen twice a week. Cotton threads were provided to serve as oviposition sites and were covered with a piece of black plastic for shelter (1 x 1 cm²). Cotton threads with eggs were placed on a new arena to obtain cohorts of similar-aged individuals. After 10-12 days, the young adult females were used for experiments. The rearing arenas were kept under controlled conditions (25 ± 2° C, 70 ± 10% RH and 12 h of photophase).

Predation and oviposition rate of *A. herbicolus* on *D. citri*

This experiment was designed to assess the predator performance on *D. citri* eggs from field and lab plants. Young adult female predators were individually tested in black plastic dishes (ø = 5.5 cm; 1.4 cm high) with a piece of wet cotton wool inside, covered with a transparent lid. Growing tips with prey eggs were obtained from orange jasmine plants from the laboratory and the field. Other species of herbivores were removed from the growing tips from the field. The number of prey eggs was assessed, and growing tips were placed in an arena with one *A. herbicolus.* In a pilot study, predators were observed to prey on eggs during 24 h to assess the numbers of prey eggs needed for ad libitum feeding. Subsequently, all replicates were done with 60-62 prey eggs, either from the lab or from the field. Every day, individuals were moved to a new arena with fresh prey eggs. The same methodology was used for oviposition experiments, but using a treatment with one mg of pollen as control. The numbers of *D. citri* eggs consumed and the predator oviposition rate were scored for 3 days, but the score of the first day of oviposition was excluded to reduce effects from the previous diet (Sabelis 1990). The numbers of females tested were 12 and 13 for prey eggs from the field and the laboratory, respectively, and 13 for the pollen treatment. All experiments were performed at 25 ± 2 ° C, 70 ± 10% RH and 12 h photophase. Predation and oviposition data were analyzed with linear mixed effects models (LME) with individual as a random factor to correct for repeated measures. Contrasts between treatments were obtained with the Tukey method (function cld of the multcomp package of R, Hothorn et al. 2008). All statistical analyses were done using the software R version 3.3.3 (R Project for Statistical Computing. http://www.r-project.org).

Preference

Because the previous experiment showed that the predators consumed many more prey eggs from the field than from the lab, we further investigated possible differences in prey eggs of these different origins by offering the predators a choice between them. Similar quantities of prey eggs were offered on two growing tips (as similar as possible); one from the field and one from the lab, each on one side of a circular arena made of a black plastic sheet (ø = 5.5 cm) on wet cotton wool and surrounded with water. The side of the arena on which the growing tips of each origin were offered was alternated to avoid possible bias of the predators for one side. The predators showed no significant preference for either side of the arena (binomial test, all $p > 0.05$). An adult female was placed in the center of each arena, and its position was checked every hour. After 6 h, the consumption of prey eggs from both origins was evaluated. The number of females tested was 13. The data were analyzed first with a binomial test per female, with the expectation that they would choose eggs of either origin with a probability of 0.5 (Siegel and Castellan 1988). Subsequently, we compared the predation of *D. citri* eggs from each origin with a generalized mixed effects model (GLMER, Bates et al. 2015) with a binomial error distribution.

Predation and oviposition rate of *A. herbicolus* on limited numbers of *D. citri* eggs

Because the previous experiments indicated a possible difference in quality of prey eggs from the field and from the lab, we further measured oviposition and predation on eggs from the field and the lab. We investigated whether offering limited numbers of eggs from the field would result in a larger reduction of oviposition than eggs from the lab because compensatory feeding would no longer be possible. Two experiments were performed, one providing 30 and the other 10 eggs of both origins per day. The number of females tested was 11 for eggs from the field and 12 for eggs from the lab with 30 eggs provided per day and 14 and 10 with 10 eggs provided per day. Differences in numbers of females tested were caused by differences in the availability of eggs from the field and the lab. The experiments were performed as described above, but without a control with pollen. The data were analyzed as above.

Juvenile development

This experiment was designed to verify whether *A. herbicolus* can develop by feeding on *D. citri* eggs only. Adult *A. herbicolus* were removed from the rearing and placed in a new arena, similar to those used in the predation and oviposition experiments, for 24 h to oviposit. Forty-eight h after removing the adults, larvae were taken from this cohort and each one was placed in a separate black dish (as above). There were three treatments: one group of larvae received one mg of pollen, another group was supplied with a surplus of *D. citri* eggs from the field and a third group with a surplus of prey eggs from the lab. Every day, individuals were transferred to new black dishes with fresh food and were checked for survival and development until adulthood. Upon observing the first adult, evaluations were done every 12 h. The number of replicates was 23-27 per treatment. Effects of diet on survival and developmental were analyzed with a Cox's proportional hazard model (Cox 1972).

Development and oviposition of *A. herbicolus* feeding on *T. cracentiseta*

This experiment was designed to assess the ability of *A. herbicolus* to feed, reproduce and develop on *T. cracentiseta*. From a cohort of predator larvae, individuals were placed each in a separate black plastic dish (as described above Half of the individuals were fed with two mg of *T. cracentiseta* and the other half was fed with one mg of cattail pollen as control (*Typha* sp.). We previously confirmed that these quantities are more than the daily consumption of the predators. Both treatment and control individuals were transferred to new black plastic dishes with fresh food and checked every day until adulthood. There were 18-20 replicates for pollen and *T. cracentiseta*, respectively. After reaching adulthood, the oviposition of each predator was recorded for three days. The number of replicates of this oviposition experiment was 11-15 for pollen and *T. cracentiseta*, respectively. Effects of diet on survival and developmental were analyzed with the Cox's proportional hazard model as above. The oviposition of *A. herbicolus* on *T. cracentiseta* was analyzed with a GLMER with a Poisson error distribution.

RESULTS

Predation and oviposition rate of *A. herbicolus* on *D. citri*

Amblyseius herbicolus consumed *D. citri* eggs and the predators obtained a yellow colour after consumption (Fig. 2). There was a significant effect of the interaction between day and origin of eggs of *D. citri* (lme: d.f. = 5, Chi² = 4.53, p = 0.033; Fig. 3). The predators consumed more prey eggs from the field than from the laboratory on all three days. In addition, the number of eggs consumed from the laboratory did not vary significantly during the three days, but consumption of eggs from field increased with time (Fig. 3). The oviposition rate did not differ significantly between days (lme: d.f. = 5, $Chi^2 = 2.01$ $p = 0.155$; Fig. 4) and did not differ significantly with pollen, eggs from the field or from the laboratory (lme: d.f. = 3, $Chi^2 = 1.81$ $p = 0.40$; Fig. 4). The mean total consumption of eggs from the field over the three days was about 3.5 times higher than that of eggs from the laboratory (lme: d.f. = 1, $Chi^2 = 122.03$, $p< 0.0001$; Fig. 5), but the oviposition during the last 2 days of the experiment did not differ significantly between these two diets (GLM: $Chi^2 = 3.60$, d.f. = 1, $p = 0.74$; Fig. 5). This suggests that there were differences between prey from the field and from the lab.

Preference

There was a significant preference for eggs from the laboratory relative to eggs from the field (GLMER, $Chi^2 = 85.0$, d.f. = 1, $p<0.0001$; Fig. 6). In general, five times more eggs from the laboratory were consumed than eggs from the field. In addition, we observed that more than half of the eggs from the laboratory were partially eaten, which was about 6 times more than observed for eggs from the field (GLMER, $Chi^2 = 79.2$, d.f. = 1, $p < 0.0001$; Fig. 6). Hence, predators consumed more eggs from the field than from the lab if they had no choice (Fig. 3 and 5), but had a clear preference for eggs from the lab when given a choice. This suggests that the eggs from the lab were of better quality than the eggs from the field, and that predators perhaps consumed more eggs from the field to compensate for low egg quality when they had no choice. To further test this, we limited the numbers of prey eggs.

Predation and oviposition rate of *A. herbicolus* on limited number of *D. citri* eggs

When 30 *D. citri* eggs were provided per day, there was no significant effect of the interaction between day and origin of eggs (Fig. 7, lme: d.f. = 1, $Chi^2 = 0.001$, $p = 0.97$). The predation of eggs from the field was higher than the predation of eggs from the laboratory (lme: $Chi^2 = 41.9$, d.f. = 1, $p < 0.0001$; Fig. 7) and there was no significant difference in predation per treatment among days (lme: d.f. = 1, $Chi^2 = 0.01$, $p = 0.91$; Fig. 7). Predation on eggs from the field was higher than of eggs from the laboratory on every day. There was a significant effect of the interaction between day and origin of eggs on oviposition (Fig. 8, lme: d.f. = 1, Likelihood ratio = 6.88, $p = 0.0087$). On the third day, the oviposition on a diet of eggs from the field was lower than oviposition on eggs from the laboratory (Fig. 8). In addition, the oviposition on eggs from the field decreased over time (Fig. 8). During the experiment, the mean total predation was lower, but oviposition was higher, for predators that fed on eggs from the laboratory than those that fed on eggs from the field, (Fig. 9), suggesting that eggs from the field were of lower quality. When 10 *D. citri* eggs were provided per day, there was no significant effect of the interaction between day and origin of eggs on predation (lme: d.f. = 1, $\text{Chi}^2 = 0.25$, p = 0.61; Fig. 10). Predation of eggs from the field was again higher than predation of eggs from the laboratory (lme, d.f. = 1, $\text{Chi}^2 = 7.75$, p = 0.0054; Fig 10). Within treatments, there was no significant difference in predation among days (lme, d.f. = 1, $\text{Chi}^2 = 1.60$, p = 0.21, Fig. 9). There was no significant effect of the interaction between day and origin of the prey on oviposition (lme: d.f. = 1, $\text{Chi}^2 = 0.21$, p = 0.65; Fig. 11). Additionally, oviposition did not vary significantly among days (lme: d.f. = 1, $\text{Chi}^2 = 1.08$, p = 0.30; Fig. 11) or between treatments (lme: d.f. = 1, $\text{Chi}^2 = 0.192$, p = 0.66; Fig. 11). During the entire experiment, the mean total oviposition of predators fed on prey eggs from both origins did not differ (lme: $\text{Chi}^2 = 0.23$, d.f. = 3, $p < 0.627$; Fig 12).

Juvenile development

There was a significant effect of diet on the developmental time of *A. herbicolus* (log-rank test: $\text{Chi}^2 = 30.01$, d.f. = 2, $p < 0.001$; Fig. 13). Development took longer when predators fed on *D. citri* eggs from the field or from the laboratory than when they fed on pollen. Survival was high on all diets (Fig. 13), and there was no significant effect of diet (log-rank test: $\text{Chi}^2 = 4.61$, d.f. = 2, p = 0.1), although there was a trend of the survival being somewhat higher on a diet of eggs from the laboratory.

Development and oviposition of *A. herbicolus* feeding on *T. cracentiseta*

The developmental time of *A. herbicolus* fed on *T. cracentiseta* did not differ significantly from that of pollen-fed predators (Likelihood ratio = 2.83, d.f. = 1, p = 0.097; Fig. 14). Survival on these two diets also did not differ significantly (Likelihood ratio = 2.16, d.f. = 1, p = 0.1; Fig. 14). There was no significant difference in oviposition rate between treatments (GLMER, $\text{Chi}^2 = 0.16$, df = 1, p = 0.687; Fig. 15) and there was no significant effect of time (GLMER, $\text{Chi}^2 = 3.37$, df = 1, p = 0.066).

DISCUSSION

The results of this study show that the generalist predator *A. herbicolus* was able to feed, develop and reproduce on eggs of *D. citri*. We also observed that the predation rates were affected by the origin of prey eggs, whereas predator oviposition rates differed only when the supply of prey eggs from the field was limited. Additionally, *A. herbicolus* was capable to reproduce and develop on the astigmatid mite *T. cracentiseta*.

In the preference experiment, about 5 times more eggs from the laboratory were consumed than eggs from the field (Fig. 6). Predators are generally known to prefer high-quality prey (Dicke et al. 1990; Bilde and Toft 1994; Naustvoll 2000), and prey preference of phytoseiid mites is often positively related to fitness parameters and reproductive success (Dicke et al. 1990; Rovesnká et al. 2005). We also observed that some eggs were partially consumed. After some hours, the partially consumed eggs dehydrated and shrunk, and it was no longer possible to see the difference between partially consumed and totally consumed prey eggs. Hence, partial consumption could only be assessed by frequent observation. We observed movements of the digestive tract of the predators when attacking prey eggs, which is a sign of taking up food, thus confirming that the predators indeed consumed eggs partially, and did not just perforate them. When satiation of predators decreases, the tendency to capture prey increases, and partial prey consumption occurs when less than the content of an entire prey is needed for full satiation (Sabelis 1990). Most of the eggs from the laboratory were partially consumed, whereas most eggs from the field were completely consumed. This suggests that the predators fed on eggs from the lab when they were more satiated than when they fed on eggs from the field.

The potential differences in the quality of eggs from different origins can be caused by several factors. The plants in the field and from the lab were of the same population: the nursery used seeds from the field to produce seedlings, hence, there was not much difference in genetic background of the plants. Moreover, no pesticides were used in the field. Differences in plant quality may not only affect herbivores but also the third trophic level (Southwood 1972; Price et al. 1980; Teder and Tammaru 2002), and it is possible that herbivores feeding on plants with lower nutritional value produce eggs that are also lower in nutritional value. For our study, this would imply that plants in the field were of lower quality for the herbivores than the plants from the lab. This may have several reasons. First, it may have been caused by differences in environmental conditions, such as differences in humidity and temperature, which may affect the eggs directly, or indirectly via the host plant. Second, plants can also be of reduced quality for herbivores by containing fewer nutrients, and plant quality is also reduced by the presence of phytotoxins and secondary metabolites (Guidolin and Cônsoli 2020). The development of constitutive defences of the plants is known to be age-dependent (Kearsley and Whitham 1989; Boege and Marquis 2005; Quintero and Bowers 2011), and the plants from the laboratory were about 6 months old, whereas the plants in the field were about 4 years old. We rarely found *D. citri* on orange jasmine plants older than 10 years in the field, which may have been caused by well-developed constitutive defences of these older plants. Hence, the older plants in the field may have had better-developed defences against herbivores than the younger plants from the lab, and this may have affected the prey quality of *D. citri* eggs.

Third, plants in the field were attacked by other species of herbivores besides *D. citri* and this will have induced defence responses in the plants (Karban and Carey 1984; Karban and Baldwin 1997; Walling 2000), which could have affected *D. citri* and its quality as prey.

Fourth, differences in nutritional quality of the eggs might in theory have been caused by differences in the HLB infection of the plants (Fereres et al. 1989; Maris et al. 2004; Mauck et al. 2010; Belliure et al. 2010; Cen et al. 2012), but a molecular analysis showed no evidence for the infection of plants and insects from the field or from the lab (Supplementary Material).

Lastly, the size of eggs from both origins did not differ significantly (eggs from the field: 2.0 ± 0.06 µ, eggs from the laboratory: 2.4 ± 0.4 µ, t-test, $p = 0.6$), although the trend is towards eggs from the lab being bigger. However, the difference was about 10%, which is not sufficient to explain the observed difference in predation rate of about 258% (Fig 5).

In conclusion, the differences in predation rate were probably caused by nutritional differences or by the presence of hazardous compounds that needed to be detoxified (Sabelis 1990). It is known that animals may compensate nutritional imbalances in their diet by consuming more food that is deficient in some aspect (Mayntz et al. 2005), and the increased consumption of eggs from the field is perhaps an example of such compensatory feeding (Slansky and Feeny 1977; Price et al. 1980; Timmins et al. 1988; Rueda et al. 1991; Cruz-Rivera and Hay 2000). This compensation, in turn, then ensured that the oviposition rate of the predators was not negatively affected by the deficient diet, if this was present in sufficient quantity.

Based on this theory of compensatory consumption on low quality resources, we expected an effect of diet on oviposition rates of *A. herbicolus* when it could feed on limited numbers of eggs. A comparison of predation and oviposition with different densities of prey eggs (Figs. 5, 8 and 11) shows that predation on eggs from the field was always higher than on eggs from the laboratory. When high numbers of eggs were provided, the increased predation of eggs from the field apparently compensated for their lower quality, resulting in similar oviposition rates (Figs. 4 and 5). With intermediate numbers of eggs (Figs. 8 and 9), total predation of eggs from the field was still higher than that of eggs from the laboratory, but this apparently did not compensate for the low quality, resulting in lower oviposition rates. When the numbers of eggs were further limited to 10 per day, eggs from the rearing also did not offer sufficient resources to maintain a high oviposition rate. Hence, these results indeed further confirm the idea that eggs from the field were of lower quality than eggs from the lab, and that the predators can maintain high oviposition rates by compensatory feeding.

Amblyseius herbicolus showed a high predation rate on *D. citri* eggs, and the oviposition rate on this prey was comparable to that on cattail pollen, which is considered a good food source (Duarte et al. 2015; Fig. 4). Although a direct comparison of our results with the other two studies of predatory mites feeding on *D. citri* is difficult because of the different methodologies utilized, *A. herbicolus* seems to be a more promising biocontrol agent than *A. swirskii*, *N. cucumeris* and *N. barkeri*. The predation rates of *A. herbicolus* on *D. citri* eggs from the laboratory or from the field were much higher than those of the other three species (Juan-Blasco et al. 2012; Fang et al. 2013). Moreover, *A. herbicolus* is capable of ovipositing when feeding on *D. citri*, whereas such information is not available for *A. swirskii* (Juan-Blasco et al. 2012). Additionally, oviposition of *N. cucumeris* was low compared to that of *A. herbicolus* (Fang et al. 2013). Furthermore, it is not known whether *A. swirskii* and *N. cucumeris* can develop into adults when feeding on *D. citri* eggs, whereas *A. herbicolus* can (Fig. 13). Hence, our results suggest that the *A. herbicolus* is a potential good biological agent of *D. citri*.

Diaphorina citri is stimulated to oviposit on uninfected plants (Mann et al. 2012; Martini et al. 2014), and this especially reinforces the preventive use and maintenance of *A. herbicolus* populations in citrus orchards as bodyguards of uninfected plants (Sabelis 1990), in order to prevent pest establishment and greening infection. A holistic approach is necessary for an effective control of HLB spread, including effects of the host plant, natural enemies, and pathogens. Furthermore, the dispersal behaviour of *D. citri* should be studied, especially if and how the presence of natural enemies affects it. Dispersion of the pest might be stimulated by the presence of predators, and cues from natural enemies can also be avoided by herbivores to reduce predation risk (Lima and Dill 1990; Kats and Dill 1998; Nomikou et al. 2003a; Helms et al. 2019). Concluding, the effects of *A. herbicolus* on the prevention of spread of HLB need and deserve further studies.

The compatibility of different natural enemies also needs to be evaluated, because natural enemies may have synergistic or antagonistic effects on biological control (Ferguson and Stiling 1996). Besides the parasitoid *T. radiata*, lacewings and ladybirds are responsible for natural mortality of *D. citri*, but coccinellids may feed on parasitized *D. citri* (Michaud 2004; Hall 2008b). It is known that *T. radiata* can detect cues associated with the predator *Harmonia axyridis* (Coleoptera: Coccinellidae) and avoids plants with this predator (Shrestha and Stelinski 2019). These interactions within the third trophic level may limit the efficiency of this parasitoid. Although we have not performed predation experiments with *D. citri* nymphs as prey, we observed that *A. herbicolus* does consume 1st instar nymphs of *D. citri* and that it did not consume 5th instar nymphs. Further experiments are needed to confirm if other instars can be eaten by the predator, especially because the 4th and 5th instar are the preferred host stage of *T. radiata* (Chien and Chu 1996; Sule et al. 2014).

In the experiment with astigmatid mites, *A. herbicolus* developed, reproduced and oviposited as well as when it fed on pollen (Fig. 14 and 15). Mass production has been considered a way to improve biological control programs, and rearing natural enemies with the pest as food is often more expensive and laborious than using alternative food (Van Lenteren 2000). Besides, the use of astigmatids for mass rearing has some advantages compared to the use of pollen. Astigmatids are easy to obtain and they need to be added to the cultures less frequently than pollen, which molds quickly. Therefore, one of the ways to make field releases of *A. herbicolus* economically viable is mass rearing it with alternative food and releasing it using sachets with astigmatid mite (Baxter et al. 2011; Calvo et al. 2015). *Thyreophagus cracentiseta* was a suitable alternative food for *A. herbicolus* and can be used for mass rearing and future releases of this predator.

In conclusion, *A. herbicolus* proved to be a promising biological agent for *D. citri*, consuming large amounts of eggs and being able to develop and survive when feeding on this pest. Thus, it deserves further research as biocontrol agent of *D. citri*, and indirectly of HLB, on entire plants and in the field. In addition, the possibility of using pollen or astigmatid mites as alternative food will facilitate mass rearing and release of this agent.

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Figure 1: Life cycle of *Diaphorina citri* from egg to adulthood. Eggs: 0.01-0.15 mm; 1° instar: 0.25 mm; 5° instar: 1.5-1.7 mm. Adults: 3-4 mm.

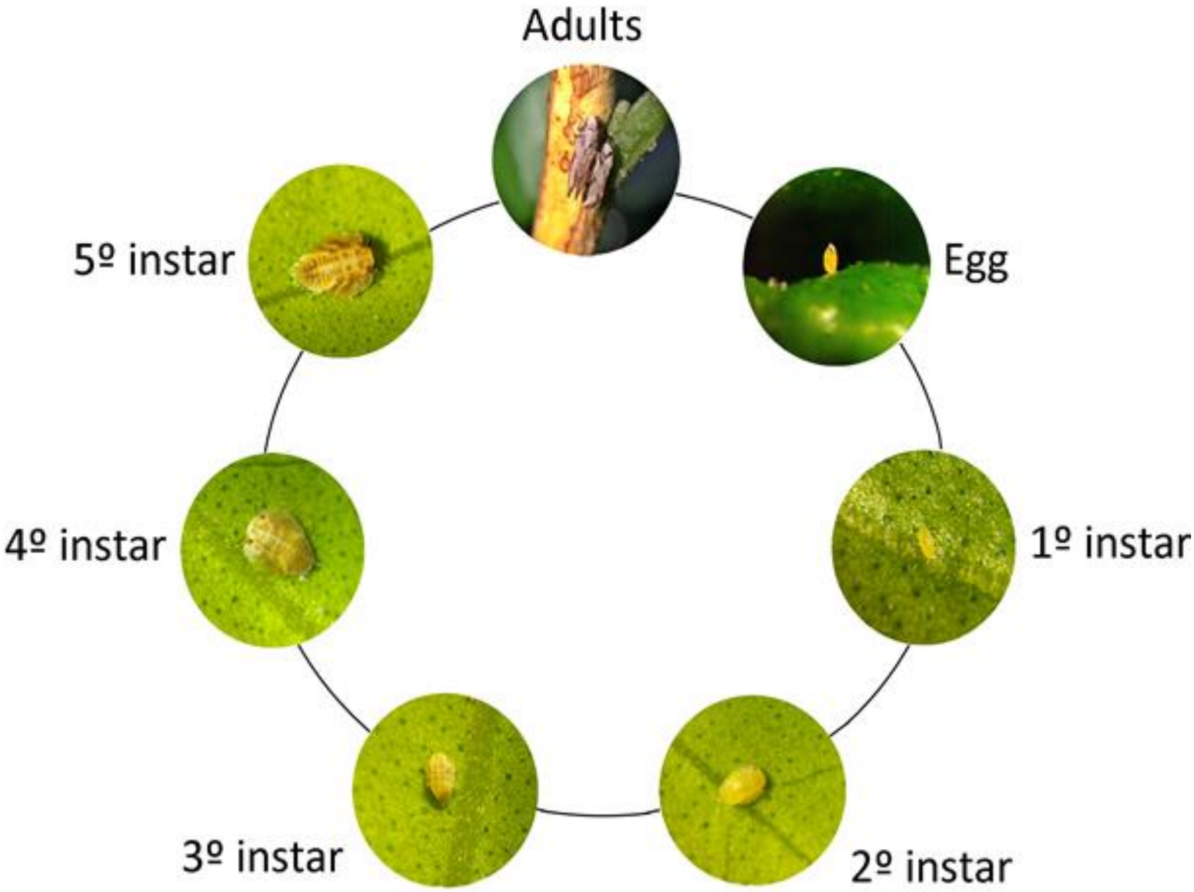


Figure 2: Adult females of *Amblyseius herbicolus* (width: 0.34 mm, length: 0.23 mm) became yellow (right) after having fed on *Diaphorina citri* eggs, compared to when it fed on pollen (left).

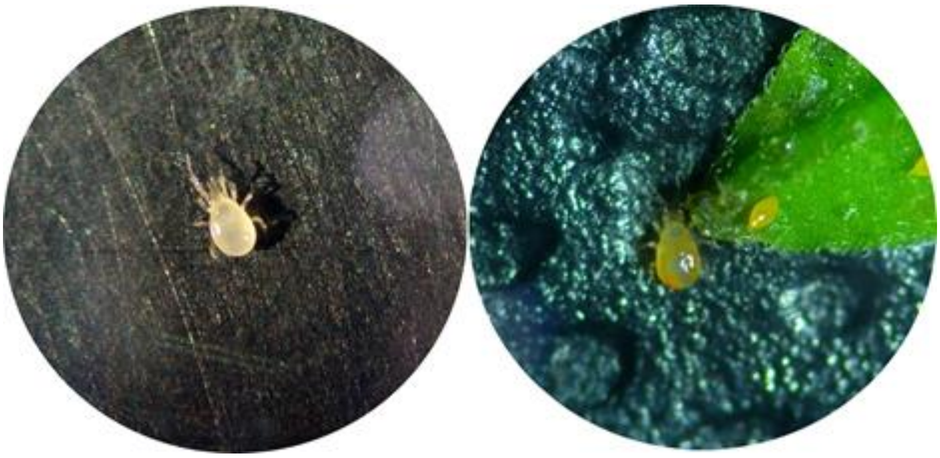


Figure 3: Mean (± s.e.) predation rate of *Amblyseius herbicolus* fed on eggs of *Diaphorina citri* from the laboratory (light grey) or the field (dark grey) during three days. Different lowercase letters and uppercase letters indicate significant differences in egg consumption among days per treatment. Asterisks indicate significant differences in consumption of eggs from the field or the laboratory per day (contrasts after lme, $p < 0.05$).

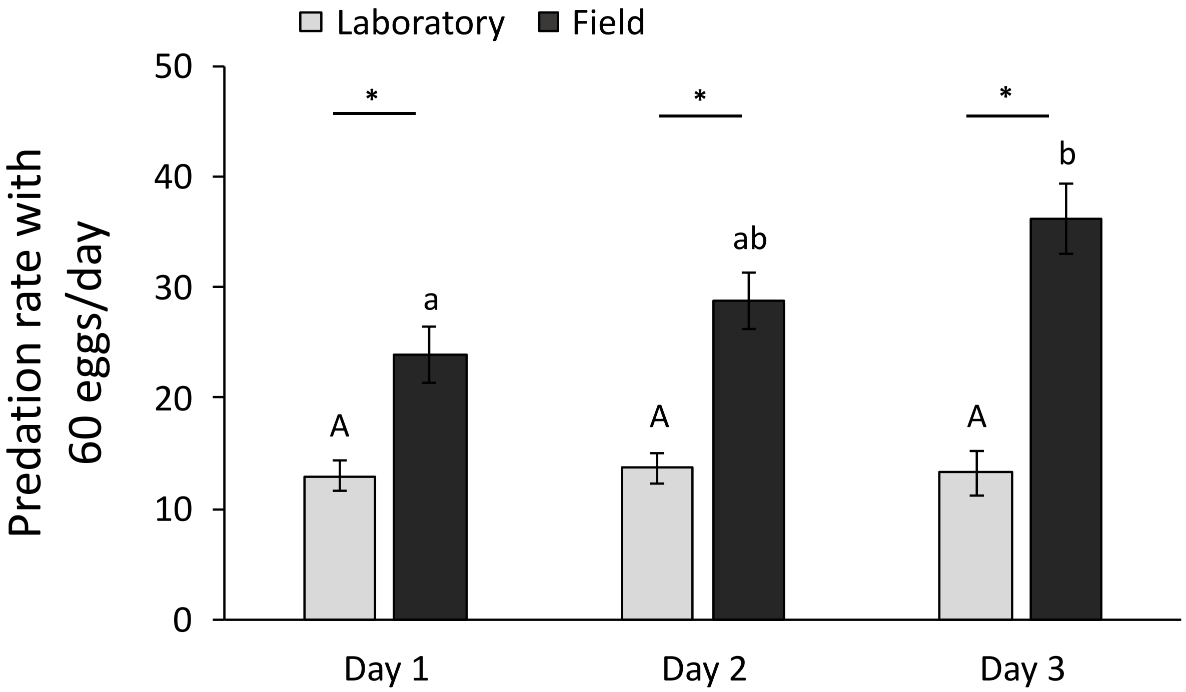


Figure 4: Mean oviposition rate (± s.e.) of *Amblyseius herbicolus* fed on pollen (medium grey) or on 60 eggs of *Diaphorina citri* from the field (dark grey) or the laboratory (light grey). Oviposition rates did not differ significantly among treatments or between days (linear mixed effects model).

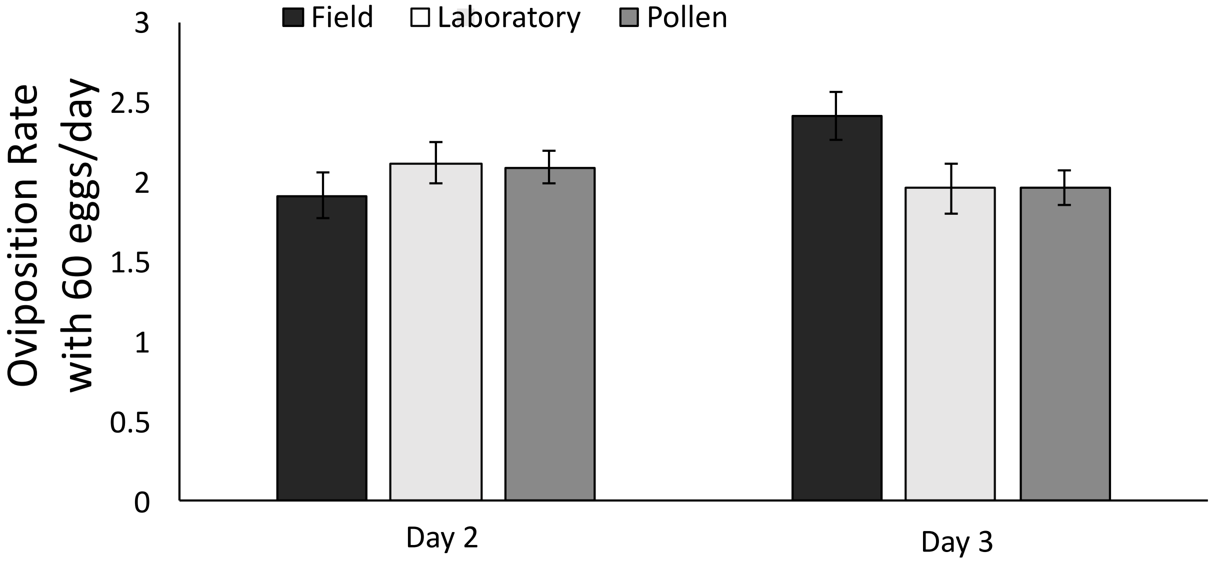


Figure 5: Total oviposition of *Amblyseius herbicolus* during two days (vertical axis) as function of total predation (horizontal axis) of eggs from the laboratory (light grey circles) or from the field (dark grey squares) when 60 eggs were provided per day. The larger symbols show the means (± s.e.), smaller symbols are individual measurements.

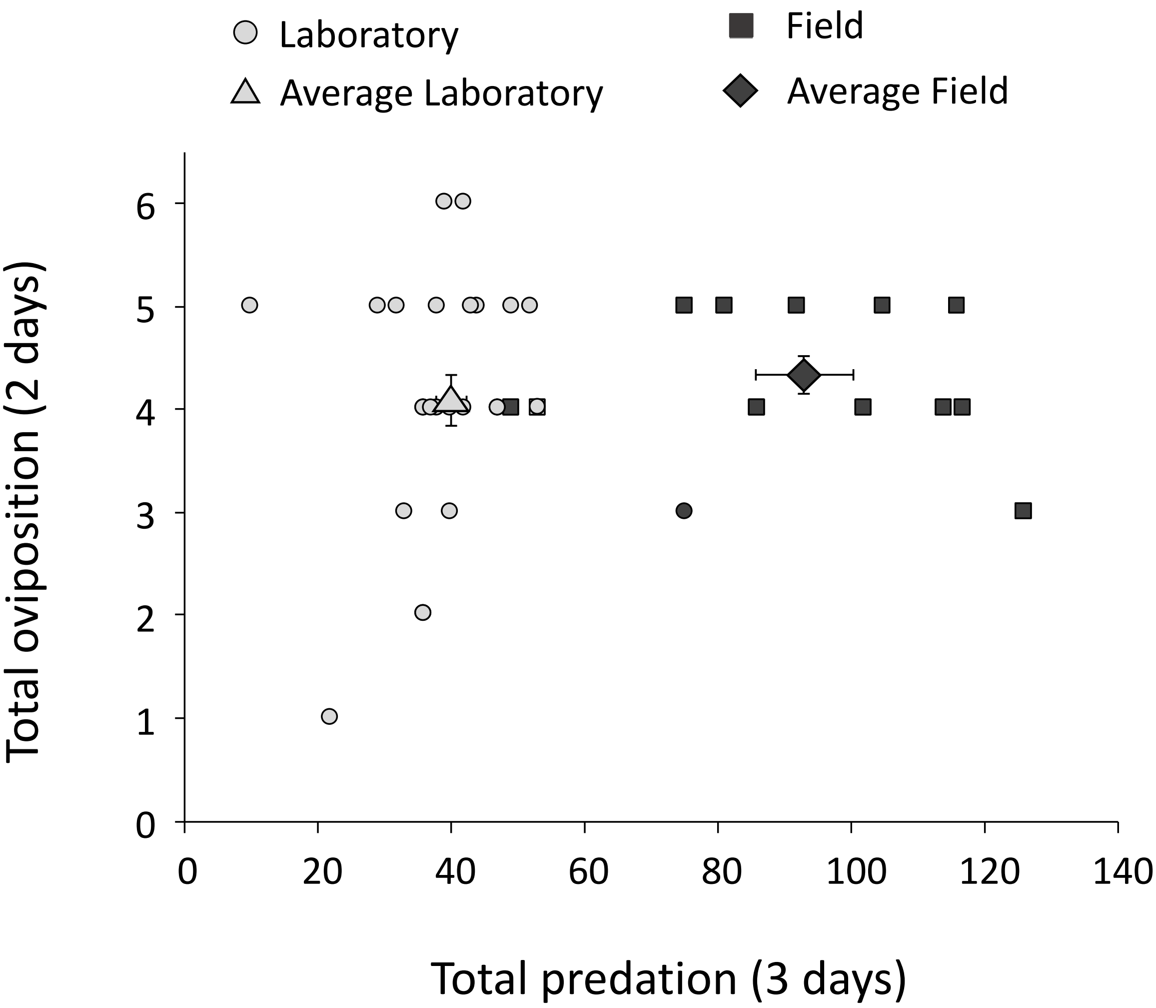


Figure 6: Mean numbers (± s.e.) of eggs of *Diaphorina citri* from the field or from the laboratory consumed by *Amblyseius herbicolus* when offered a choice. Light gray bars refer to partially consumed eggs; dark gray to completely consumed eggs. Different letters indicate significant differences in partial and total consumption (totally eaten plus partially eaten) of eggs from the both origins.

Figure 7: Mean predation rate (± s.e.) of *Amblyseius herbicolus* fed on 30 eggs of *Diaphorina citri* per day from the laboratory (light grey) or the field (dark grey) during three days. The same lowercase and uppercase letters indicate no significant differences in consumption of eggs among days per treatment. Asterisks indicate significant differences in consumption of eggs from the field or the laboratory per day (contrasts after lme, $p < 0.05$).

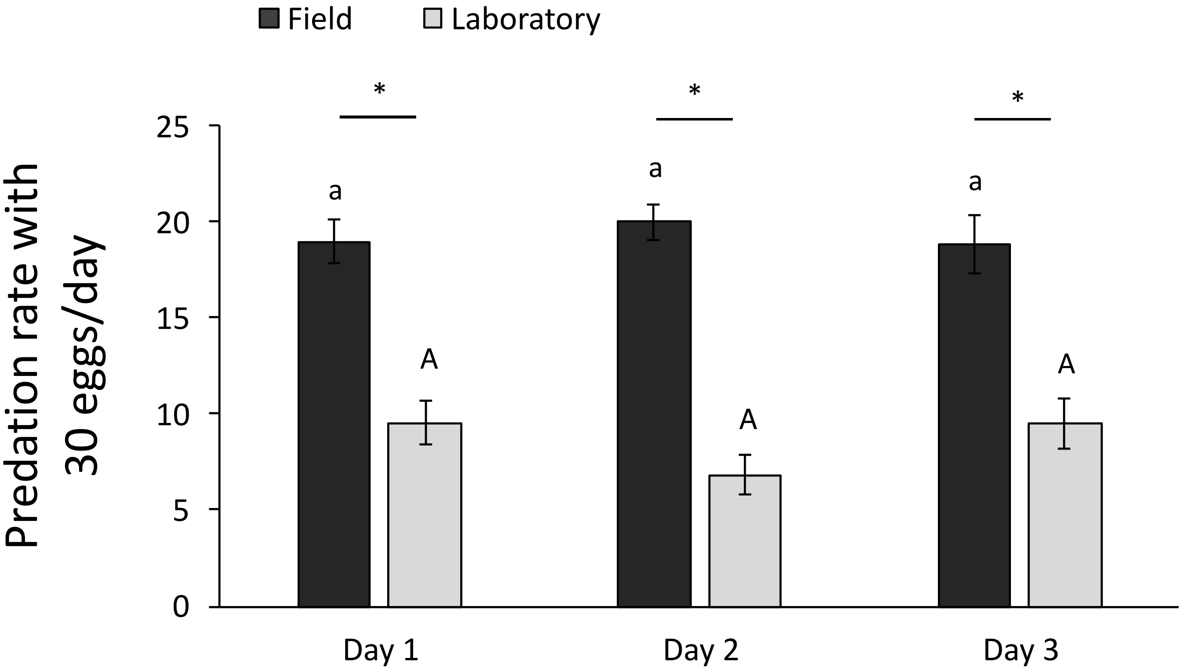


Figure 8: Mean oviposition rate (± s.e.) of *Amblyseius herbicolus* fed on 30 eggs of *Diaphorina citri* from the field (dark grey) or the laboratory (light grey). Different lowercase letters and uppercase letters indicate significant differences in egg consumption among days per treatment. Asterisks indicate significant differences in oviposition between treatments per day ($p<0.05$).

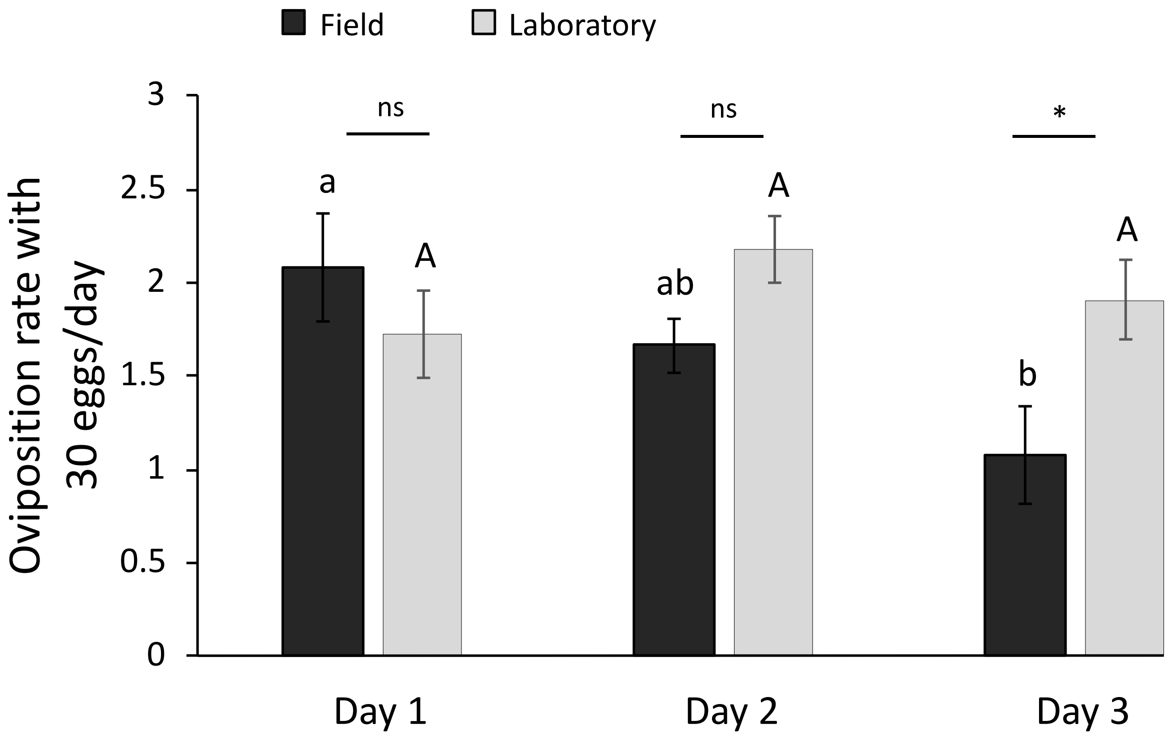


Figure 9: Total oviposition of *Amblyseius herbicolus* during two days (vertical axis) as function of total predation (horizontal axis) of eggs from the laboratory (light grey circles) or from the field (dark grey squares) when 30 eggs were provided per day. The larger symbols show the means (± s.e.), smaller symbols are individual measurements.

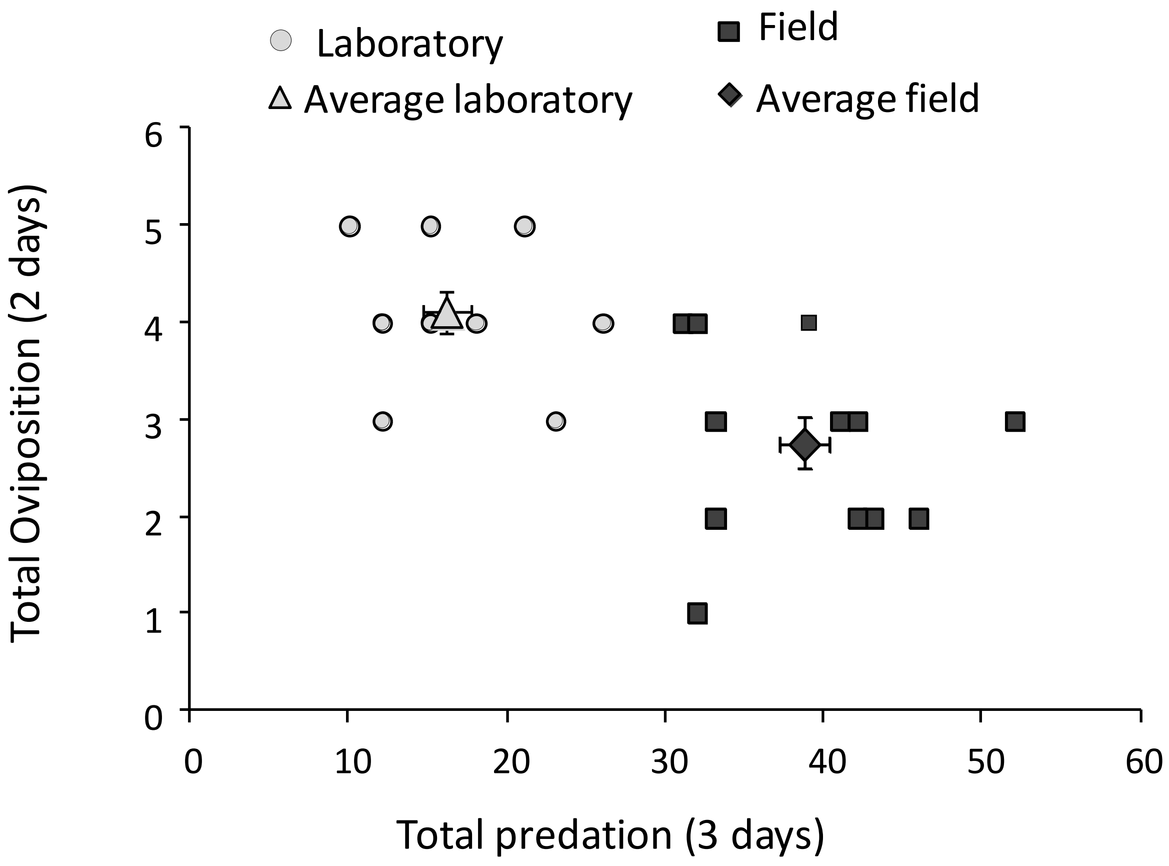


Figure 10: Mean predation rate (± s.e.) of *Amblyseius herbicolus* fed on 10 eggs of *Diaphorina citri* per day from the laboratory (light grey) or the field (dark grey) during three days. The same lowercase and uppercase letters indicate no significant differences in consumption of eggs among days per treatment. Differences in consumption of eggs from the field or the laboratory per day were not significant (p<0.05).

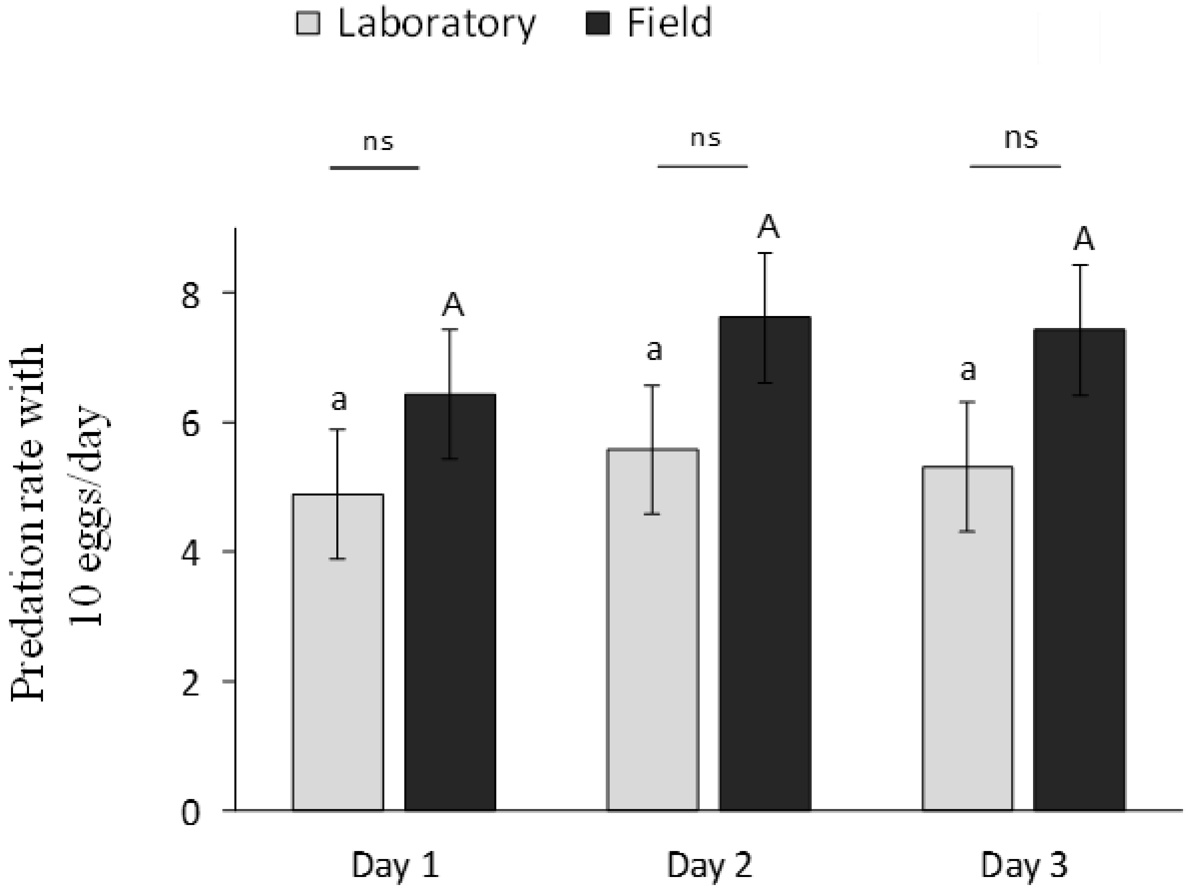


Figure 11: Mean oviposition rate (± s.e.) of *Amblyseius herbicolus* fed on pollen or on 10 eggs of *Diaphorina citri* from the field (dark grey) or the laboratory (light grey). Differences in consumption of eggs from the field or the laboratory per treatment and per day were not significant ($p<0.05$).

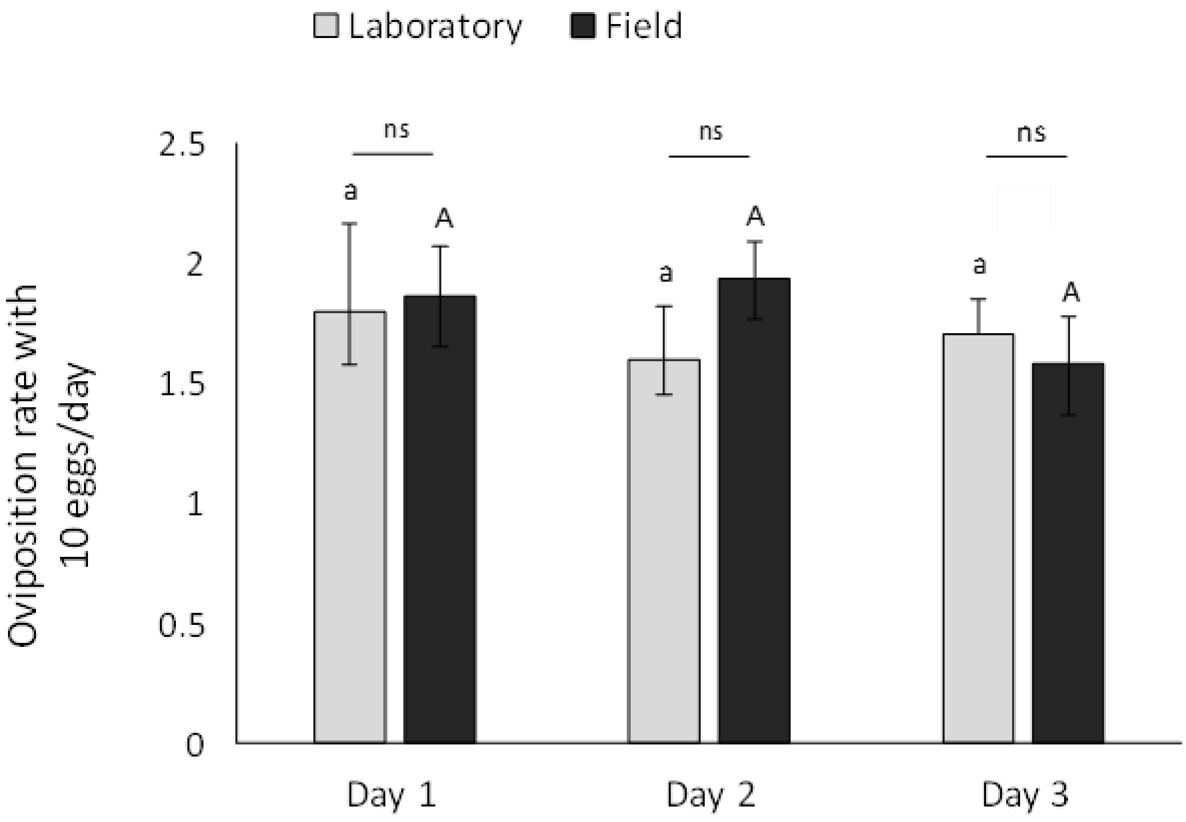


Figure 12. Total oviposition of *Amblyseius herbicolus* during two days (vertical axis) as function of total predation (horizontal axis) of eggs from the laboratory (light grey circles) or from the field (dark grey squares) when 10 eggs were provided per day. The larger symbols show the means (± s.e.), smaller symbols are individual measurements.

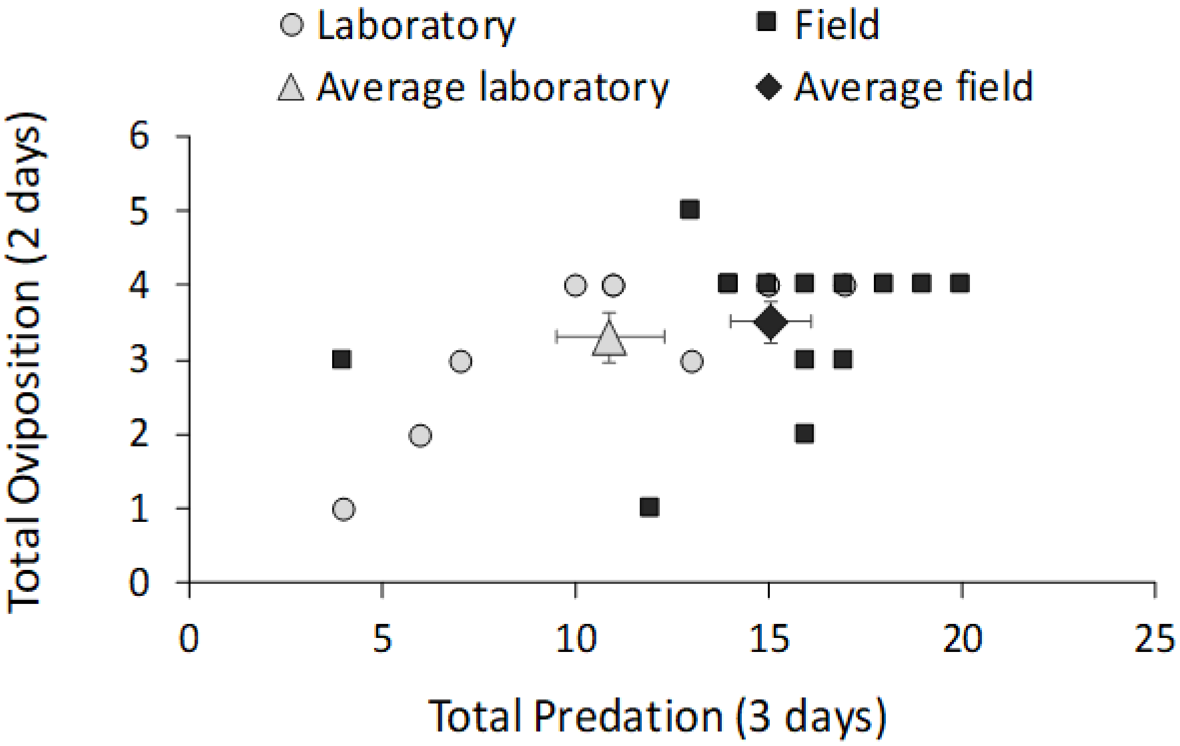


Figure 13: Juvenile development and survival of *Amblyseius herbicolus* fed *Diaphorina citri* eggs from the field or from the laboratory or *Typha sp.* pollen. Shown is the cumulative proportion of adults as a function of time. Total survival is the final cumulative proportion that reached adulthood. Survival was high on all diets, but developmental rate was significantly higher on pollen than on eggs of *D. citri* (contrasts after developmental rate and survival analyses are represented by lowercase and uppercase letters, respectively, in the legend). Error bars represent standard errors.

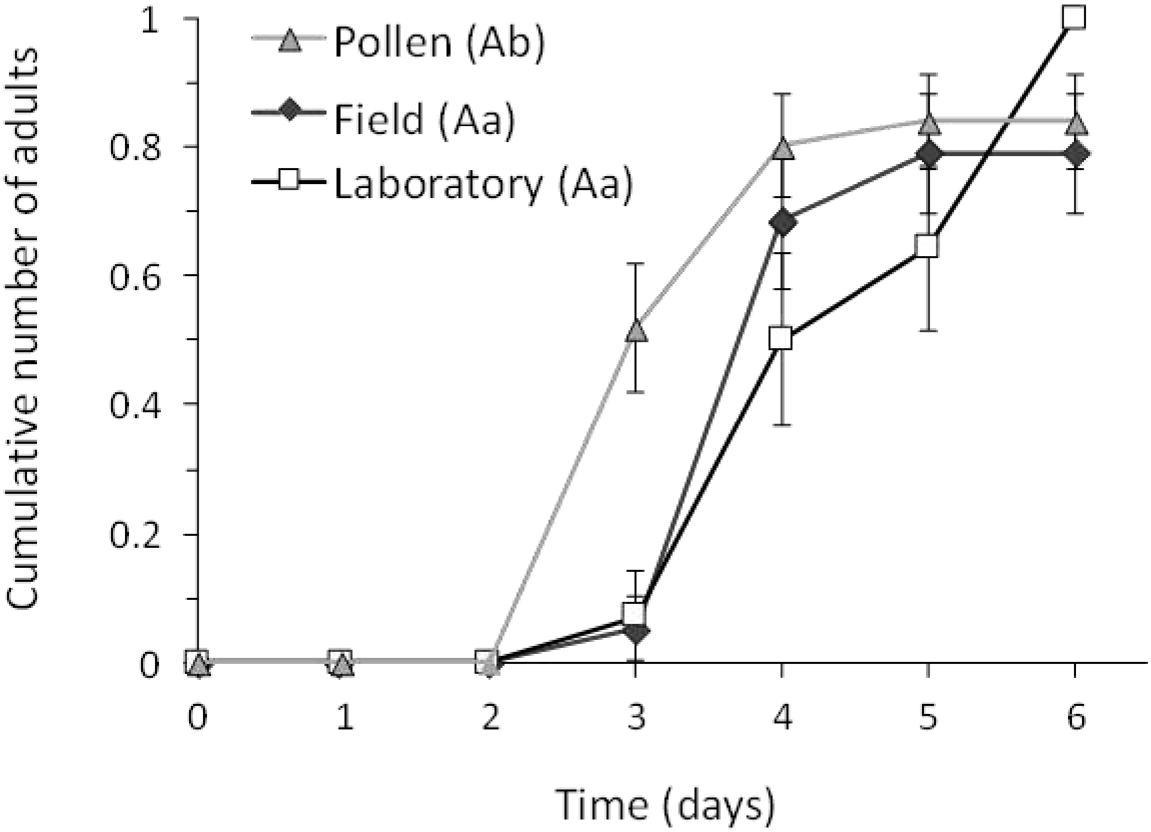


Figure 14: Juvenile development and survival of *Amblyseius herbicolus* fed on *Thyreophagus cracentiseta* (black line) or pollen (grey line). Shown is the mean cumulative proportion of adults as a function of time. Total survival is the final cumulative proportion that reached adulthood. Developmental rate and survival were similar on pollen and on *T. cracentiseta* (significance of contrasts after developmental rate and survival analyses are represented by lowercase and uppercase letters, respectively, in the legend). Error bars represent standard errors.

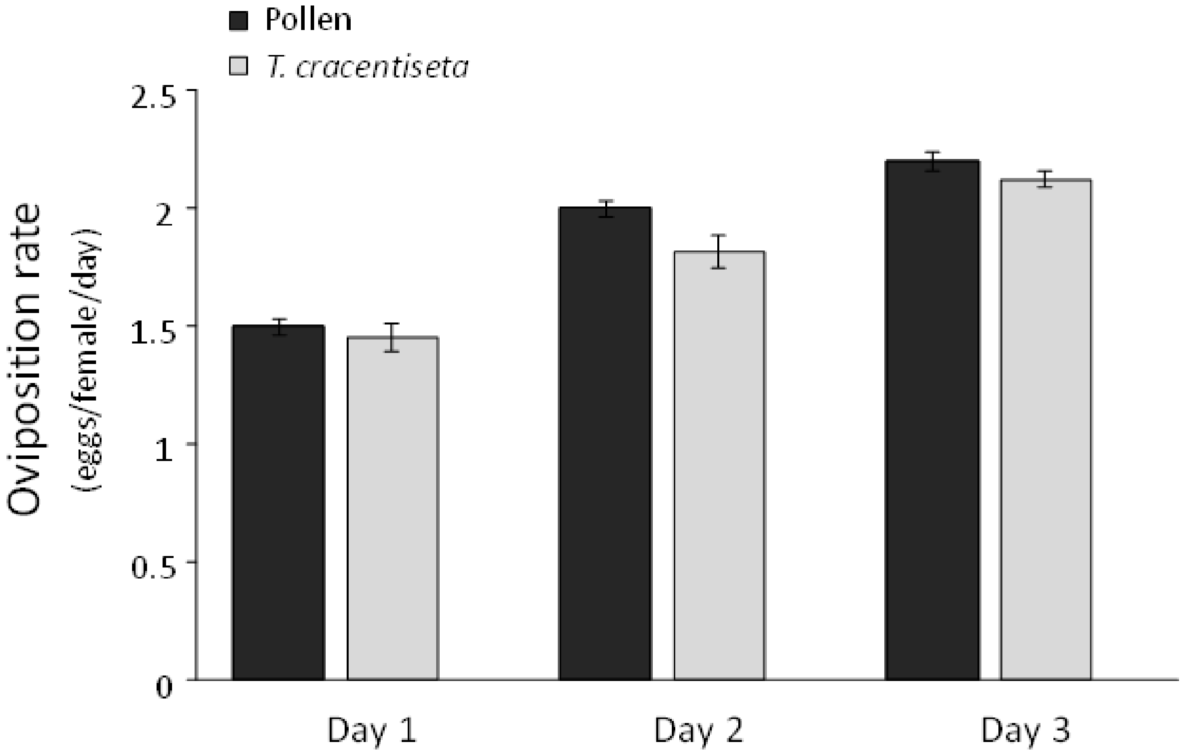
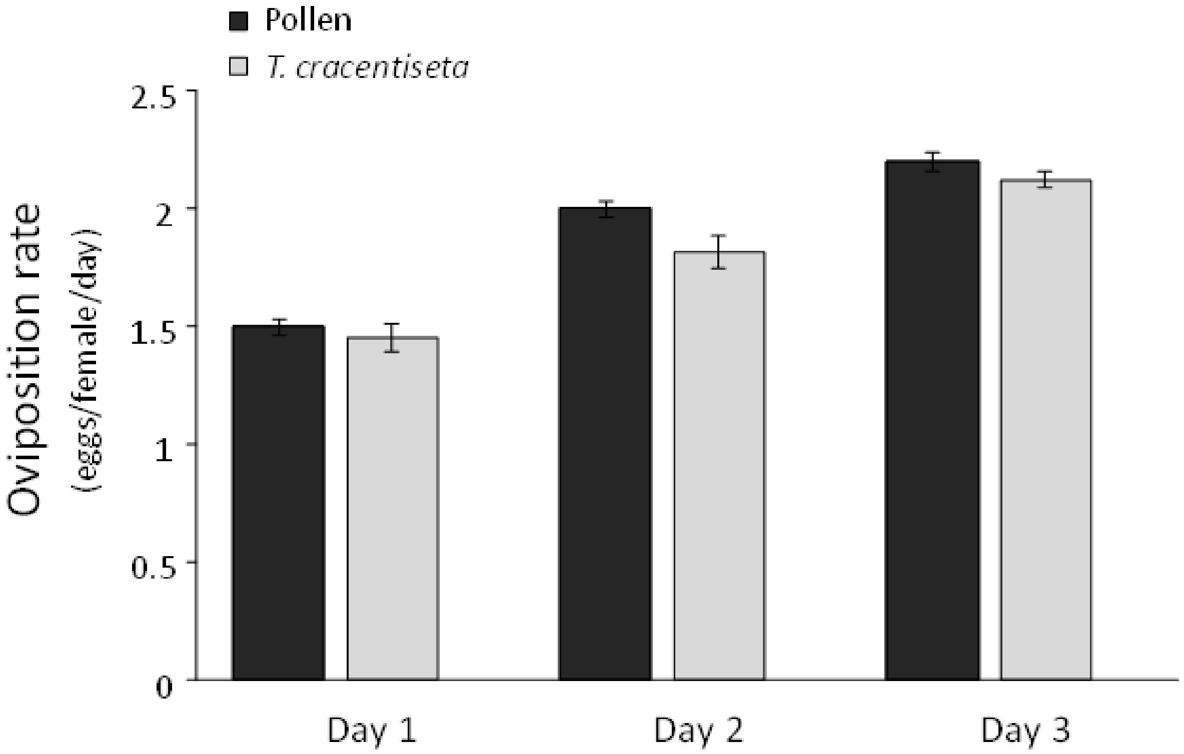


Figure 15: Mean oviposition rate (± s.e.) of the predatory mite *Amblyseius herbicolus* on *Typha sp*. pollen (black) or on *Thyreophagus cracentiseta* (grey). Differences in oviposition on pollen or on *T. cracentiseta* per day were not significant (p<0.05).



Chapter 2:

Amblyseius herbicolus Chant (Acari: Phytoseiidae) suppress populations of *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) on orange jasmine plants with alternative food

ABSTRACT

Thus far, no natural enemy is commercially available to control *Diaphorina citri*. Some predatory mites were investigated as potential agents, however none of them are sold in Brazil. Previous experiments in the laboratory showed that *Amblyseius herbicolus* is able to develop and reproduce on a diet of *Diaphorina citri* eggs. However, this ability is not sufficient to guarantee that the predator will be able to control the pest and more realistic experiments are needed. We therefore released predatory mites on isolated orange jasmine plants one week before the release of adults of *D. citri*. *Typha* sp. pollen was provided as food on twines, which served as oviposition sites, twice per week to enable survival and persistence of the predators, even in the absence of the pest. The density of *D. citri* on plants without predators was twice as high as on plants with predators. We observed that the addition of pollen was sufficient to keep *A. herbicolus* on the plants, even without the pest. Our results show that *Amblyseius herbicolus* is a promising biological control agent of *D. citri* and the use of alternative food helps to maintain the predators on plants before pest invasion. Thus, they could prevent infestation of plants with this vector of an important plant disease.

INTRODUCTION

Predators can serve as bodyguards of plants (Sabelis and van Rijn 1997), and plants can increase the effects of natural enemies on pests in several ways. For example, they can produce alternative food for the predators, such as nectar and pollen, and they can offer shelter through morphological structures, such as hollow thorns and domatia (Price et al. 1980; Dicke and Sabelis 1987; Marquis and Whelan 1996; Sabelis et al. 1999; Nomikou 2003). Alternative food and shelters increase the reproduction or survival of natural enemies on the plant, and consequently, their effect on plant pests (Norton et al. 2000; van Rijn et al. 2002; Nomikou et al. 2002; Adar et al. 2014; Schmidt 2014). If plants lack such structures or do not supply alternative food for the natural enemies, one way of improving control of plant pests with natural enemies is to add alternative food and shelter to the plants (van Rijn and Sabelis 1999; Agrawal et al. 2000; Nomikou et al. 2002, 2010; Adar et al. 2014; Delisle et al. 2015). We used this method here to study the possibility of controlling an important citrus pest and the plant disease it vectors.

The citrus disease Huanglongbing or Greening is quickly spreading through the orchards worldwide and is already established in more than 40 countries (Croxton and Stansly 2014; Blaustein et al. 2018). The major management method is controlling the vector but mitigating HLB is still a challenge and effective and environmental-friendly control strategies against the pest are urgently required (Blaustein et al. 2018). In Brazil, the largest citrus-producing country, the incidence rate in 2015 was 159% higher than 2012 in citrus orchards of São Paulo and Minas Gerais (Fundecitrus 2015; Adami et al. 2019). To try to control *Diaphorina citri* and limit the spread of the disease, some Brazilian growers spray insecticides about 40 times a year, which does not solve the problem, but did increase production costs (Adami et al. 2019).

Biological control can be a good alternative to try to limit the spread of Greening without causing the environmental problems associated with pesticide use (Adami et al. 2019). In Brazil, biological control alternatives used so far are the parasitoid *Tamarixia radiata* Waterson (Hymenopeta: Eulophidea) and two entomopathogenic fungi, *Isaria fumosorosea* and *Beauveria bassiana* (Concesqui et al. 2016; Adami et al. 2019). However, the disease continues to spread, especially on small farms (Fundecitrus 2020). It is known that the transmission of the bacteria among psyllids can be sexual, transovarial and by feeding on infected plants (Mann et al. 2012; Pelz-Stelinsky et al. 2010). However, the probability of acquiring the bacteria by feeding on infected plants is greater than by transovarial and sexual transmission (Pelz-Stelinsky et al. 2010; Kelley and Pelz-Stelinsky 2019). Besides, the psyllids are more competent to infect plants when they acquire the bacteria at the nymphal stage and feeding on uninfected plants may cause decrease in the proportion of infected *D. citri*. The successful acquisition of *Candidatus* bacteria to both *D. citri* and plants is dependent on persistence and time of feeding by *D. citri* (Pelz-Stelinsky et al. 2010; Kelley and Pelz-Stelinsky 2019). Additionally, the infection of plants is also dependent on the quantity of psyllids feeding: multiple infected individuals reinforce the transmission (Lee et al. 2015). Thus, controlling the eggs of *D. citri* is important because it is the only phase that does neither damage the plants directly, nor transmit HLB (Qureshi and Stansly 2009; Juan-Blasco et al. 2012). Therefore, discovering a predator that can control *D. citri* by feeding on their eggs is crucial, especially if it can be used together with other biological control agents in an integrated pest management program (Zhang et al. 2015).

Insects and predatory mites cause mortality of *D. citri*, especially of immature stages (Qureshi and Stansly 2009; Juan-Blasco et al. 2012; Fang et al. 2013). Previous studies with whiteflies and thrips showed that predatory mites are effective biological control agents of these pests (Nomikou et al. 2002; Nomikou 2003; Messelink et al. 2008). *Diaphorina citri*, thrips and whiteflies show some similarities. They have stages that are invulnerable to predation by predators and adult predatory mites are smaller than the adults of these pests. Although large predators can consume more prey, the intrinsic rate of population increase is inversely proportional to body size (Murdoch 1971; Sabelis and van Rijn 1997). Being small, the generation time of the phytoseiids is often relatively short, allowing for a quick numerical response to increased pest densities (Sabelis and van Rijn 1997). Additionally, *D. citri* oviposits on developing leaves, where they are difficult to reach by large predators, but not by predatory mites (Juan-Blasco et al. 2012). The use of predatory mites may also be advantageous because it attacks other stages than the parasitoid *Tamarixia radiata*, but it is still necessary to test whether the presence of the predator can disrupt control by the parasitoid.

Until now, three species of commercially available predatory mites were studied to control *D. citri*. They are *Amblyseius swirskii* (Juan-Blasco et al. 2012), *Neoseiulus cucumeris* (Oudemans) and *Neoseiulus barkeri* (Hugues) (Acari: Phytoseiidae) (Fang et al. 2013). The predation and oviposition rates of *A. swirskii* were higher than those of the other two species, but further experiments are needed to verify the feasibility of using *A. swirskii* to control *D. citri* populations. Fang et al. (2013) studied population dynamics on citrus plants, but they only observed reduction in *D. citr*i densities when releasing a high number of *N. cucumeris*, and the feasibility of its use to control *D. citri* has yet to be evaluated. The three species are not commercially available in Brazil and are exotic. Although the use of exotic natural enemies may be effective in classical and augmentative biological control, their introduction can cause risks to the environment and may have negative effects on non-target organisms (De Clercq et al. 2011). Nowadays, more strict regulations to reduce the risks of releasing exotic natural enemies also reduce the implementation of this control method and the commercialization of exotic biocontrol agents (Van Lenteren et al. 2012). Thus, classical biological control is only adopted to try to control invasive pests that cannot be controlled otherwise (Hajek et al. 2016). We therefore first investigated whether there are a potentially effective predatory mites that already occur in Brazil.

Our previous results showed that the indigenous predatory mite *Amblyseius herbicolus* Chant (Acari: Phytoseiidae) was able to reproduce and develop when feeding on *D. citri* eggs (Chapter 1). This species occurs on several plant species in Brazil, including citrus plants. However, the effective application of augmentative biological control depends not only on predation, reproduction and development of natural enemies on the pest, but on the population dynamics of the pest-natural enemy system (Ridgway 2013), and more realistic experiments, on a larger temporal and spatial scale are therefore necessary to confirm the potential of *A. herbicolus* to control *D. citri*. In this study we verified the ability of this predatory mite to suppress populations of *D. citri* on isolated orange jasmine plants which are the preferred alternative host of this pest (Halbert and Manjunath 2004). This plant is widely used as an ornamental, which facilitates the dispersal of the vector and the disease, so *D. citri* also needs to be controlled on orange jasmine plants, especially because they can be used as a trap crop to manage *D. citri* (Tomaseto.et al. 2019).

Plants of the family Rutaceae, such as citrus and orange jasmine, are usually glabrous, so do not offer many structures for shelter or oviposition by predatory mites (Schmidt 2014), and although several citrus species can flower several times a year (Krajewski and Rabe 1995), the availability of nectar and pollen is not permanent. We therefore used pollen as alternative food and twines (Adar et al. 2014) as oviposition substrate and shelter for *A. herbicolus* in a population-dynamical experiment on isolated plants.

MATERIALS AND METHODS

Predator sampling

To verify the presence of predatory mites on citrus and orange jasmine, plants of these species were sampled on the campus of the Federal University of Viçosa (20º46’9’’ S, 42º87’02’’ W) in the state of Minas Gerais, Brazil. Two methods of collection were used; the first was beating the leaves above a black tray, which was subsequently covered with plastic film. For the second method, we removed the branches with *D. citri* eggs in the growing tips, which then were placed in paper bags. Tray and paper bags were taken to the laboratory, where slides of predatory mites were made for morphological identification using a phase contrast microscope and a dichotomous key (Chant and McMurtry 1994, 2007). The predatory mite *A. herbicolus* was found on both citrus and orange jasmine plants, in the last case together with *D. citri*.

Acquisition and maintenance of clean plants

Orange jasmine plants (4-6 months old) were obtained from Viveiro Antuérpia in the vicinity of Viçosa, Minas Gerais, Brazil. Subsequently, the plants were kept in an insect-proof greenhouse (25 ± 2 ºC), where they were fertilized every two months with a mixture of NPK (4/14/8). The plants were pruned regularly to induce the development of growing tips, which are oviposition sites of *D. citri*. We assessed the presence of the three *Candidatus* Liberibacter species in plant material as well as in *D. citri* (see Supplementary Material).

Diaphorina citri rearing

Adults of *D. citri* were collected from orange jasmine trees of about 6 years old on the campus of the Federal University of Viçosa. They were incubated in a BugDorm-4F insect cages (0.5 x 0.5 x 1.0 m) to oviposit on pesticide-free orange jasmine plants (see above) and formed the basis of what will be referred to as the lab population. These insects were kept under laboratory conditions. The plants were watered two times a week and adults were regularly transferred to new plants. Besides eggs from this lab strain, eggs were also obtained directly from trees on the campus; they are referred to as the field strain.

Pollen

The pollen used for the predatory mite rearing and experiments was collected from *Typha sp.* plants from rural areas around Viçosa, Minas Gerais. This pollen was chosen because previous studies showed high oviposition and population growth rates of *A. herbicolus* when feeding on it (Duarte et al. 2015; Chapter 1). It was dried in an oven at 60º C for 48 h and was stored in a container in the freezer. Periodically, small amounts of pollen were removed from the container and placed in 1.5 ml microtubes (Eppendorf), dried at 60° C for an additional 48 h and then stored in the freezer.

Population dynamics experiment

The capacity of *A. herbicolus* to control *D. citri* was tested on *M. paniculata* plants (15-20 cm high) with similar numbers of growing tips to allow oviposition by *D. citri*. The plants were placed inside tubular cages (ø = 61 cm; 50 cm high) made of transparent acetate and covered with a mesh (90 µm) glued to the top end of the tube to provide air circulation (Fig. 1). An opening at the centre of the side of the cage (10 cm X 10 cm) also allowed for air circulation to avoid excess of humidity. The base of the tube was closed with a plastic tray (ø = 60 cm), and cages were placed inside a tray with water and detergent to prevent invasions of control cages. In previous tests we observed that after the release of *D. citri,* some individuals were found dead in the moist soil. To prevent this, a black plastic sheet was placed at the base of the plant, we subsequently observed that the predatory mites walked under this sheet, close to the soil, perhaps to protect themselves from light during the day, and the sheet was therefore kept during the experiment. Half of the plants received 6 adult females of *A. herbicolus,* collected randomly from a laboratory rearing with females of similar ages (10-12 days old), one week before infestation with *D. citri*. The predatory mites were transferred to the plants using a fine brush. The other plants did not receive predators and served as control. To facilitate oviposition by *A. herbicolus*, one fibrous string of cotton (5 cm long) with pollen was suspended from all plants, including the controls (Adar et al. 2014) before the release of predators. We observed that the mites oviposited on this string during the first weeks. Adults of *D. citri* of similar age were obtained from the rearing by removing plants with fifth instar nymphs, which were placed in a new cage until adulthood. From the field, growing tips with fifth instar nymphs were collected, wrapped in moistened cotton wool and placed in the cage with the other fifth-instar nymphs. After reaching adulthood, the *D. citri* were allowed to become sexually mature for 5–10 days (Shivankar et al. 2000). Subsequently, they were individually sucked into a pipette tip (1000 µl; Nichiryo, Japan) connected to a transparent hose (ø = 1 cm) with a mesh (90 µm) at the interface of the hose and the tip to restrain the *D. citri*. Subsequently, the pipette tip was detached from the hose and closed on both sides with Parafilm "M" laboratory film (Bemis Flexible Packaging, Neenah, WI 54956) and the insect was sexed based on the abdominal tip. For release of *D. citri*, the tubular cage was lifted, and the pointed part of the pipette tip was inserted into the soil. Subsequently, the Parafilm was removed so that the insects could move onto the plant and the tubular cage was replaced. In this way, three males and seven females of *D. citri* were released per plant (5-10 days old). This reflects the sex ratio observed in the rearing and the field population. More individuals were released weekly until all plants had eggs of *D. citri*. In total, each plant received seven males and 17 females of *D. citri*. This was done because the release of pairs of *D. citri* only at the beginning of the experiment in previous attempts did not guarantee oviposition and populations did not even increase on the control plants.

Twice per week, the presence of predators and eggs of *D. citri* were registered, and the numbers of adults and 4th-5th instars of *D. citri* nymphs were counted by inspecting the plant systematically by naked eye during 100 days. All plants were irrigated and received about three mg of *Typha sp.* pollen twice a week. The pollen was applied through the mesh in the top of the cage, so that it was spread over the plant. The number of replicates was five plants per treatment. After 26 days, two control cages were invaded by predators and were therefore evaluated as treatment cages from that moment on, resulting in seven plants with predators and three plants without predators from that moment onwards. The experiment was conducted under controlled temperature and humidity conditions (25 ± 2 ° C, 70 ± 10% RH and 12 h photophase).

Statistical analysis

The numbers of *D. citri* on plants with and without predators were analyzed with a linear mixed effect model (LME) (Bates 2005) with replicate as a random factor and the presence of predators and time as fixed factors. Contrasts between treatments were obtained with the function cld (package multcomp of R) (Hothorn et al. 2009).

RESULTS

Population dynamics experiment

In the two first weeks, predators oviposited on the twine with *Typha* sp. pollen suspended from the plant. When aged pollen accumulated on the twine throughout the experiment, we observed that predatory mites stopped ovipositing on the twine. Later during the experiment, the predators oviposited near *D. citri* nymph exuviae (Fig 2). The effect of the interaction between treatment and time on *D. citri* densities was significant (lme: d.f. = 1, $Chi^2 = 22.7$, $p < 0.0001$; Fig. 3). This was because the treatments started with the same number of *D. citri* and finished with fewer *D. citri* on plants with predators than on plants without predators. The difference in densities of *D. citri* between plants with and without *A. herbicolus* was significant from the 52th day until the end of the experiment on day 100. At the end of the experiment, on average about 50% fewer *D. citri* were found on plants with *A. herbicolus* than on plants without them (Fig. 3). Moreover, we observed *A. herbicolus* sucking body fluids of first instar nymphs.

DISCUSSION

We previously observed that *A. herbicolus* can develop and reproduce feeding on *D. citri* eggs (Chapter 1), but it was still unclear whether it can control this pest in plants where it normally occurs. In the current experiment, we observed that *A. herbicolus* suppressed *D. citri* populations by about 50% of compared to plants without this predator (Fig. 3). To our knowledge, this is the first study to describe control of *D. citri* by an indigenous predatory mite in Brazil.

A direct comparison of our results with those with other predatory mites (*A. swirskii* or *N. cucumeris*) is difficult because of the different host plants and methodologies utilized. Juan-Blasco et al. (2012), did not study population dynamics, but evaluated predation of eggs and juveniles on plants. Fang et al. (2013) released very high numbers of *N. cucumeris* on caged citrus plants. Compared to this, our results are more complete and promising.

Although pest eradication is perhaps not necessary to reduce disease spread, eradication can be achieved and is dependent on the initial predator/prey ratio (Janssen and Sabelis 1992; Nomikou 2003). A high predator/prey ratio can be achieved by releasing high numbers of predators or by releasing much lower numbers and providing alternative food such as pollen (van Rijn and Sabelis 1990; Nomikou, 2002). In our case, we released a low number of predators, provided pollen twice a week and one twine per plant, which was sufficient to obtain a significant reduction of the pest population. Perhaps providing more twines or new twines will further increase predator densities, resulting in further reductions of the pest population.

We released *A. herbicolus* before *D. citri*, thus the predator had access to pollen in the first week and to both *D. citri* and pollen subsequently. The pollen allowed the predators to survive when there were no *D. citri* eggs to be consumed yet and enabled the persistence of predator populations on the plants. Although we did not have a treatment with predators without pollen for logistical reasons, our results indicate that alternative food can be used to maintain predators on plants before the pest arrives. Considering that *A. herbicolus* already occurs on citrus and orange jasmine plants in the field, a strategy to control *D. citri* may be to increase existing predator populations by providing alternative food such as pollen or storage mites and oviposition sites such as twines. In general, preventive releases of predators are shown to be effective to control various crop pests (Calvo et al. 2012; Leman et al. 2019). The maintenance of predators in the crop can be seen as a preventive measure that is not possible with populations of specialist predators and parasitoids, which decrease when pest densitiesare low, and this may favor new pest invasions and outbreaks (Janssen and Sabelis 2015).

Understanding the persistence and regulation of populations has been challenging for ecologists for years (Cappuccino and Price 1995). Prey persistence on plants with predators was also observed in whitefly dynamics experiments (Nomikou et al. 2002) and can be explained by the presence of stages that are invulnerable to consumption by predators. Adult stages can fly when disturbed and they are less vulnerable to predation than eggs and the presence of invulnerable stages can prevent the extinction of pest (Murdoch et al. 1987; Nomikou et al. 2002). However, the addition of an extra food source for the predator can help to reduce pest populations (Holt 1977; Nomikou et al. 2002). Experiments with more than one generation, such as those reported here, are essential because they show not only if pest densities decrease, but also whether this decrease is persistent. Moreover, the reduction of densities observed during one generation may be ambiguous due to the preponderance of vulnerable stages (Harrison and Cappuccino 1995). Our experiment was conducted for 100 days, covering about five generations of *D. citri* (Liu and Tsai 2000) showing that the reduction of *D. citri* densities was persistent (Fig. 3).

The consumption of eggs of *D. citri* can prevent the spread of HLB (Fang et al. 2013), which can be acquired by nymphs and transmitted to other plants by adults. We observed previously that predation rates of *D. citri* eggs may be affected by the host plant (Chapter 1) and population dynamics of the predators and prey may depend on prey quality as food for predators (Nelson et al. 2001). However, this can be compensated by providing alternative food and even if the prey is of low quality, control can be achieved. Besides the effect of host plants on prey quality, there can also be effects of microbial endosymbionts of the prey on its quality. An intracellular bacterium *Candidatus* Profftella armatura (*Betaproteobacteria*) is a defensive symbiont of *D. citri* and can synthesize a polyketide called diaphorin (Yamada et al. 2019). Diaphorin has negative effects on the survival of *Harmonia axyridis* (Coleoptera: Coccinellidae), one of the main predators of *D. citri* (Michaud 2004; Yamada et al. 2019). Information about the susceptibility of *A. herbicolus* to diaphorin is still lacking, but deserves further attention.

We observed a significant effect of the interaction between time and treatment and the differences in pest density became significant starting on day 50. The lack of significant differences before this was caused by the delayed establishment of a population of *D. citri* in one of the control cages. Future experiments with larger numbers of replicates with pollen on twine should further confirm our results, but the use of the astigmatid mite *T. cracentiseta* as alternative food also deserves further research. Additionally, the predators invaded two control cages, and although this should have been avoided, the fact that the predators found these plants with high prey density is further confirmation of the suitability of this predator (Harrison and Cappuccino 1995).

Much remains to be investigated to achieve successful biological control of *D. citri*. In agroecosystems for example, honeydew is the prevalent source of sugar (Wäckers et al. 2008) and it is known to favour natural enemies and increase the effectiveness of biological control (Wäckers et al. 2008; van Rijn et al. 2013; Beltrà et al. 2017). The honeydew produced by *D. citri* can also be an alternative food source for natural enemies (Juan-Blasco et al. 2012), and its use by *A. herbicolus* should be studied. Moreover, the quality of honeydew is dependent of the quality of the plant (Walkers et al. 2008). When *D. citri* fed on infected plants, for example, it produced low-quality honeydew, because HLB changes the compounds available in the phloem (Hijaz and Killiny 2016). It is not known yet how the consumption of honeydew of *D. citri* may affect predatory mites and biological control.

Mortality caused by natural enemies is crucial to regulate *D. citri* populations (Qureshi and Stansly 2009), especially because of the increased pesticide resistance of this pest (Tiwari et al. 2011; Chen and Stelinski 2017; Chen et al. 2020). Besides the control exercised through the predation of eggs and nymphs, there is the possibility that predatory mites can be used together with some biopesticides in citrus orchards to increase mortality of *D. citri*. For example, *A. swirskii* and *N. cucumeris* were used to disseminate spores of *Beauveria bassiana,* an entomophagous fungus, to kill nymphs of *D. citri*. The nymphs showed high mortality, and the mites not, and this approach can be used to improve biological control of this pest (Zhang et al. 2015).

The compatibility between different biological control agents of *D. citri* will need to be established to enable integrated pest management. Thus, it is necessary to verify if this predator can disrupt control by the parasitoid *T. radiata*, for example by competition, or if the actions of these two natural enemies are synergetic. Citrus growers of the state of São Paulo, Brazil, already reported that the intensive use of pesticides kill natural enemies that previously occurred in their crops. The main parasitoid, *T. radiata* is used especially in abandoned or organic orchards (Adami et al. 2019), but to limit the spread of HLB, the use of predators and parasitoids, if compatible, will need to be increased.

We also do not know yet how the presence of the predator on the plant affects the behavior of *D. citri*. On the one hand, studies indicate that the presence of predators may cause the pest to leave the plant when disturbed, and this may increase disease transmission. On the other hand, the presence of predators can reduce the transmission of the pathogen to plants by altering the feeding behavior of vectors. Leafhoppers, for example, fed more in xylem than in phloem in the presence of a predator, but the pathogen vectored by the leafhoppers occur in the phloem. This change in vector behavior reduced the transmission of pathogens (Tholt et al. 2018). On orange jasmine plants on the field, both predators and *D. citri* were present. Understanding how the presence of *A. herbicolus* may affect both the population densities and behavior of *D. citri* is particularly important for understanding how to reduce the spread of HLB and the effects of predator-induced changes in vector behaviour on disease transmission should be studied.

Promising species of predatory mites for biological control programs should be easy to rear and effective in reducing pest populations (Van Lenteren 2000). We showed that *A. herbicolus*, a native predatory mite, was capable to reproduce and develop on a diet of the pest eggs, pollen and to reduce population densities of *D. citri* on orange jasmine plants. The next step is to produce *A. herbicolus* on a large scale, release and evaluate its performance in in the field.

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Figure 1: Tubular cage used in population dynamics experiment of *D. citri*. A plastic tray was placed at the top and bottom of the cage to seal it. A circular hole in the top plastic tray was closed with a mesh to allow ventilation. An additional mesh was glued over an opening in the middle of the cage for the same purpose.

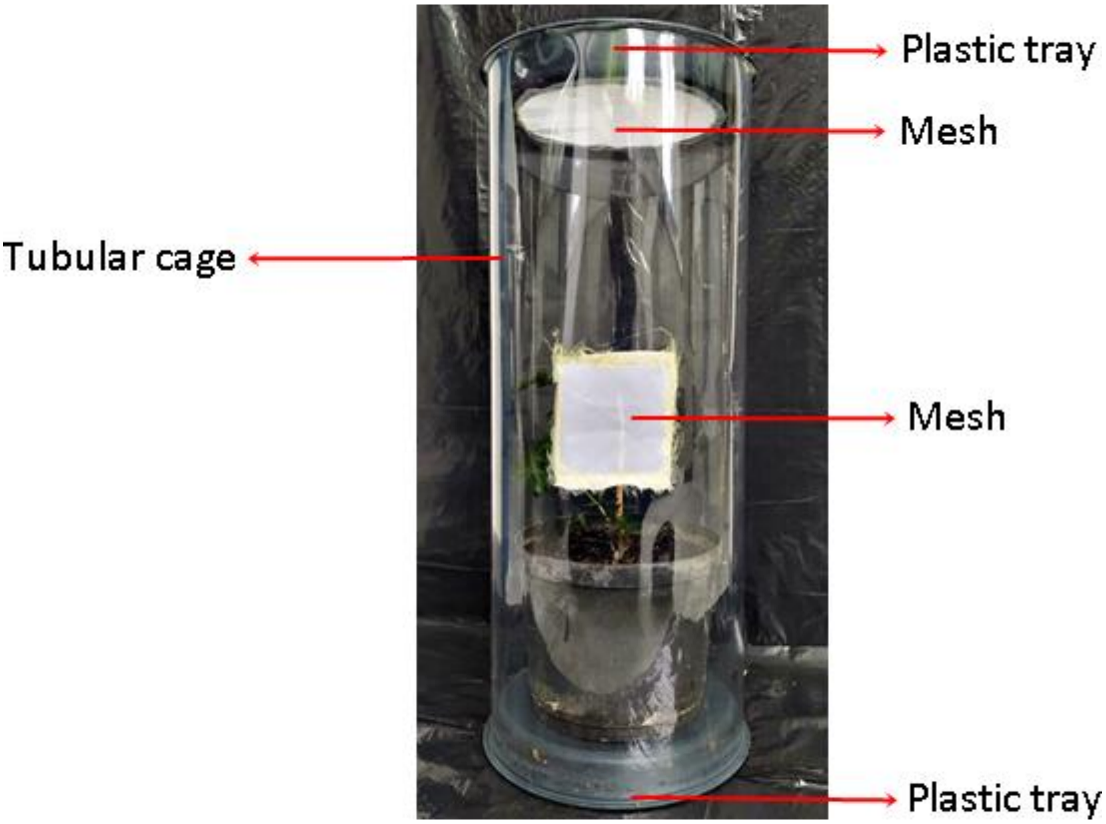
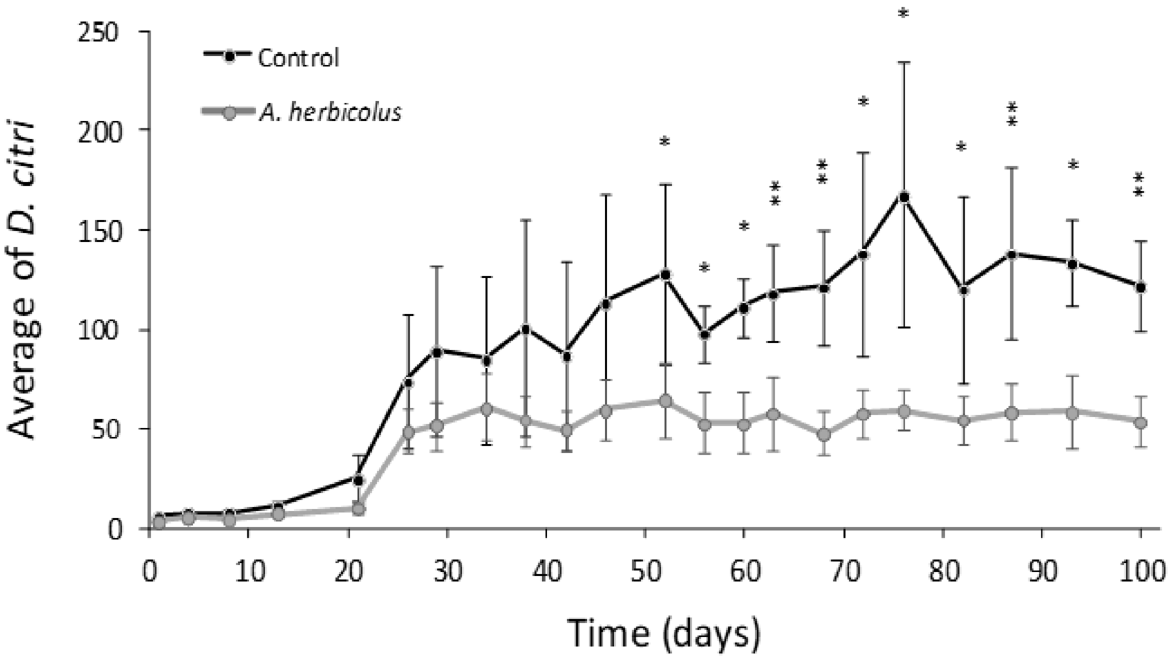


Figure 2: Eggs of the predatory mite *A. herbicolus* oviposited under an exuvium of a *D. citri* nymph in the population dynamics experiment.

Figure 3: Mean numbers (± s.e.) of *Diaphorina citri* on orange jasmine plants with (grey line) and without (black line) predators during three months. Asterisks (*) indicate significant differences between treatments per observation day (* p<0.05, ** p<0.001).



GENERAL DISCUSSION AND CONCLUSIONS

From the work presented in this thesis, it can be concluded that the predatory mite *Amblyseius herbicolus* has potential as a biological control agent of *Diaphorina citri* and could potentially decrease the spread of the citrus greening disease, vectored by this herbivore. The predator is able to feed, develop and reproduce on *D. citri* eggs. Additionally, the predator was found to be affected by the nutritional quality of the prey, depending on the origin, size or age of the plant on which the pest fed. The predator consumed more eggs when offered only low-quality eggs from the field than when offered only high-quality eggs from a lab laboratory, hence, appeared to compensate the low quality by increasing the consumption such that its oviposition rate was not reduced. The cause of the differences in egg quality is still unknown, but could be related to differences in herbivory-induced plant defences between plants from the field and small plants from the lab. Although the plants in my experiments were not infected with HLB, infected plants are known to be of lower quality than healthy plants, and this may then also affect the quality of *D. citri* eggs as prey. As a consequence of the effects of HLB on plant and vector, the third trophic level can also be affected (Martini et al. 2014). Infected plants have a low amount of essential amino acids available because the bacteria seem to remove nutrients from the host (Hijaz and Killiny 2016; Tholt et al. 2018). Additionally, a defensive response of infected plants is the accumulation of starch to limit further spread of the bacteria in the phloem, but this also reduces host quality for the herbivore by impairment of the translocation of photo assimilates (Wang and Trivedi 2013; da Graça et al. 2015). HLB infected plants have suboptimal quality for *D. citri* (Martini et al. 2014; Hijaz and Killiny 2016), which subsequently induces their dispersion to uninfected plants (Zhao et al. 2013; Pelz-Stelinski and Killiny 2016). Moreover, it is known that plant infection causes changes in volatiles produced by infected plants, which affects the attraction of parasitoids and parasitism rates under field conditions (Mann et al. 2012).

In conclusion much remains to be investigated about the effects of HLB infection on the third trophic level. At the moment, we do not know how the infection can affect the control exercised by the predator. In this context, the effects of plant-pathogen interactions on the predator, especially their foraging behaviour, need to be studied. For example, it is not known whether the quality of *D. citri* eggs is indirectly affected by the pathogen. If eggs produced by females that fed on infected plant are of lower quality for the predator than are eggs of uninfected plants, this may induce compensatory predation, resulting in higher predation rates of eggs from infected plants. However, if predators have a choice, they might prefer eggs from uninfected plants. Preference is dependent on prey quality but also on prey availability and we do not know how eggs of different qualities are distributed in citrus orchards, but we can infer from our results that the predators probably will prefer to consume eggs from plants with high nutritional quality if they are available. If predators show the same compensatory predation of eggs infected with the pathogen, this may have two opposite effects. Because predators will prey more on infected eggs, the effect of this compensatory feeding would limit the spread of HLB. However, if the predators have a choice between infected and uninfected eggs, they are expected to prefer uninfected eggs, which will not stop the spread of HLB.

In a population dynamics experiments, we released a few predators on plants and observed a reduction of 50% of the densities of *D. citri*. The plants used were of higher quality than plants from the field. Because of the compensatory feeding observed with *D. citri* from plants in the field, control on plants in the field may be better than observed here in the lab, especially if the populations of predators in the field are increased by adding alternative food. I showed that populations of *A. herbicolus* can establish on orange jasmine plants with alternative food and oviposition sites, and thus reduce densities of *D. citri*. Furthermore, the current thesis shows that this predatory mite is also able to develop and reproduce by feeding on *T. cracentiseta*, which may further facilitate mass-rearing. The use of alternative food may not only promote the establishment of *A. herbicolus* on host plants of *D. citri*, but also limit the spread of the disease, which is the objective of management programs against this pest. Future experiments should focus on the dynamics of this predator using the astigmatid mites in sachets and on large plants.

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SUPPLEMENTARY MATERIAL – Molecular analysis

Detection of *Candidatus* Liberibacter species

Plants (*Murraya paniculata*) and insects (*Diaphorina citri*) were tested for the presence of *Candidatus* Liberibacter spp. using PCR. The presence of *Ca*. Liberibacter asiaticus (Las), *Ca*. Liberibacter africanus (Laf) and *Ca*. Liberibacter americanus (Lam) bacteria was assessed with 'touchdown' PCR (Don et al. 1991) of the 16S rDNA gene (Teixeira et al. 2005, 2008). Positive controls for Las and Lam were donated by João Roberto Spotti Lopes (ESALQ, Universidade de São Paulo, Brazil) and Nelson Wulff (Fundecitrus, Brazil), respectively.

We extracted the total DNA of entire insects (10-20 adult psyllids per sample, three samples from the field and three from the lab population) and orange jasmine leaves of 26 plants (petiole, midrib, and parts of leaf blade of 10 leaves/sample) from the field (7) and of plants used in the laboratory (19). We used the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's protocols, but with some extra steps to improve the purity of the extracted total DNA. Briefly, we extended the incubation step for cellular lysis to 1 h at 65 ºC and the isopropanol step for DNA precipitation and desalting to overnight instead of a few minutes. We also added one more step for deproteinization before DNA precipitation with 700 µl of cold 24:1 chloroform:isoamyl-alcohol, then vortexed this solution for 20 s and centrifuged it for 10 min at 14,000 rpm. After this step, we transferred the aqueous phase (600 µl) to a new microtube with 600 µl of cold isopropanol and incubated it overnight at -20 ºC. Lastly, the DNA pellet was washed twice in 500 µl of cold 70% alcohol instead of once, and the pellet was re-suspended in 50 µl of DNA Rehydration Solution plus 3 µl of RNAse Solution after drying at room temperature, and was then incubated for 1 h at 37 ºC. We examined the purity and concentration of the extracted total DNA using a spectrophotometer.

For the amplification of the 16S rDNA we performed a duplex PCR using the primer pairs listed in Table S1. The PCR mix solution (28 µl) was composed of 12.5 µl of DreamTaq™ PCR Master Mix (2X) (Thermo Scientific™), which contains a standardized premix of Taq DNA polymerase, dNTP, $MgCl_2$, and buffer; plus 1.45 µl of each 10 µM primer (for a final concentration of 0.5 µM); 0.2 µl of DMSO; and 7.5 µl of ultra-pure water. To enhance DNA amplification, we added an extra 2 µl of 25 mM $MgCl_2$ (i.e. 1.7 mM final concentration). We then transferred the PCR mix solution to a 200 µl microtube and added 1 µl of DNA template (ca. 100 ng, total volume = 29 µl).

The following thermocycling conditions were used with the ‘touchdown’ procedure: 2 min at 94 °C for initial denaturation; 10 cycles of 30 s at 94 °C, 30 s at 65 °C (up to 55 °C in a ‘touchdown’ annealing, reducing 1 ºC per cycle), and 1 min at 72 °C; plus 30 standard cycles of 30 s at 94 °C, 30 s at 62 °C, and 1 min at 72 °C; and a final extension at 72 °C for 10 min, followed by an end-step at 4 ºC until removal from the thermocycler. PCR products were inspected with 1.2% agarose gel electrophoresis in 1 X TBE buffer pH 8.3 (0.89 M Tris-base, 0.89 M boric acid, 0.02 M EDTA) using the nucleic acid dye GelRed™ (Biotium) and a 1 kb DNA Ladder (band size markers) under UV transillluminator.

Positive and negative controls were used in PCRs and electrophoresed to compare the results. We used four positive controls, two for Las (isolated from a psyllid and plant), and two for Lam (isolated from different HLB-positive plants). As negative controls, we used an isolate of a healthy plant and another consisting of a PCR reaction without DNA template.

As expected, the positive controls resulted in fragments of 703 bp (Las) and 1027 bp (Lam) of the 16S rDNA gene. Despite the leaves of some orange jasmine plants showing symptoms similar to those of HLB, none of the plant or insect samples showed a band on the gel (see Figure S1). In conclusion, *Ca.* Liberibacter could not be detected in any sample.

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Table S1. Primers used in duplex PCR for detection of *Candidatus* Liberibacter asiaticus (Las), *Ca*. L. africanus (Laf) and *Ca*. L. americanus (Lam) in psyllid and plant samples

Primer	**Primer sequence (5' – 3')**	**Target sp.**	**Amplicon**	**Reference**
A2	TATAAAGGTTGACCTTTCGAGTTT	Las	703 bp	(Hocquellet et al. 1999)
J5	ACAAAAGCAGAAATAGCACGAACAA	Laf	669 bp	
GB1	AAGTCGAGCGAGTACGCAAGTACT	Lam	1027 bp	(Teixeira et al. 2005)
GB3	CCAACTTAATGATGGCAAATATAG			

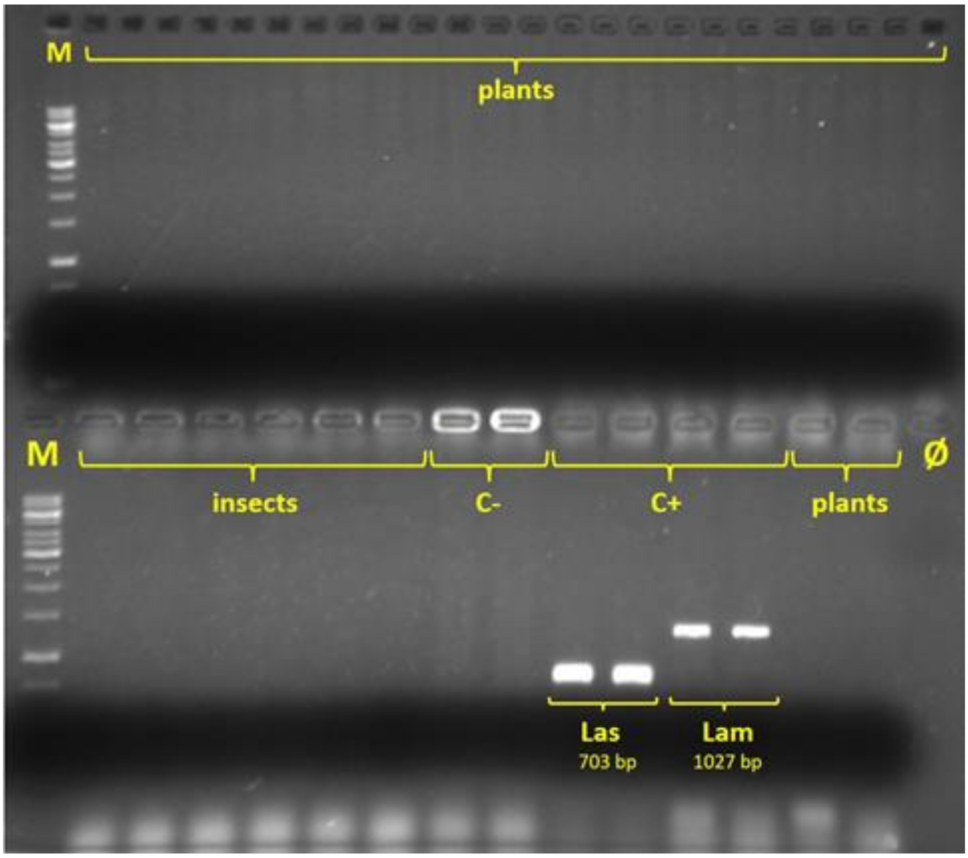

Fig. S1 Electrophoretic profiles in an 1.2% agarose gel. Profiles of the 16S rDNA gene fragment amplified by A2/J5 and GB1/GB3 primer pairs (Table S1). Loading samples arranged from left to right: (i) upper lanes = marker "M" (1 kb) and 24 non-infected plant samples from the UFV campus and lab; (ii) lower lanes = marker "M", six insect samples, two negative controls ("C-" lanes, i.e. one of a plant without HLB and one of a PCR mix without DNA), four positive controls ("C+" lanes) two of *Candidatus* Liberibacter asiaticus (Las; 703 bp) and two of *Ca.* Liberibacter americanus (Lam; 1027 bp), and two plant samples from the UFV campus, near each other, but one of them with high number of *Diaphorina citri* eggs. Band sizes are in base pairs (bp).