Improving the efficiency of biological control with parasitoids for *Tuta absoluta* pest management

Necremnus tutae (left), *Tuta absoluta* (right), picture M. Waldburger

PhD Thesis

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Agroscope

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Inaugural dissertation
of the Faculty of Science,
University of Bern

presented by
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Summary

New sustainable strategies to control pest insects are required to reduce harmful effects on the environment and the rise of pesticide resistance. *Tuta absoluta*, also known as the tomato leafminer, is a major pest of tomato native to South America. It has since spread to other parts of the world, including Europe, Asia, Africa, and the Middle East, causing significant economic losses to tomato growers. The pest is highly destructive, with the potential to cause up to 100% loss in tomato crops if left untreated. It is extremely challenging to control due to its high population increase, wide host range, cryptic behavior, and pesticide resistance. Managing this pest requires an integrated approach that combines different control methods. Regarding biological control, parasitoids can be very effective agents; however, their use presents several challenges that remain to be addressed.

Trichogramma spp. are generalist egg parasitoids widely used as biological control agents and have been proposed to control *T. absoluta*. *Trichogramma* wasps are commercially available and can be released in tomato fields at the early stages of crop development to prevent or reduce infestations. Nevertheless, their efficacy is limited, partially due to the low attraction to infested tomato plants. Studies demonstrated that female *Trichogramma* spp. could learn odor cues affecting their preference. In the first part of the project, we assessed whether learning increases the response to tomato odor and improves the parasitism rate of three species of *Trichogramma*. Associative learning with the tomato odor (leaves or extract) increased the searching time of *T. achaeae* and *T. evanescens* strongly. Rearing the parasitoids on *T. absoluta* for one generation increased the response of *T. dendrolimi* strongly and that of *T. evanescens* slightly. Concerning parasitism improvement, only associative learning with *T. achaeae* resulted in an increased parasitism rate in small-scale cage experiments. Therefore, the learning capacity of *T. achaeae* could be further explored to improve its biological control efficacy against *T. absoluta*.

Besides *Trichogramma* spp., several larval parasitoids have been found attacking *T. absoluta*, including *Necremnus tutae* in Europe and *Dolichogenidea gelechiidivoris* in South America. *N. tutae* is particularly abundant around the Mediterranean basin, and its role in naturally controlling the pest population is well studied. *D. gelechiidivoris* is an essential agent against *T. absoluta* in its native range and has recently been introduced intentionally into Kenya to help control *T. absoluta*. Recently, *D. gelechiidivoris* established unintentionally in Spain and Algeria.

Combining parasitoids with entomopathogen microorganisms such as baculoviruses is an emerging strategy to improve efficiency. Baculoviruses are a family of insect viruses successfully used as a biological control agent against several lepidopteran pests. Specifically, the Phthorimaea operculella granulovirus (PhopGV) has shown promising results in controlling *T. absoluta* populations in the field. Baculoviruses are highly specific to their target pests, killing the larvae of *T. absoluta* but not infecting other beneficial insects or non-target organisms. However, the efficacy

of baculoviruses is highly dependent on the right timing and is sometimes limited, calling for complementary measures. Combining them with macroorganisms like parasitoids can improve the control efficiency, especially if each agent targets different host instars.

Therefore, in the second part of the project, we studied the interactions between larval parasitoids and baculoviruses. The aim was to evaluate the compatibility of PhopGV and the release of larval parasitoids to control *T. absoluta*. First, indirect non-target effects of baculoviruses on parasitoids can result from overlapping resource requirements and resource competition. Hence, we evaluated whether ovipositing parasitoid females avoided virus-treated hosts and the impact of within-host competition between two parasitoid species, *N. tutae* and *D. gelechiidivoris*, and PhopGV. The results showed that female *D. gelechiidivoris* avoided virus-treated hosts, but *N. tutae* did not discriminate. The virus had limited indirect effects on parasitoid offspring, with outcomes varying depending on the parasitoid species, sex, and timing of virus treatment. Overall, the virus had a low impact on parasitoid offspring, and the adverse effects detected are unlikely to reduce their fitness significantly. Therefore, both parasitoid species are compatible with the baculovirus as a control strategy for *T. absoluta*.

Large-scale experiments are crucial for evaluating the effectiveness of new control strategies and provide essential information to understand the effect over time at the population level. The importance of additive effects between parasitoids and baculovirus on pest population ─ and the relevance of such effects for pest control ─ remain largely unexplored. Therefore, we evaluated the efficacy of combining the baculovirus with the parasitoid *N. tutae* in greenhouse experiments. In term of pest reduction and plant damage, we found no significant reduction in the combination compared to each agent alone, although the combination constantly resulted in the the strongest absolute reduction. The parasitoid *N. tutae* alone or in combination with the baculovirus reduced the adult pest density equally, but less reduction was found for the baculovirus alone over the whole trial. Nevertheless, using the virus alone resulted in the strongest reduction of adult density in the third generation. Finally, each agent achieved the same plant damage reduction alone or in combination.

Modeling is an important tool for evaluating and improving biological control programs because it allows for predicting the outcome of different control strategies. Models can help to identify the most effective and sustainable approach by simulating the interactions between pests, natural enemies, and the environment. Parasitoids' efficiency is highly sensitive to the timing and amount of release. Thus, in the third part of this project, we developed a modeling framework to determine the optimal timing and frequency of interventions and the potential impact of baculovirus on pest populations and parasitoid efficiency. By integrating data from the greenhouse experiment, our model provides a comprehensive understanding of the dynamics of pest populations and the effectiveness of different control strategies. For instance, we demonstrate with our model that baculovirus application reduces the number of required parasitoids by almost 80%.

Finally, high host-specificity reduces the risk of ecological disturbance and enhances the parasitoid's efficiency in finding and attacking its host species. Thus, in the last chapter, we focused on understanding the trophic connections between the parasitoids *D. gelechiidivoris* and *N. tutae*, the target host *T. absoluta* and different non-target hosts. We evaluated the potential risks of using the parasitoid *N. tutae* and *D. gelechiidivoris* as biological control agents by conducting host-specificity testing in the laboratory. We also assess parasitization preference in the field. According to our findings, *N. tutae* is quite polyphagous, whereas *D. gelechiidivoris* is more specific. In the greenhouses, the parasitism rate for *N. tutae* was 27%, while for *D. gelechiidivoris*, it reached 35%. Our study highlights the significance of host affinity in comprehending the possible non-target impacts and underscores the potential value of these two parasitoids as a classical biological control.

Overall, this work provides promising ways for developing sustainable biological control of *T. absoluta* using parasitoids. We highlighted the importance of using multiple agents in the studied agroecosystem and clarified the role of each in the success or failure of biological control programs. The efficacy of these agents can be limited, but various strategies exist and have a large potential for improvement. In addition, a tool is provided that can aid future research: a modeling framework to test various combinations of micro and macroorganisms.

General introduction

Tuta absoluta, picture M. Waldburger

A worldwide threat to tomato production

Ecology and pest status

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae), recently reinstated “*Phtorimaea absoluta*” (Chang and Metz 2021), also known as the tomato leafminer or the South American tomato pinworm, is a major pest of tomato crops worldwide (Desneux et al. 2010; Guedes and Picanço 2012; Mansour et al. 2018; Han et al. 2019). It threatens other economically important solanaceous crops, including potato, eggplant, pepper, and tobacco (Desneux et al. 2022). While Solanaceous species are the main host plants, *T. absoluta* can also oviposit and develop on several plants belonging to the Amaranthaceae, Convolvulaceae, Fabaceae, and Malvaceae (Bawin et al. 2016).

The yellow-green larvae of *T. absoluta* mine into leaves, stems, and fruits, causing extensive damage. The pest can hide in the plant structures during an early infestation, damaging young plants significantly. Additionally, its feeding activity on fruits at high density directly impacts the harvest (Biondi et al. 2018). *T. absoluta* has a high reproductive potential, with females producing, on average, 150 eggs during a lifetime (Krechemer and Foerster 2015). The pest can cause complete crop failure if no control methods are implemented (Desneux et al. 2010; Bawin et al. 2016). In addition, subsequent quarantine restrictions imposed (Idriss et al. 2018) and secondary infestations with fungi (Campos et al. 2017) intensify the damages and costs caused by the pest. The life cycle duration varies from 26 to 75 days, with an optimal temperature for its development at 30°C (Martins et al. 2016). *T. absoluta* can undergo up to ten generations annually and survive during cold seasons. It can even subsist during short periods in temperatures below freezing (Van Damme et al. 2015) and enter facultative diapause, significantly enhancing its invasive potential in colder countries (Campos et al. 2020a). Besides its high generation rate and cryptic behavior, the pest is challenging to control due to its ability to develop insecticide resistance and its high dispersal capacity (Desneux et al. 2022). As such, new effective management strategies for *T. absoluta* are crucial to minimizing its impact on tomato production.

Distribution

Native to South America (Tropea Garzia et al. 2012), *T. absoluta* was first detected in Spain in 2006 (Biondi et al., 2018) and spread throughout Europe, reaching Switzerland in 2009 (Agroscope, press release). Since then, the pest has established in several regions of the world, including the Middle East, India, Bangladesh, and Africa (Biondi et al. 2018), as well as China, the largest global producer of tomatoes (Zhang et al. 2020). The pest is known to spread at approximately 800 km per year (Campos et al. 2017), and North America, Australia, and New Zealand are at high risk of being invaded by *T. absoluta* (Biondi et al. 2018; Han et al. 2018). Overall, *T. absoluta* has invaded over 100 countries outside South America (EPPO; https://gd.eppo.int).

The non-biological control options

Insecticides: Use, resistance, and other concerns

Chemical control is the primary method for managing *T. absoluta*, especially in open-field tomato production (Biondi et al. 2018). Shortly after the pest invasion, insecticide usage typically increases to prevent the spread of invasive populations and to minimize the potential crop losses that may occur (Guedes and Siqueira 2012).

Using synthetic insecticides to control *T. absoluta* can lead to several problems. The major concern is environmental contamination risk and human health hazards (Chandra Yadav and Linthoingambi Devi 2017). Synthetic insecticides, especially those with a broad spectrum, can negatively impact non-target organisms, including beneficial insects such as soil microorganisms or natural enemies (Desneux et al. 2007; Abbes et al. 2015; Soares et al. 2019b). These organisms are important in maintaining ecosystem health and can be essential for sustainable agriculture. For example, the loss of natural enemies can cause secondary pest outbreaks (Dutcher 2007). Synthetic pesticides can also long-term impact other ecosystem functions such as pollination (Devine and Furlong 2007, Kevan and Viana 2003).

Another major concern is the pest population's resistance development over time, which can make the insecticides less effective (Guedes and Siqueira 2012). It can lead to higher doses of insecticide required to achieve the same level of control, which increases the environmental and human health risks. In 2012, the first chemical control failures in eradicating *T. absoluta* infestations in greenhouse tomato crops were reported in Sicily (Giorgini et al. 2019). Subsequently, many other countries dealing with *T. absoluta* invasions reported insecticide resistance (Guedes et al. 2019). According to the Arthropod Pesticide Resistance Database (https://www.pesticideresistance.org), almost 60 cases of populations exhibiting resistance to 17 active ingredients exist worldwide. Resistance has been mainly found in the organophosphates, carbamates, pyrethroids, and spinosyns classes (Guedes et al. 2019), which disrupt the insect's nervous system, causing paralysis and death (Nauen 2006). It has become increasingly clear that relying solely on chemical insecticides to control *T. absoluta* will not be a sustainable long-term solution.

Alternatives to synthetic insecticides

A variety of methods other than chemical insecticides have been developed and used to control *T. absoluta*. This includes agronomic practices such as using resistant cultivars, routine surveillance, and removing infested leaves. For greenhouse tomatoes, physical barriers (netting and double doors), removal of alternative host plants, crop rotation, and exposure to ambient temperatures in winter can aid in controlling the pest (Reviewed by Desneux et al. 2022).

Botanical insecticides derived from essential oils extracted from plants are being developed to control *T. absoluta* (Soares et al. 2019a). However, the practical use of essential oils in integrated pest management programs is still limited by factors such as the need for authorized formulations and the compatibility of essential oils with biocontrol agents (Pavela and Benelli 2016). In addition, cost, efficacy, and reliability are other factors that may discourage growers from using essential oils as a control option (Campos et al. 2021)

Mass trapping methods for controlling *T. absoluta* population densities involve using semiochemicals to attract males (Witzgall et al. 2010). Light sources may also be included to improve attraction (Cocco et al. 2012). Unfortunately, the mating behavior and reproduction characteristics of *T. absoluta* present challenges to mass-trapping efficacy. *T. absoluta* males are polygenic (Wang et al. 2021), and females can lay viable eggs through parthenogenesis (Caparros Megido et al. 2012), which may limit the effectiveness of mass trapping. Mass trapping is rarely sufficient and should be combined with other control measures (Cherif et al. 2018).

The mating disruption strategy uses synthetic female pheromones to hinder the ability of males to find mating partners (Caparros Megido et al. 2013; Cocco et al. 2013). However, pest density, migration of mated females, and parthenogenesis can limit the effectiveness of this strategy (Michereff Filho et al. 2000; Caparros Megido et al. 2012). Studies using this technique against *T. absoluta* in open fields and protected tomato crops have shown mixed results (Michereff Filho et al. 2000; Vacas et al. 2011; Cocco et al. 2013), with larger farms having better results.

Biological control

Biological control is generally recognized as exploiting organisms, such as insects or pathogens, to control pest populations (Stenberg et al. 2021). Biological control, also called biocontrol, has successfully been used for centuries. This approach has several advantages over traditional chemical control methods.

First, biological control is less likely to lead to the development of resistance in the pest population (Bale et al. 2008). Biological control agents tend to have a lower selection pressure on the pest population, which reduces the likelihood of resistance developing (Holt and Hochberg 1997). Second, biological control can target pests in areas where pesticides cannot reach them. For example, pests that feed on plant roots or burrow into the soil can be difficult to control with pesticides. However, some natural enemies, such as nematodes, can effectively control these pests.

Additionally, mobile biological control agents, such as parasitoids, actively seek out their hosts (Waage and Hassell 1982) and can locate and attack them in areas that are difficult to access with pesticides, such as larvae hiding inside mines. While biological control has many advantages, some risks and challenges associated with its use should be considered (Collatz et al. 2021). One of the main challenges is regulatory issues surrounding the release of non-native biological control agents (Barratt et al. 2021) that can have unintended environmental consequences, including impacts on non-target organisms and ecosystem functioning (Collatz et al. 2021).

To mitigate these risks, some jurisdictions strictly regulate biocontrol agents (Collatz et al. 2021). Regulatory agencies often require extensive testing of biological control agents before release to predict environmental risk (Guijarro et al. 2020), which is time-consuming and costly. Another challenge is the compatibility of biological control with other pest management methods, such as chemical control (Gentz et al. 2010). Pesticides can sometimes harm or kill natural enemies, reducing their effectiveness (Gentz et al. 2010). Additionally, some natural enemies may have specific requirements for their survival, such as access to certain plant species or environmental conditions (Heimpel and Jervis 2005), which can limit their use in some settings. A further challenge is the limited predictability of biological control outcomes. Unlike chemical control methods, the effectiveness of biological control can vary depending on a range of factors, including environmental conditions, pests, natural enemy interactions, and the availability of alternative hosts (Lamichhane et al. 2017). Hence biological control may not always provide consistent or predictable results, making it challenging to plan for and implement in certain settings.

Despite these challenges, biological control remains an important tool for integrated pest management. To maximize the benefits of biological control while minimizing its risks and challenges, it is important to carefully evaluate the potential impacts of natural enemies before their release (Collatz et al. 2021). It is equally important to use multiple complementary pest control methods and, where possible, carefully monitor the effectiveness of biological control over time to ensure that it remains a viable and sustainable approach (Barzman et al. 2015).

Types of biological control

Different types of biological control exist. Conservation biocontrol aims at enhancing the efficacy of present natural enemies of pests in agricultural ecosystems via modification of the environment or farming practices (Eilenberg et al. 2001). This strategy is based on recognizing that a diverse and stable ecosystem, with healthy populations of natural enemies, can help maintain a balance between pests and their predators, parasites, and pathogens (Shields et al. 2019). Conservation biocontrol set up good farming practices for promoting and protecting specific natural enemies species (Eilenberg et al. 2001).

When the naturally present natural enemies fail to colonize the pest habitat each season, colonize the habitat too late, or fail to reach sufficient numbers to control the pest, augmentative biocontrol (van Lenteren 2000) may be necessary. Augmentative biocontrol is the repetitive release of natural enemies into the crop where the pest is present (Perez-Alvarez et al. 2019). It is repeated yearly because the natural enemies do not establish or establish but in too low numbers. Different types of augmentative biocontrol exist (Eilenberg et al. 2001). Seasonal inoculation is considered the release of natural enemies to inoculate a new crop, where the control is provided later by the offspring of the released natural enemies (Crump et al. 1999). When predominantly the released natural enemies provide the control, it is called inundative (Eilenberg et al. 2001). The natural enemies are usually produced in the laboratory and then sold by a company to the farmers or other distributors. Augmentation biocontrol has been used successfully against many pest species, especially greenhouse pests, due to the comparatively small treatment area, the closed and stable environment, and the high cash value of the greenhouse-cultivated plants (van Lenteren 2000). In addition, the greenhouse system favors retention and, therefore, a population increase of the released natural enemies. Finally, greenhouse crops such as tomatoes, pepper, or strawberry often rely on commercial bumblebees for pollination (Velthuis et al. 2006), requiring farmers to use few or no chemical insecticides. Because most of the significant open-field crops do not have these characteristics, fewer opportunities exist to employ augmentation biocontrol in these systems. However, when natural enemies can be mass-reared at low costs, such as the egg parasitoids of the genus *Trichogramma*, augmentative biocontrol can be very successful in open-field (Zang et al. 2021).

When no efficient endemic natural enemies are present, releasing exotic enemies for long-term establishment might be considered the so-called classical biocontrol (Stenberg et al. 2021). Nowadays, it is used against exotic pests where natural enemies from the area of origin are used. The main distinction from inundation or inoculation biocontrol is that the goal is to permanently establish self-sustained long-term control (Eilenberg et al. 2001).

Microorganisms: Viruses, bacteria, and fungi

Biopesticides with entomopathogens increase in importance as sustainable control strategies. Numerous microorganisms can cause the mortality of *T. absoluta*, including fungi, bacteria, and viruses. Microbial biopesticides are generally less harmful to the environment than synthetic insecticides, pose less risk to humans and other vertebrates, and are compatible with other groups of beneficial organisms, such as parasitoids or predators (González-Cabrera et al. 2011; Mansour and Biondi 2021). *Bacillus thuringiensis* (Berliner) (Bt) (Bacillaceae), which produces Cry toxins with insecticidal properties, has been tested against *T. absoluta* with promising results (Giustolin et al. 2001; González-Cabrera et al. 2011; Jamshidnia et al. 2018). Regarding fungi, *Beauveria bassiana* (Bals.-Criv.) Vuill. was evaluated against *T. absoluta*, causing up to 50% mortality in all instars (Klieber and Reineke 2016). In terms of viruses, granulovirus isolates from *Phthorimaea operculella* have been evaluated for their insecticidal activity (Gómez Valderrama et al. 2018; Ben Tiba et al. 2019) against *T. absoluta*. One challenge with virus particles and other microorganisms is that they are quickly degraded by UV radiation (Moore and Jukes 2019; Reid et al. 2022). In consequence, farmers must treat repeatedly, which increases the costs.

Mirids predators

Mirid predators are highly effective biocontrol agents in controlling *T. absoluta* in the Mediterranean basin (Giorgini et al. 2019). *Nesidiocoris tenuis* (Reuter), *Macrolophus pygmaeus* (Rambur) (Hemiptera: Miridae), and some species of the genus *Dicyphus* (all Hemiptera: Miridae) are generalist predators endemic to the Mediterranean area, which are long-known natural enemies contributing to the control of different pests associated with tomato crops (e.g., aphids, whiteflies) (Giorgini et al. 2019). Not surprisingly, they quickly started to prey upon the eggs and young larvae of *T. absoluta* after its arrival (Arnó et al. 2010; Urbaneja et al. 2012; Jaworski et al. 2015). Nevertheless, their zoophytophagous behavior can cause secondary damage to the plants (Moerkens et al. 2016; Biondi et al. 2016) and questions their use in the tomato crop. Mirid nymphs and adults can feed on different plant parts, causing necrotic rings and consequent abortion of flowers and small fruits and reducing vegetative growth (Castañé et al. 2011). Especially when insect prey availability is low, and mirid populations increase, there is a high risk of crop damage associated with mirid plant-feeding; in these cases, insecticide sprayings against the mirids are sometimes needed to avoid economic losses (Pérez-Hedo et al. 2018).

Parasitoids as biological control agents

Within a long history of good biocontrol services, parasitoid wasps are efficient natural enemies of agricultural and forest pests. Most parasitoids selected for the biological control of agricultural pests belong to a few families of Diptera and Hymenoptera (Greathead 1984). The life cycle starts with the adult female parasitoid foraging for a host. The female will then oviposit eggs through an ovipositor in the alive host (endoparasitoid) or on the hosts previously paralyzed (ecotparasitoid). The larvae, after emergence, begin feeding on host tissues and pass through several developmental stages either within the host or on the host (Waage and Hassell 1982), usually leading to the host's death. Some parasitoids show even nonreproductive host-killing behavior, increasing the number of hosts a female can kill (Zhang et al. 2022).

Parasitoids can efficiently find their hosts even in complex environments (Wäschke et al. 2013; Greenberg et al. 2022). These insects use a variety of cues to locate their hosts, including chemical, visual, and tactile (Wäschke et al. 2013). The host produces direct cues (Colazza et al. 2010), which the parasitoid can detect, including chemical cues, such as host-specific pheromones (Colazza et al. 2010). Indirect cues are produced by the host plant or other organisms associated with the host. These include, in particular, plant volatiles released in response to herbivore damage (Rani et al. 2017). The response to cues can be innate or learned (Vet and Groenewold 1990; Wilson and Woods 2016). Innate responses are genetically programmed and do not require prior experience to locate their hosts, whereas learned responses are acquired through experience and may help find other hosts more easily (Papaj and Vet 1990).

Almost 100 hymenopteran parasitoids from Chalcidoidea, Chrysidoidea, and Ichneumonoidea genera have been associated with *T. absoluta* worldwide, mainly in South America (Salas Gervassio et al. 2019). Since the pest invasion in the Eastern Hemisphere, 53 parasitoid species have formed new associations with *T. absoluta*, indicating a pattern of natural enemy adaptation (Gabarra et al. 2014; Salas Gervassio et al. 2019). However, only a few species have been considered for developing biological control strategies, including conservation, augmentative, and classical biological control options (Desneux et al. 2022).

Egg parasitoids

Trichogrammatidae egg parasitoids are the only ones used in augmentation to control *T. absoluta* (Desneux et al. 2022). Parasitoids belonging to the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) are generalist egg parasitoids, mainly of Lepidoptera. Different *Trichogramma* species are now commercially sold to support *T. absoluta* management – *T. pretiosum* Riley in South America (Parra and Zucchi 2004; Medeiros et al. 2009) and *T. achaeae* (Nagaraja and Nagarkatti) in the Mediterranean countries (Giorgini et al. 2019). Even though parasitism rates can be high in laboratory (Do Thi Khanh et al. 2012; Chailleux et al. 2012) and under greenhouse conditions (Chailleux et al. 2012), *T. achaeae* mass releases have not been considered economically sustainable, and a combination with other biological control methods (i.e., mirid predators, mating disruption) has been suggested (Desneux et al. 2010).

Larval parasitoids

Many different fortuitous parasitoids have been documented in regions newly invaded by *T. absoluta*, mainly ectoparasitoids, with the most dominant ones belonging to the families of Eulophidae and Braconidae (e.g., Spain: Gabarra et al. 2014; Italy: Ferracini et al. 2012). Only a few species of parasitoids have been considered for biocontrol against the pest, including *Dolichogenidea* (= *Apanteles*) *gelechiidivoris* Marsh, *Pseudapanteles dignus* (Muesebeck) (both Hymenoptera: Braconidae), *Dineulophus phthorimaeae* (de Santis) and *Necremnus tutae* Ribes and Bernardo (both Hymenoptera: Eulophidae) (Desneux et al. 2022).

Native to South America, *D. gelechiidivoris* has recently been discovered in Spain (Denis et al. 2022). In South America, it is considered an essential agent against *T. absoluta* (Salas-Gervassio et al. 2019), and mass-rearing protocols have been developed to release this parasitoid for *T. absoluta* control (Morales et al. 2013). Furthermore, in 2017 the species was imported to Kenia from Peru to contribute to controlling *T. absoluta* in Africa (Aigbedion-Atalor et al. 2020).

The European larval parasitoid *N. tutae* is among the most abundant larval parasitoids of *T. absoluta* in the Mediterranean region (Zappalà et al. 2012, 2013; Gabarra et al. 2014; Giorgini et al. 2019), reaching parasitization rates as high as 75–80% (Giorgini et al. 2019). *N. tutae* has been shown to be highly efficient in the laboratory (Biondi et al., 2013; Chailleux et al., 2013; Idriss et al., 2018; Bodino, 2019; Ferracini et al., 2019) while mixed results have been obtained under semi-field conditions (Calvo et al., 2016; Chailleux et al., 2017; Campos et al., 2020). To date, *N. tutae* is promoted through conservation biocontrol methods.

Combination of different agents

Combining different pest control methods, including biological control agents, offers several advantages over relying on a single control method. First, the effectiveness can be enhanced (Dainese et al. 2017). Different control methods may have different strengths and weaknesses, and by combining them, it may be possible to overcome some of the limitations of each method. For example, a combination of different natural enemies may be more effective at controlling a pest population than a single natural enemy species, as each species may target different life stages or have different foraging behavior (Dainese et al. 2017). Second, it reduces the risk of resistance development by reducing the pest's selection pressure (Barzman et al. 2015).

However, combining different pest control methods also presents challenges (Suckling et al. 2014), including ensuring compatibility between different control methods and managing potential negative interactions between different biological control agents. Careful planning and implementation are necessary to optimize the effectiveness of different control methods and avoid unintended consequences (Barzman et al. 2015).

Regarding *T. absoluta*, an Integrated Pest Management (IPM) program based on the conservation of the parasitoid *N. tutae*, releases of the mirid *N. tenuis* and the egg parasitoid *T. achaeae*, as well as mating disruption is currently recommended in Southeast Spain (Desneux et al. 2022). IPM generally combines biological, physical, cultural, and chemical practices to control insect pests in agriculture (Ehler 2006). "Integrated" suggests incorporating natural enemies into these programs and using compatible, non-harmful methods to preserve these agents (Ehler 2006). Biological control is a vital component of IPM (Giles et al. 2017), and the need for biological control will likely grow as IPM programs establish worldwide.

Thesis outline and aim

While parasitoids can be an effective biological control method against *T. absoluta*, their use presents several challenges that must be addressed to control this pest successfully. The aim of this thesis is to provide a basis of knowledge to improve the control potential of parasitoids against *T. absoluta* in different management scenarios: a) Augmentation biocontrol of *Trichogramma* in open fields or greenhouses – can specific conditioning before the release increase parasitoid efficiency?; b) Augmentation or conservation biocontrol using two larval parasitoids in combination with an entomopathogenic virus to combine the advantages of each type of agent; c)

Understanding the trophic connections of two larval parasitoids in the context of the accidental introduction of one and their potential use in augmentation of classical biocontrol.

Chapter I examined the lack of attraction and retention of *Trichogramma* spp. by *T. absoluta* and tomato plants, which hampers their search efficacy. The study identified relevant conditioning processes to improve their attraction to the *T. absoluta* tomato plant system, which can enhance their parasitism success.

Chapter II evaluated a new strategy consisting of the augmentative release of parasitoids and the application of a virus-biopesticide. In the first step, the potential negative effects of the baculovirus on two *T. absoluta* larval parasitoids with very distinct life histories were assessed. This study contributes to a better understanding of interactions between micro- and macro-organisms in biocontrol to support the control of *T. absoluta* and pests in general.

In the second step, the effectiveness of the combination under semi-field conditions was evaluated. Various factors, including environmental conditions, may cause the strategy to fail. Therefore the potential of the combination with the biopesticide for controlling the pest over several generations was tested in greenhouses. Consequently, it was determined whether using both agents together has a positive, neutral, or negative effect on the control of *T. absoluta* compared to each agent used alone.

Finally, a modeling framework to predict host-parasitoid dynamics was developed. The best timing and dosage of parasitoids for efficient control were assessed when combined with the biopesticide. This tool will help practitioners worldwide to design integrated pest management strategies according to their needs.

Chapter III studied the interaction between a parasitoid accidentally introduced into Spain, the target pest, *T. absoluta*, a non-target pest, and a native parasitoid. The results are essential to assess the parasitoids' value as augmentative or classical biological control agents and to promote their establishment in conservation biocontrol.

Table 1. Questions of the PhD project by chapter and respective acronym.

Chapter	Acronym	Questions
1	Egg Parasitoid	Which factors restrict the efficiency of egg parasitoids against *T. absoluta*, and how can the efficiency be improved?
2	New Combination	Is the combination of larval parasitoids and an entomopathogenic virus against *T. absoluta* compatible?
		Does the combined use in the field show better pest control than each agent?
		Can a population model improve the efficiency of the combination under different management scenarios?
3	Trophic Connections	How specific are two promising larval parasitoids of *T. absoluta*? What can it tell us about their value as biocontrol agents.

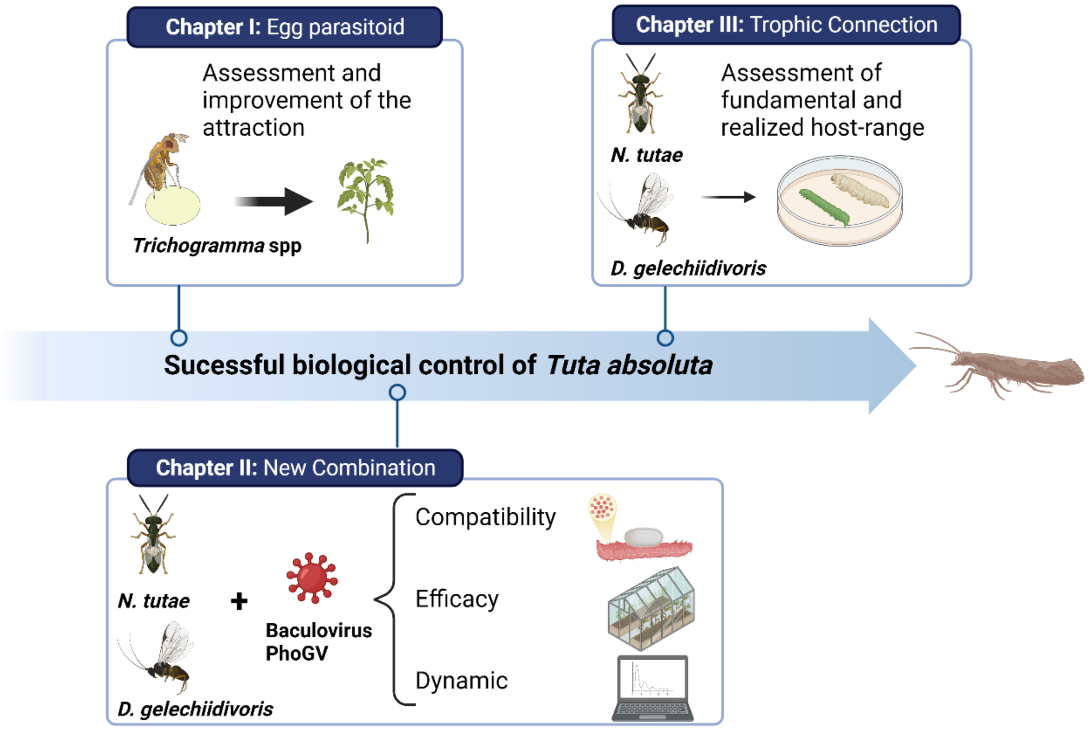

Figure 1. Concept model of the PhD plan. Several ways to improve the efficiency of parasitoids and their integration into tomato farming systems have been evaluated.

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Chapter I: Improving the efficiency of egg parasitoids against *Tuta absoluta*

Trichogramma evanescens, picture M. Waldburger

Article 1: Odor learning improves efficacy of egg parasitoids as biocontrol agents against *Tuta absoluta*

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Abstract

Trichogramma (Hymenoptera: Trichogrammatidae) are generalist egg parasitoids widely used as biological control agents, yet with limited efficacy against *Tuta absoluta* (Meyrick; Lepidoptera: Gelechiidae), a key pest of tomato. Olfactory cues related to the host-plant complex influence the searching behavior in parasitoids. Studies have shown that female *Trichogramma* spp. can learn odor cues affecting their preference. We assessed if learning increases the response to tomato odor and whether it improves the parasitism rate of *Trichogramma achaeae, Trichogramma evanescens,* and *Trichogramma dendrolimi*. The parasitoids were either naive, reared for one generation on *T. absoluta* eggs, or conditioned associatively with an oviposition experience on a factitious host in the presence of tomato odor. Subsequently, their arrestment response (searching time) to *T. absoluta*-infested tomato leaves was observed in a static four-chamber olfactometer. Rearing the parasitoids on *T. absoluta* increased the arrestment time of *T. dendrolimi* strongly and that of *T. evanescens* slightly but not that of *T. achaeae*. In contrast, oviposition experience on a factitious host with the tomato odor (leaves or extract) increased the searching time of *T. achaeae* and *T. evanescens* strongly but did not improve the response of *T. dendrolimi.* Concerning parasitism improvement, only *T. achaeae* showed an increased parasitism rate after associative learning. Hence, the learning capacity of *T. achaeae* should be further explored to improve its biological control efficacy against *T. absoluta*.

Keywords

Tomato pinworm, biological control, odor conditioning, olfactometer, *Trichogramma* efficiency

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Introduction

Hymenopteran parasitoids are contributing to the reduction of important arthropod pest species worldwide. Parasitoid wasps lay their eggs in or on the body of other arthropods, where the immatures will develop, resulting in the host's death. The efficiency of host searching behavior is determinant for the reproductive success of the female parasitoid wasps. For parasitoids of herbivorous hosts, their searching ability often relies on their response to odor cues from the host and the host plant (Bjorksten and Hoffmann 1998; Takasu and Lewis 2003; Vet and Groenewold 1990). This response may be affected by genetic mechanisms (Prevost and Lewis 1990; Gu and Dorn 2000) or learning (Kruidhof et al. 2019). Learning can occur through different processes and at diverse moments during the life cycle of a parasitoid. When emerging, for instance, a wasp can learn the cues of its environment (Kester and Barbosa 1991; Hastings and Godfray 1999). Adult wasps can establish an association between two stimuli or between a stimulus and a response through experience, the so-called associative learning (Kruidhof et al. 2019). Learning behavior can be exploited for manipulating the efficacy of augmentative biological pest control as parasitoids can be conditioned to become more efficient in the different phases involved in host searching and host acceptance (Giunti et al. 2015; Kruidhof et al. 2019).

Generalist egg parasitoids of the genus *Trichogramma* (Hymenoptera: Trichogrammatidae) are common natural enemies of many Lepidoptera species. They are relatively easy to mass-rear and are successfully used in inundative and inoculative biological control programs worldwide (van Lenteren 2000). It has been shown that *Trichogramma* spp. can learn volatile plant compounds (Bjorksten and Hoffmann 1995; Pashalidou et al. 2010; Wilson and Woods 2016). Recent attempts have been made to use *Trichogramma* spp. against *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) in Europe (Urbaneja et al. 2012, Zappalà et al. 2013). This key pest of tomato has become a significant threat to tomato production in Europe and Africa (Biondi et al. 2018; Mansour et al. 2018) and has reached China in 2017, the worldwide biggest tomato producer (Zhang et al. 2020a). Populations of *T. absoluta* established first in the province Xinjiang (Zhang et al. 2020a), followed by Yunnan (Zhang et al. 2020b), Guizhou, Sichuan, Hunan, Guangxi, and Chongqing, causing severe damage in Xinjiang (yield losses up to 86 %) and Yunnan (Zhang et al. 2021). To control the pest, Chinese tomato growers currently rely on insecticides, including Emamectin benzoate, Avermectin-chlorobenzoyl, Methoxy fenozide, and Chlorantraniliprole (Amire et al. 2020), as well as on the microbial pesticide *Bacillus thuringiensis* (Bt-G033A) (Zhang et al. 2020c). However, *T. absoluta* has developed high levels of resistance to several of the most popular insecticides, thus implying that the invading individuals may already bear or will develop resistance to those insecticides (Han et al. 2018, Guedes et al. 2019, Desneux et al. 2021). Therefore, new tools that are safe for human health and the environment are highly needed for managing this invasive pest. The egg parasitoids *Trichogramma* spp. are one possible option. Unfortunately, in

Europe, several factors limit the success of *Trichogramma* spp. in augmentative biological control against *T. absoluta*, such as the low attraction to infested tomato plants (Milonas et al. 2019) and the fact that *T. absoluta* eggs are not laid as egg patches (Tropea et al. 2012). Our study aimed to evaluate if learning can improve the success of *Trichogramma* spp. Against *T. absoluta*.

We focused on three commercially available species, namely *Trichogramma achaeae* Nagaraja & Nagarkatti, *Trichogramma evanescens* Westwood, and *Trichogramma dendrolimi* Matsumura. We selected *T. achaeae* since this species is currently used for biological control of *T. absoluta* in Europe (Desneux et al. 2021) and *T. evanescens* and *T. dendrolimi,* as both are commonly occurring in China (Zang et al. 2021). In China, *T. dendrolimi* is mass-reared and widely used in maize fields against the Asian corn borer (ACB) *Ostrinia furnacalis* Guenée (Lepidoptera: Crambidae) and to a lesser extent on rice fields against *Chilo suppressalis* Walker (Lepidoptera: Crambidae) (Zang et al. 2021).

The aims were to explore different mechanisms by which experience increases parasitoid attraction towards tomato plants and whether this differs between *Trichogramma* species. To address this, three questions were investigated: (i) What is the innate response of the parasitoids to volatile cues of the tomato plant? (ii) Does experience of cues from host remains and tomato plants at emergence improve this response? (iii) Does an oviposition experience in the presence of tomato plant cues result in associative learning and increased preference to the plant-host complex? Finally, we explored whether the behavior modified by learning in the three *Trichogramma* species translates into differences in parasitism efficacy.

Materials and methods

Plants and insects

Tomato plants, *Solanum lycopersicon* cv. Rentita, were grown in greenhouses at 25±5°C and 60±10% RH. Plants were grown in pots (10 cm dia.) with long-term fertilizer mixed with the soil (3 kg/m^3, Manna Cote 4M, Hauert Düngerwerke GmbH, Germany). Eggs of *T. absoluta* were provided by Andermatt Biocontrol (Switzerland) and a colony was established in a climate chamber at 25±1°C, 70±10% RH and a 16:8 h L:D photoperiod). Adults were kept in mesh cages (50x50x50 cm) (bug dorm, MegaView Science Co., Ltd., Taiwan) and provided cotton soaked in honey-water (10% v/v) placed on the top of the cage and tomato plants for egg laying. After seven days, plants with eggs and young larvae were moved to a another cage to start a new colony. Moth eggs used in the experiments were between 4 and 16 h old.

The commercially available egg parasitoids, *T. evanescens*, *T. achaeae and T. dendrolimi* were obtained from AGROLINE Bioprotect (Switzerland), Bioline AgroSciences (France), and AMW Nützlinge (Germany), respectively. Colonies of parasitoids were established on UV-irradiated eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) from AGROLINE Bioprotect

(Switzerland) in climate chambers (22±1°C, 70±10% RH, 16:8 h L:D). Rearing was established in large acrylic glass tubes (10 cm dia.; 30 cm length), and the parasitoids were fed on honey-agar (water:agar:honey, 1 l:8 g:0.1 l). Two to three times per week, food and a post-it® with pasted fresh host eggs were added to the rearing and parasitized eggs were removed and placed in incubation containers (5 cm dia.; 10 cm length). After about 10 days, newly emerged adults from these containers were transferred back into the rearing tube. The parasitoid species were maintained for at least 50 generations on *E. kuehniella* eggs before experimentation.

For the experiments, only female wasps were used. Females and males were distinguished under a stereomicroscope by their dimorphic antennae. In females, the last flagellomeres are usually prominently enlarged, creating an apical club. In males the antennomere number is often higher and the shape filiform (see Colazza et al. 2010). Females were between 6 and 12 h old and mated. In *Trichogramma*, mating takes place just after the female emerges (Waage and Ming 1984). One hour before the start of the experiment, females were isolated in small glass tubes. All experiments took place in climate chambers (22±1°C, 70±10% RH, 16:8 h L:D).

Parasitism capacity of *Trichogramma* spp.

We first characterized the parasitism capacity of the three *Trichogramma* species. The experiments were conducted in glass vials, where single females recieved approximately 400 eggs of *E. kuehniella* daily for 9 days. Parasitism, fertility and sex ratio of offspring were recorded. A detailed description of the experiments and the results can be found in the supplementary electronic material.

Conditioning procedures

The *Trichogramma* spp. females used in the experiments were allowed to gain experience with tomato and host cues. Different conditioning procedures were implemented (Fig. 1).

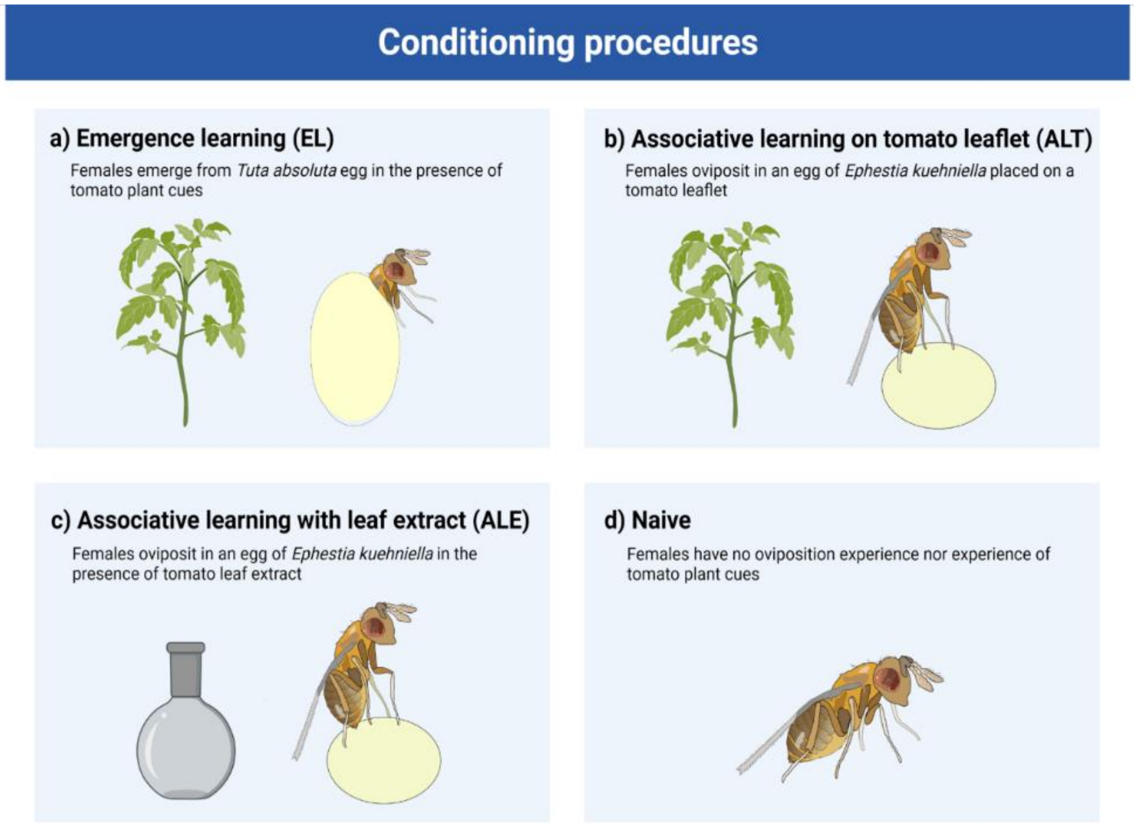

Figure 1 The *Trichogramma* spp. females were allowed to gain experience with tomato and host cues before the experiments. Different conditioning procedures were used. **a)** Emergence learning (EL): Parasitoids were reared for one generation on *T. absoluta* eggs on tomato leaflets. **b)** Associative learning on tomato leaflet (ALT): female wasps experienced oviposition on *E. kuehniella* eggs placed on a tomato leaflet. **c)** Associative learning with a tomato leaf extract (ALE): female wasps experienced oviposition on *E. kuehniella* eggs placed on a filter paper with tomato leaf extract. **d)** Naive: control female wasps were reared on *E. kuehniella* eggs without any exposure to *T. absoluta* or tomato cues. This figure was created with with BioRender.com.

Emergence learning: (EL)

Parasitoids were reared in containers covered with a fine mesh (5 cm dia.; 10 cm length) for one generation on *T. absoluta* eggs on tomato leaflets (5±2 cm length, freshly cut from 3 weeks old plants). The eggs had been laid by *T. absoluta* onto the tomato leaves within 24 h before the assay. To assure the presence of cues from freshly infested tomato plants during the entire parasitoid development, the containers were placed inside cages with *T. absoluta*-infested tomato plants until parasitoid emergence. After emergence, the parasitoids remained in the cage for 6 to 12 h until used in the experiments.

Associative learning on tomato leaflet (ALT)

0.5 to 1 h before the experiment, female wasps experienced oviposition on *E. kuehniella* eggs placed on a tomato leaflet. Approximately 30 eggs of *E. kuehniella* were placed on a freshly cut leaflet from an uninfested tomato plant in a Petri dish (5 cm dia., 1 cm height). Single parasitoid females randomly selected from the rearing were placed on the leaflet and allowed to oviposit. Oviposition (i.e., egg stung with the ovipositor) was confirmed under the stereo-microscope.

Immediately after the first oviposition, the females were individually collected with a glass aspirator and transferred into a glass vial until used in the experiments. Females that did not oviposit within 15 min. were discarded (approximately 5%). When testing the memory retention in a separate experiment, experienced females were kept for 24 h before used.

Associative learning with a tomato leaf extract (ALE)

The same procedure as described above was conducted except that, instead of a tomato leaflet, a filter paper (5 cm dia.) with tomato leaf extract was used. The extract was prepared by soaking 3.5 g of tomato leaves for 12 h in 20 ml of solvent (diethyl ether or hexane, Sigma-Aldrich Chemie Gmbh, Germany ≥ 95% purity). The solution was filtered through a cotton to remove the leaves and gently heated in a water bath at 60°C to reduce the volume to 3.5 ml, corresponding to 1 g of tomato leaf per 1 ml extract. The filter paper was placed under the fume hood, 400 µl of extract were added and the solvent was allowed to evaporate for 5 min before the conditioning. The filter paper was exchanged between each female conditioning process.

Naive

Parasitoids were reared on *E. kuehniella* eggs without any exposure to *T. absoluta* or tomato cues. They served as control in the experiments.

Olfactometer assays

Experimental set-up

To test the arrestment response of the *Trichogramma* spp. females to the cues of infested tomato leaves, we used a static four-chamber olfactometer (10 cm dia.), which consisted of a bottom with four chambers, which were covered with a fine mesh providing a walking arena (height 0.5 cm), and a lid made of a glass plate (Collatz et al. 2006). The walking arena was divided in four sectors corresponding to the four olfactometer chambers. The mesh prevented access of the parasitoids to the chambers. The experiments took place in a climate chamber at 22°C and 70% RH. A freshly cut tomato leaflet infested with approximately 30 *T. absoluta* eggs (< 24 h old) was placed in one sector of the olfactometer. Factitious leaflets made of green paper of the same size were placed in each of the other sectors. The olfactometer was placed in a cardboard box illuminated from the top to avoid any directional light attraction. One *Trichogramma* spp. female at a time was placed on the walking arena and allowed to settle for 5 sec. Subsequently, the time spent on each of the four chambers was recorded for 5 min using the Software the Observer XT11 (Noldus, Netherlands). The position of the factitious and tomato leaflets were swaped between each replicate to minimize location bias. Every hour, new tomato and factitious leaflets were used. After each series of observation the chambers were cleaned with 98% Ethanol to remove any remaining volatiles.

Experimental design

In the first experiment, we tested the arrestment response of female *T. achaeae* after the following conditioning procedures: a) EL; b) ALT; c) ALT after 24 h. We evaluated in parallel if ALT females discriminate between infested and uninfested leaves. We arranged a choice test by placing an infested tomato leaflet into the test chamber and an uninfested leaflet in the control chamber of the olfactometer. In the second experiment, we tested the response of female *T. achaeae* after the following conditioning procedures: a) ALE with diethyl ether; b) ALE with hexane. Finally, in the last experiment, we tested the response of female *T. evanescens* and *T. dendrolimi* after the following conditioning procedures: a) EL b) ALT. For each assay, naive wasps were tested as control.

Just after being used in the experiment, all naive and EL females were offered *E. kuehniella* eggs for a maximum of 15 minutes. Females that did not oviposit were discarded (approximately 5% across groups). This was done to minimize the bias in the associative learning treatments by selecting only females motivated to oviposit during the first 15 minutes conditioning period before the experiment - the final sample sizes range from 16 to 28 individuals across groups.

Efficacy assays in tubes

Experimental set-up

Experiments were conducted with all three *Trichogramma* species to test whether the conditioning leading to an increased arrestment response in the olfactometer also increases parasitism efficacy compared to naive females. Mated parasitoid females of the conditioning treatments that resulted in arrestment response in the olfactometer were placed individually in glass tubes (3 cm dia., 12 cm height) closed with a lid ventilated with a fine mesh and containing a single tomato leaflet with ten *T. absoluta* eggs. The eggs were collected from infested leaves and transposed at an equal distance (approximately 5 – 10 mm) on the abaxial side of a fresh tomato leaflet directly by hand using a fine wet pencil. The leaflet stem was placed into a solution of agar-water (8 g:1 l) to provide moisture.

Experimental design

Based on the results from the olfactometer experiments, we tested ALT females for *T. achaeae* and *T. evanescens* and EL females for *T. dendrolimi*. *Trichogramma achaeae* females were allowed for 2 h to parasitize the offered *T. absoluta* eggs, whereas, for *T. evanescens* and *T. dendrolimi,* the period was extended to 6 h since they required more time to start parasitization. In the end, the females were removed, and the tubes were incubated. After five days, the eggs parasitized (visible as blackened eggs) were counted. Between 25 and 30 replicates were conducted per parasitoid species and treatment. The experiments took place in a climate chamber at 22°C, 70% RH, and artificial light. For each assay, naive wasps were tested as control.

Efficacy assays in mesh cages

Experimental set-up

Mesh cage assays were conducted with *T. achaeae* to assess whether the conditioning leading to increased parasitism in the tube also increased the efficacy at a larger scale. Ten mated ALT females were placed together in a mesh cage (15 cm dia.; 30 cm height) containing a tomato compound leaf with seven leaflets. Twenty *T. absoluta* eggs were transposed on each leaflet (2 to 3 eggs per leaflet at an equal distance) on the abaxial side directly by hand using a fine wet pencil. The leaf stem was placed into a solution of agar water to provide moisture.

Experimental design

T. achaeae females were allowed four days to parasitize the eggs. The eggs parasitized were counted four days later. Twenty replicates each were conducted for ALT females and naive females as control. The experiments took place in a climate chamber at 22°C, 70% RH and 16: 8 h L:D.

Statistical analysis

For statistical analysis, the computer software NCSS 2020 (NCSS, LLC, US) was used. Data were tested for normal distribution using the Shapiro–Wilk test, and visual inspections of the data were made using Q-Q- plots. Since data were not normally distributed, we used a Wilcoxon signed-rank test to analyze the arrestment response of parasitoids to infested tomato leaves, i.e., to compare the time spent above the chamber with the infested leaflet to the time spent above the opposite chamber of the four-chamber olfactometer. For the efficacy tests, data were normally distributed, and variance was homogenous (Modified-Levene equal-variance test). We considered the females laying zero eggs during the time allowed as not responding. A chi-square test of independence was performed to examine the relation between the treatment and the number of females not responding during the time allowed. For females that had oviposited, a two-sample unpaired t-test of equal variance was used to compare the mean number of eggs parasitized between the treatments. For all analyses, we considered a p-value lower than 0.05 as statistically significant.

Result

Olfactometer assays

In all three *Trichogramma* species, naive females spent more time on the control chamber than the chamber containing the infested tomato leaflet (Fig. 2-4). However, this deterrence from the tomato leaflet was only significant in two trials with female *T. achaeae* (Wilcoxon signed-rank test; $z = 2.05$, $p = 0.039$, Fig. 2b; $z = 2.46$, $p = 0.013$, Fig. 3a).

Trichogramma achaeae

EL females spent more time on the control chamber than on the test chamber containing the infested tomato leaflet (Fig. 2a). This difference, however, was not significant. In contrast, ALT females spent significantly more time on the test chamber than on the control chamber (z = 3.12, p = 0.002; Fig. 2b). When given a choice between an infested and an uninfested tomato leaflet, ALT females did not show any difference in searching time (z = 0.37, p = 0.708; Fig. 2b). When tested 24 h after the learning experience, ALT females spent numerically but not significantly more time on the test chamber than on the control chamber (z = 1.34, p = 0.178; Fig. 2c).When tomato leaves were extracted with diethyl ether, ALE females spent significantly more time on the test chamber containing the leaf than on the control chamber (z = 2.21, p = 0.002; Fig. 3a). However, the females did not respond to the test chamber when tomato leaves were extracted with hexane (Fig. 3b).

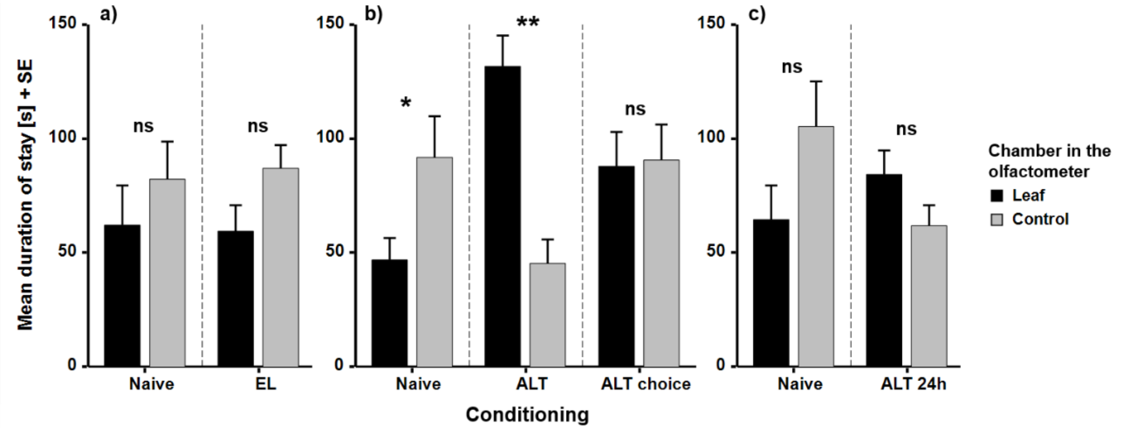

Figure 2 Walking time (mean + SE) of female Trichogramma achaeae in the odor chambers of a four-chamber olfactometer during 5 min. after different conditioning. One chamber contained a tomato leaflet infested with *Tuta absoluta* eggs, and the opposite control chamber a factitious leaflet made from green paper. a) Naive = control (n = 18); EL = Emergence learning, (n = 20). b) Naive = control (n = 20); ALT (n = 28) = Associative learning on tomato leaflet; ALT choice (n = 20) = Associative learning on tomato leaflet. Instead of a factitious paper leaflet, an uninfested tomato leaflet was placed in the control chamber of the olfactometer. c) Naive = control (n = 20); ALT 24h (n = 20) = Associative learning on tomato leaflet happened 24 h before the experiment. ns. not significant; * P < 0.05; ** P < 0.01 (Wilcoxon signed-rank test).

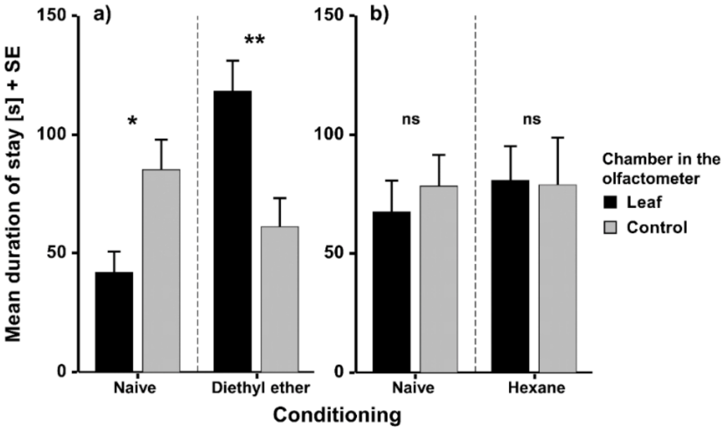

Figure 3 Walking time (mean + SE) of female *Trichogramma achaeae* in the odor chambers of a four-chamber olfactometer during 5 min. after different conditioning. One chamber contained a tomato leaflet infested with *Tuta absoluta* eggs, and the opposite control chamber a factitious leaflet made from green paper. **a**) Naive = control (n = 20); Diethyl ether = Associative learning on tomato extract (ALE females) using diethyl ether as solvent (n = 20). **b**) Naive = control (n = 22); Hexane = Associative learning on tomato extract (ALE females) using hexane as solvent (n = 15). ns. not significant; * P < 0.05; ** P < 0.01 (Wilcoxon signed-rank test).

Trichogramma evanescens and T. dendrolimi

ALT female *T. evanescens* spent significantly more time on the chamber containing an infested tomato leaflet than on the control chamber (z = 2.68, p = 0.007; Fig. 4a), while EL female *T. evanescens* spent similar time on the test and control chambers (Fig. 4a). On the opposite, ALT female *T. dendrolimi* spent similar time on the test and control chambers (Fig. 4b), while EL female *T. dendrolimi* spent significantly more time on the test chamber than on the control chamber (z = 3.09, p = 0.002; Fig. 4b).

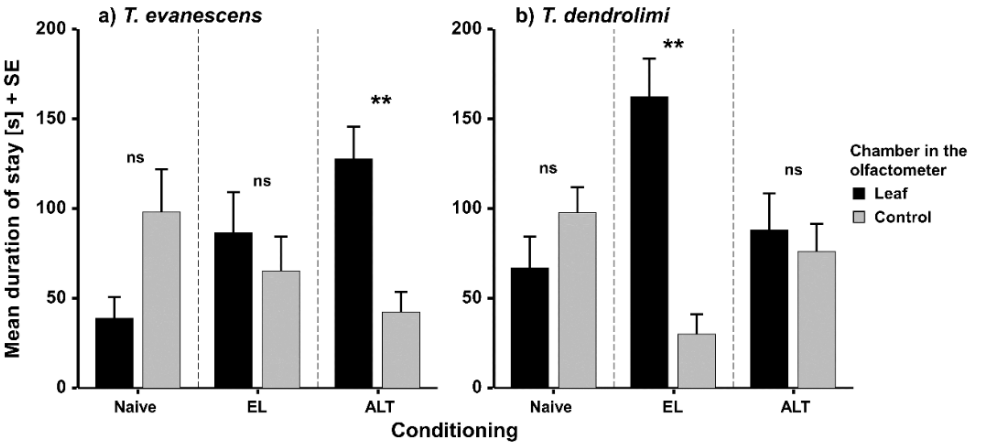

Figure 4 Walking time (mean + SE) of female Trichogramma evanescens and dendrolimi in the odor chambers of a four-chamber olfactometer during 5 min. after different conditioning. One chamber contained a tomato leaflet infested with *Tuta absoluta* eggs, and the opposite control chamber a factitious leaflet made from green paper. a) Naive = control (n = 20); EL = Emergence learning (n = 16); ALT = Associative learning on tomato leaflet (n = 20). b) Naive = control (n = 20); EL = Emergence learning (n = 16); ALT = Associative learning on tomato leaflet (n = 20). ns. not significant; ** P < 0.01 (Wilcoxon signed-rank test).

Efficacy test

Tube assays

When *Trichogramma* spp. females were allowed to parasitize *T. absoluta* eggs on a tomato leaflet, ALT female *T. achaeae* were more likely than naive females to parasitize at least one egg (X^2 = 9.36, df = 1, *N* = 52, *p* = 0.002); of ALT females, 88% parasitized one or more eggs whereas only 48% of the naive females did. In addition, ALT females parasitized significantly more eggs than the naive ones (unpaired t-test of equal variance, t = 2.24, *N* = 33, p = 0.03; Fig. 5a). In contrast, ALT female *T. evanescens* did not parasitize more eggs than the naive females (t = -0.19, *N* = 34, p = 0.842; Fig. 5b); 48% ALT females and 56% naive females parasitized one or more eggs (X^2 = 0.138, df = 1, N = 61, p = 0.709). Also, no difference was observed in the number of eggs parasitized by the naive and EL female *T. dendrolimi* (t = 0.57, *N* = 40, p = 0.567; Fig. 5c); 66% EL females and 73% naive females parasited one or more egg (X^2 = 0.317, df = 1, N = 60, p = 0.573).

Mesh cage assay

When *T. achaeae* were allowed to parasitize *T. absoluta* eggs on a compound tomato leaf, ALT females parasitized significantly more eggs than the naive ones (t = 2.9, *N* = 31, p = 0.007; Fig. 5d). Overall, 80% of females in both treatments (ALT and naive) parasitized at least one egg.

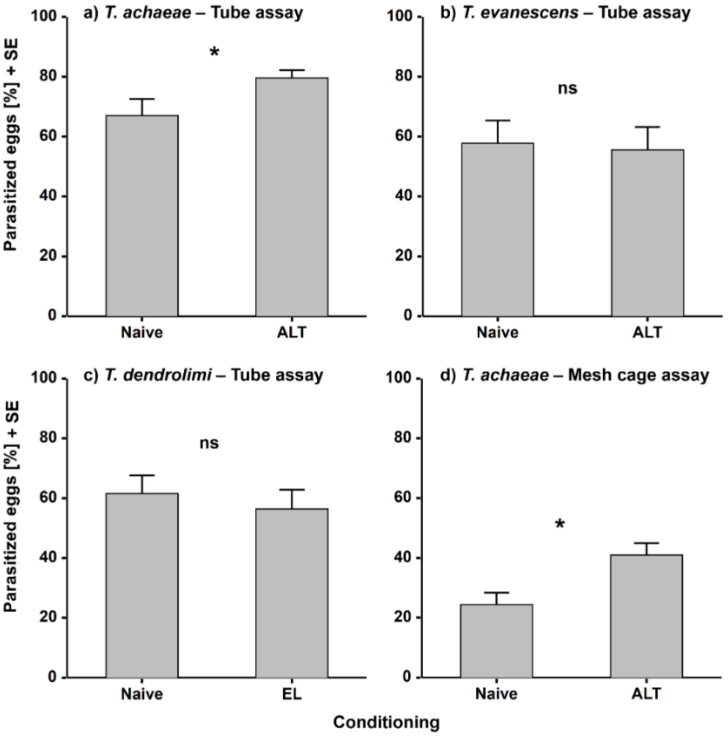

Figure 5 Parasitism of *Tuta absoluta* eggs under laboratory conditions. Mean percentage (+ SE) of parasitized *T. absoluta* eggs per *Trichogramma* species and conditioning. The tube assay consisted of a single tomato leaflet (with 10 eggs) and a single female per replicate; the mesh cage assay of a compound tomato leaf (seven leaflets with 20 eggs) and 10 females per replicate. **a)** Number of eggs parasitized after 2 h by *Trichogramma achaeae* in tube assay*;* Naive = control (n = 27); ALT = associative learning on tomato leaflet (n = 25). **b)** Number of eggs parasitized after 6 h by *Trichogramma evanescens* in tube assay*;* Naive = control (n = 31); ALT = associative learning on tomato leaflet (n = 30). **c)** Number of eggs parasitized after 6 h by *Trichogramma dendrolimi* in tube assay*;* Naive = control (n = 30); EL = Emergence learning (n = 30). **d)** Number of eggs parasitized after 4 days by *T. achaeae* in mesh cage assay*;* Naive = control (n = 16); ALT = associative learning on tomato leaflet (n = 16). ns. not significant; * P < 0.05 (two-sample unpaired t-test of equal variance).

Discussion

Our study provides clear evidence for three species of *Trichogramma* that they can learn odor cues from the host-plant complex of tomato plants infested with *T. absoluta* eggs. While in *T. achaeae* and *T. evanescens,* associative learning resulted in an increased arrestment response in a four-chamber olfactometer, the primary learning mechanism in *T. dendrolimi* was emergence learning. Furthermore, in *T. achaeae,* but not in the other two species, the increase in arrestment response translated into a higher parasitism efficiency of *T. absoluta* eggs on tomato in small-scale laboratory assays.

The odor of tomato leaves infested with *T. absoluta* eggs innately repelled naive females of all three *Trichogramma* species. This is likely one reason explaining the low suitability of the tomato crop for supporting *Trichogramma* spp. parasitoids (Giorgini et al. 2019). That naive parasitoids are repelled by particular plant odors has been reported before. For example, Romeis et al. (1997) showed that female *Trichogramma chilonis* Ishii were repelled by volatiles from pigeon pea (*Cajanus cajan*) plants in the reproductive stage. It has been hypothesized that repelling odors will likely be found in systems where the interactants have not coevolved closely (Romeis et al. 1997). In our study, the three *strains* tested likely have no common origin with *T. absoluta*, which is native to South America (CABI, 2021, Invasive Species Compendium). Corroborating this hypothesis, *Trichogramma pretiosum* Riley, from the area of origin of *T. absoluta*, have been reported to innately respond to volatiles from tomato (Nordlund et al. 1985). Therefore, repellent volatiles might be a limiting factor for using new associations in biological control.

Previous studies in y-tube olfactometers with *Trichogramma* spp. and tomato plants infested or not by *T. absoluta* have reported contradictory results. Gontijo et al. (2019) found that naive *T. achaeae* females were attracted to volatiles produced by tomato, regardless of their infestation status. This, however, could not be confirmed by Milonas et al. (2019). Another study has found that *T. evanescens* were innately attracted to cues of infested tomato plants (Alsaedi et al. 2016). Our results cannot directly be compared to those previous studies since entirely different set-ups were used. A repellency or arrestment response is difficult to detect in a y-tube olfactometer since non-responsive individuals are excluded. Moreover, since multiple females were tested simultaneously in the studies by Alsaedi et al. (2016) and Gontijo et al. (2019), an aggregation effect cannot be excluded. Another explanation for the observed differences resides in the different strains used. It is acknowledged that biological traits can differ significantly among strains of the same *Trichogramma* species (Hoffmann et al. 2001; El-Wakeil 2007; Chailleux et al. 2012).

Our olfactometer assays showed that the learning behavior is species-specific. Female *T. dendrolimi* learned the cues of the host-plant complex at emergence; female *T. evanescens* likewise, but to a lesser extent. However, female *T. achaeae* did not learn the cues from the host-plant complex during development or emergence. This latter result contrasts with a study by Cascone et al. (2015), who reported that rearing *T. achaeae* on *T. absoluta* eggs laid on tomato plants for one generation significantly improved the attack rate against *T. absoluta*. However, in this study, the attack rate was tested in small-scale assays, and the eggs were not provided on tomato leaves.

It is generally accepted that egg parasitoids can learn odors from the host-plant complex at the time of emergence (Dukas and Duan 2000; Meiners et al. 2005; Pashalidou et al. 2010). However, few studies have compared this learning process between species. Like our result, Bjorksten and Hoffmann (1995) found that the rearing host's effects were weak or absent in the Australian egg parasitoid, *Trichogramma* nr. *ivelae* Pang & Chen. In contrast, the host chosen initially for oviposition (associative learning) substantially impacted the later host preference. However, another study found that when reared on a tomato plant, *Trichogramma brassicae* Bezdenko modified their behavior, prolonging searching time compared to individuals that emerged from the same host eggs placed directly in glass vials (Bjorksten and Hoffmann 1998) - demonstrating that even in the same genus, species may show very different learning mechanisms. In our study, it is possible that another rearing host than *E. kuehniella* may have influenced the response of *T. dendrolimi* since this species was attracted by the tomato plants after having been reared for just one generation on *T. absoluta* eggs. We think that this is less likely for the two other species since they did not response positively to host’s change.

Associative learning increased the arrestment response of female *T. achaeae* and *T. evanescens* to *T. absoluta*-infested tomato leaves. Since naive females showed no arrestment response and did not learn the host plant's odor at emergence, associative learning seems to be the key to improving their arrestment response to the tomato plant's odor. This finding is consistent with the results from previous studies. *Trichogramma* species have been reported to learn different components of host plant volatiles by associative learning and to adapt their behavior accordingly (Pashalidou et al. 2010; Wilson and Woods 2016). *Trichogramma* spp. have many different hosts. Therefore, their learning ability corresponds to the generalist theory, which declares that generalist parasitoids, compared to specialists coevolved with a specific host, rely more strongly on learning plant cues to adjust their foraging behavior (Vet and Dicke 1992; Steidle and Van Loon 2003). Overall, the low innate response to tomato plants and the learning ability match the environmental unpredictability hypothesis, suggesting that it is best to learn odor cues unique to a specific time and place (Dukas 2008) rather than evolve innate responses (Papaj and Prokopy 1989). In contrast to *T. achaeae* and *T. evanescens*, the behavior of *T. dendrolimi* was not affected by associative learning. For associative learning, the small *E. kuehniella* eggs might have been considered a low-quality host for the relatively large *T. dendrolimi*. Indeed, female *Trichogramma* spp. discriminate between high- and low-quality hosts. They often visit and oviposit in fresh eggs of high-quality and use the old ones of low-quality for feeding (Kishani Farahani et al. 2015). We assume all eggs used in our experiments were adequately fresh.In our study, the associative learning experience in *T. achaeae* females was weaker after 24 hours than after 6-12 hours. Memory is sensitive to differences in conditioning, such as the number and type of conditioning trials, and more subtle differences, such as reward value (Kruidhof et al. 2012). Thus, only one conditioning trial is likely not enough to produce a long-lasting memory. Numerous and spaced training often leads to longer forms of memory in parasitic wasps, but there are also exceptions where a single training resulted in memory lasting for five and more days (Collatz et al. 2006).

Many parasitoids of herbivorous insects rely on odors emitted by plants (volatile organic compounds - VOCs) to find hosts (McCormick et al. 2014). Firstly, plants are in general large and produce more volatiles compared with host insects. Secondly, plants may produce volatiles in response to herbivory and egg deposition to alert natural enemies (Heil 2008). Interestingly in our study, female *T. achaeae* showed no difference in arrestment response between *T. absoluta* egg-infested and uninfested tomato leaves. This finding corresponds to a recent study by Gontijo et al. (2019). The same result was also found for other natural enemies of *T. absoluta*, such as the mirid predator *Nesidiocoris tenuis* (Reuter) (Hemiptera: Miridae) (Naselli et al. 2017). However, there is evidence that egg deposition from *T. absoluta* on tomato induces volatiles (Faria et al. 2008; Gontijo et al. 2019). Likely, the low amount of volatiles produced by a single *T. absoluta* egg-infested leaflet or the fact that the leaflet used in our experiment had been cut off from the plant may have impaired the ability of *T. achaeae* females to distinguish between infested and uninfested leaves. Laboratory studies have shown that a higher density of *T. absoluta* infestation results in releasing larger amounts of VOCs (Silva et al. 2017), which arrested more *Trichogramma* spp. parasitoids (Alsaedi et al. 2016).

To disentangle the different stimuli learned within the host-plant complex was not the particular aim of our study. During emergence learning, females may have learned the tomato plant's cues from remainders of the host, induced volatiles from egg deposition, or everything simultaneously. Also, to conduct associative learning, we used factitious eggs and not eggs from *T. absoluta.* Therefore, we might have obtained a better result in our efficacy test if eggs of *T. absoluta* were used for the conditioning. However, we think this is unlikely and that, in general, *Trichogramma* spp. are more prone to learn stimuli of the plant than the host egg. For instance, Kaiser et al. (1989) showed that *T. brassicae* (as *Trichogramma maidis* Pintureau and Voegelé) can learn the odor of maize extract through associative learning but cannot learn the odor of host eggs or the sex pheromone of the maize pest, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae). Since eggs produce no long-range cues and parasitoids rely primarily on the host plant cues for long-range detectability (reviewed by Colazza et al. 2010), we speculate that the tomato plant's odor is the strongest or only stimuli in this learning process.

While primarily laboratory-based evidence shows that parasitoids can learn cues of the plant-host complex, few attempts have been made to link it to efficacy (Kruidhof et al. 2019). Our result demonstrates that for *T. achaeae*, associative learning increases efficacy at the leaf and plant level under small-scale, confined conditions. Conditioning the parasitoids before their release in a biological control program might thus improve their efficiency against *T. absoluta* in the field. However, even under the confined conditions, *T. achaeae* did not achieve more than 50% parasitism of host eggs within a four days period. Whether this results from a lack of short range host detection cues as discussed above or points to a limited efficacy of *T. achaeae* in the tomato system in general remains to be determined. A comparable link between conditioning and efficacy was reported by Papaj and Vet (1990) where *Leptopilina heterotoma* (Thomson) (Hymenoptera: Figitidae) experiencing the host habitat odor in the presence of *Drosophila* spp. host larvae had a 3–4 times higher chance of finding a host habitat than inexperienced females. Furthermore, *T. achaeae* females responded to diethyl ether extracts of tomato leaves, but not to hexane extracts, indicating that the plant odor cues involved have a low molecular weight and are water-soluble compounds (Schultz et al. 1977). Therefore, future studies should prefer diethyl ether as solvent to extract the tomato plant odor cues. The plant extracts might ultimately be used to prime parasitoids before their release in a biological control program to increase their efficacy.

In contrast to *T. achaeae*, associative learning did not increase the parasitism rate of *T. evanescens* in our tube experiment, nor did emergence learning for *T. dendrolimi*. Possibly, these two species have a lower capacity to cope with the tomato trichomes than *T. achaeae* or are more prone to reject *T. absoluta* eggs as host. *Trichogramma* spp. are known to be less effective on plants bearing trichomes (Romeis et al. 2005); therefore, females may have been arrested by the tomato leaf in the olfactometer but may have avoided foraging on tomato leaves or being hampered in their movement when released into tubes. Furthermore, Roriz et al. (2006) showed that *Trichogramma* spp. prefer hosts with larger eggs. Eggs of *T. absoluta* are much smaller than the *E. kuehniella* eggs used in the rearing. Thus, *T. evanescens* and *T. dendrolimi* may have avoided *T. absoluta* eggs for the simple reason that they were too small.

Currently, *T. achaeae* is mass-reared and commercially available for use against *T. absoluta* in Europe. However, the low suitability of the tomato - *T. absoluta* system for supporting *Trichogramma* spp. parasitoids results in high costs limiting their use (Cabello et al. 2009; Chailleux et al. 2012, 2013). Furthermore, the effectiveness of parasitoids to locate their host might be constrained by an initially weak response to foraging cues emanating from the crop environment and an innate tendency for dispersal upon release (Kruidhof et al. 2019). Our findings confirm the tomato plant system's low suitability for commercial *Trichogramma* spp. releases in its current form. Since our efficacy tests with associative learning show promising results, we can conclude that learning is a possible means of improving this suitability and will likely help retain the parasitoid *T. achaeae* in the field after release. However, we showed that learning is species-dependent and must therefore be adapted for each parasitoid species. Moreover, we showed that *T. achaeae and T. evanescens* do not learn the host-plant complex's odor at emergence, which is considered an attractive characteristic for mass rearing since it does not condition them on factitious hosts (Bjorksten and Hoffmann 1995).

Trichogramma spp. have been used effectively in various parts of the world through inundative releases. China has achieved successful wide-scale use thanks to its *Trichogramma* spp. manufacturing. The country has industrialized mass-rearing systems for cost-effective *Trichogramma* spp. production, which depends on mechanized processes, complex expertise to manipulate diapause, optimal use of factitious hosts, genetic methods, and novel application approaches. In the country's biggest maize production region, 35% of the area relies on *Trichogramma* spp. releases to control corn borers (reviewed by Zang et al. 2021). So far, up to 24 species have been identified as potential biological control agents and can target over 120 different pests in China (Wang et al. 1998). Meanwhile, at least ten species have been further developed for field applications, such as *T. dendrolimi* (maize, rice, cotton), *Trichogramma japonicum* Ashmead (rice), *T. chilonis* (sugarcane, rice, cotton), *Trichogramma ostriniae* Pang & Chen (maize), and *Trichogramma leucaniae* Pang & Chen (soybean) (Lin 1994, Wang et al. 1998). China is the largest tomato producer worldwide, and *T. absoluta* has made its apparition (Zhang et al. 2020a). The country will likely develop a *Trichogramma*-based control strategy against this devasting pest (Zang et al. 2021). Since the three species tested in the present study are commercially reared in China (Li 2007), our findings can be implemented to develop this strategy.

We believe that besides assessing associative learning on a larger scale, future research should look at how this associative process can be implemented for mass production and release. Since *Trichogramma* spp. parasitoids are shipped as pupae in parasitized eggs, conditioning of adults can only occur at the release site providing access to factitious hosts, i.e., sterile eggs or alternative hosts that are not harmful to the crop. Plant extracts can be a successful way of mimicking the crop odor if this is missing or not strong enough to elicit an associative response. For example, the producer could provide the farmers with the parasitoid pupae and a conditioning package that contains an alternative reward and plant extract (reviewed by Kruidhof et al. 2019).

In conclusion, our study provides strong support for the learning ability of *Trichogramma* spp., thereby contributing to our understanding of learning processes for the improvement of biological control programs. A substantial increase in the arrestment response to the odor of tomato plants can be achieved through conditioning; however, the learning process differs between *Trichogramma* species. Overall, associative learning is very likely to increase the efficiency of *T. achaeae* against *T. absoluta*. Our observations nevertheless create demand for field studies on the efficiency of such conditioning at a larger scale.

Declarations

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Electronic supplementary material

The online version of this article contains supplementary material, which is available to authorized users.

Raw data

The complete raw data can be found at the Figshare repository. See (DOI: 10.6084/m9.figshare.14747949.)

Authors' contributions

JG, JR and JC designed the research. JG led the trials. YZ and GZ provided insight information regarding the pest and the parasitoids in China. JG, JR and JC analysed the data. JG, JR and JC wrote the manuscript. All authors read, edited and approved the manuscript.

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Supplementary Information

Parasitism capacity of *Trichogramma* spp.

We characterized the parasitism capacity of the three *Trichogramma* species by assessing for nine days the survival and the daily eggs laid. The number of individuals successfully emerging and the sex ratio of offspring was finally recorded.

Experimental set-up

The experiments were conducted in small glass tubes (1 cm dia.; 7 cm height) with a mesh cover. One single female *Trichogramma* was placed inside at day 0 with approximately 400 *E. kuehniella* eggs attached to a 1 cm wet filter paper disc. A droplet of honey-agar (water:agar:honey, 1 l:8 g:100 ml) was placed inside. Every day the droplet and the discs with eggs were changed. The discs were incubated in small boxes for 15 days, then the number of eggs parasitized and the number of females and males emerged were counted. Females that died in the first day were excluded. After nine days, the experiments were stopped.

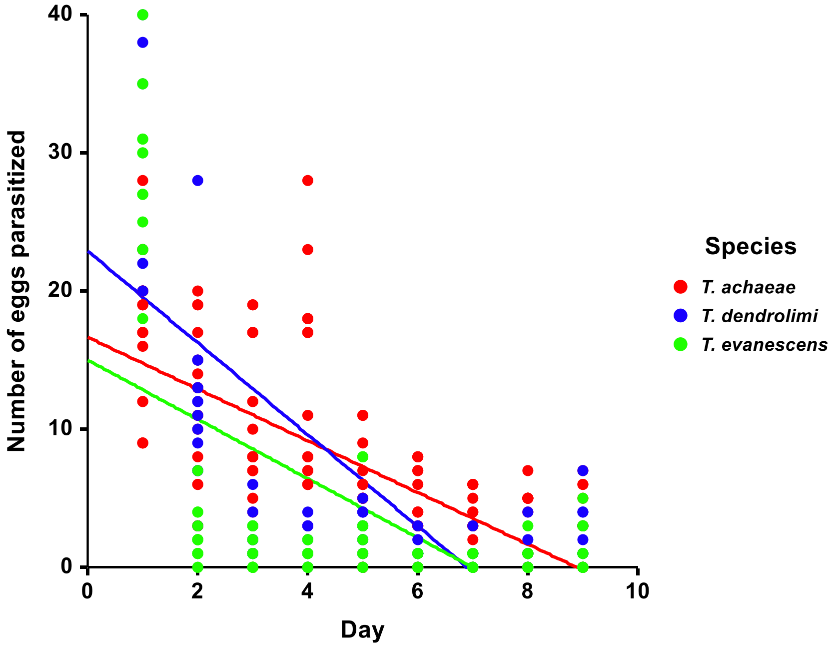

Figure 1 Regression curve of the daily number of eggs laid by the three *Trichogramma* species over the experimental time.

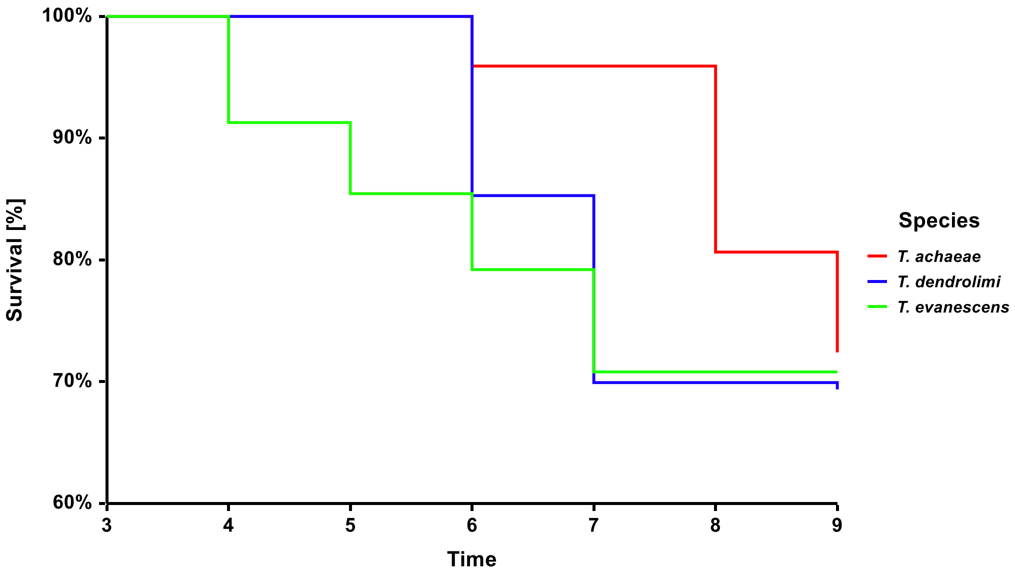

Figure 2 Kaplan–Meier survival curves of the three *Trichogramma* species over the experimental time.

Table 1 Daily fecundity, fertility, and male proportion (mean + SE) of females *Trichogramma* over the first five days. The fecundity was considered as the daily number of eggs laid, the fertility as the number of *Trichogramma* that emerged over the total number of eggs parasitized and the male proportion as number of males over the number of males and females together. We calculated the mean of the first five days only since the fertility and the fecundity dropped after this period and because *Trichogramma* life expectancy in the field is not likely to exceed more than five days.

Species	Daily fecundity	Daily Fertility	Male proportion
T. achaeae (N=20)	11.02±0.69	0.82±0.02	0.54±0.05
T. evanescens (N=20)	7.54±1.36	0.70±0.05	0.70±0.05
T. dendrolimi (N=20)	11.24±1.9	0.59±0.05	0.55±0.05

Chapter II: Combining larval parasitoids with a baculovirus to control *Tuta absoluta*

Necremnus tutae, picture M. Waldburger

Article 2: Few indirect effects of baculovirus on parasitoids demonstrate high compatibility of biocontrol methods against *Tuta absoluta*

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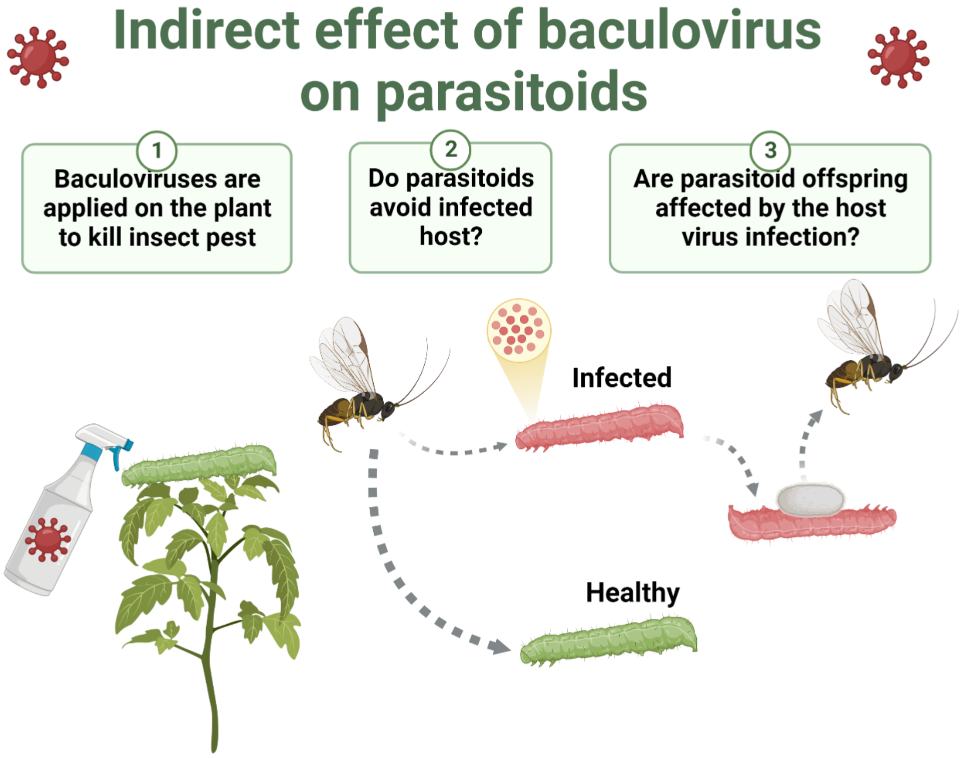

Keywords

Non-target effect, Baculovirus, Braconidae, Eulophidae, Tomato leafminer, Host discrimination, Compatibility

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Abstract

Combining different biocontrol agents, particularly micro- and macroorganisms, can contribute to new and sustainable pest control approaches. *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is one of the most destructive pests of solanaceous crops. An emerging management strategy consists of biological control using microbial insecticides such as baculoviruses, but with limited efficacy. Thanks to their high target specificity, baculoviruses can be used simultaneously with natural enemies such as parasitoids for improved control of *T. absoluta*. However, potential non-target effects of baculoviruses on parasitoids can result indirectly from overlapping resource requirements. We assessed whether ovipositing parasitoid females discriminated against virus-treated hosts and examined the outcome of within-host competition between the hymenopteran parasitoids *Necremnus tutae* (Reuter) (Eulophidae) and *Dolichogenidea gelechiidivoris* Marsch (Braconidae) and the Phthorimaea operculella granulovirus (PhopGV, Baculoviridae) that infects *T. absoluta* larvae.

Female *D. gelechiidivoris* discriminated against virus-treated hosts, whereas *N. tutae* did not. We found few indirect virus-related effects depending on the species, the sex, and the time of virus treatment. Effects were ambivalent for *D. gelechiidivoris* offspring and ranged from increased male longevity when infection occured before parasitization to reduced emergence and male longevity when infection occured after parasitization. *N. tutae* offspring showed a longer development time and shorter male longevity when they developed in virus-treated hosts.

The virus had a low impact on parasitoid offspring. In rare cases, adverse effects were detected; however, the low magnitude of these effects is unlikely to reduce the parasitoid offspring's fitness. Therefore, both parasitoids seem compatible with the baculovirus for control of *T. absoluta*.

Introduction

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) is a destructive pest of tomato plants that also attacks other solanaceous species such as eggplant or tobacco. The pest has become a serious threat to tomato production worldwide,[1–5] invading more than 90 countries outside of its endemic region (EPPO; https://gd.eppo.int). *T. absoluta* is very difficult to control due to the cryptic behavior of its larvae, the increasing resistance to synthetic insecticides, its high reproduction rate with many generations per year and high dispersal capacity.[6–8] Therefore, new management tools that are safe for human health and the environment are urgently needed for managing this invasive pest.[9]

As biological insecticides, baculoviruses can help control lepidopteran pests.[10] The baculovirus family includes a group of arthropod-specific viruses that infect insects from the orders Lepidoptera, Hymenoptera, and Diptera. These invertebrate pathogens are characterized by the presence of two different phenotypes during their infection in susceptible hosts, budded viruses

(BVs) and occlusion derived viruses (ODVs). Infection occurs when a susceptible host feeds on plants contaminated with the ODVs. After ingestion, the ODVs are dissolved in the gut, and the ODVs infect the midgut epithelial cells. BVs then disperse and replicate in other susceptible tissues. When a lethal infection occurs, the tissue of the host larva disintegrates, releasing new ODVs on the plant surface.[11,12] Baculoviruses have been used very successfully in the past, such as against the soybean pest, *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Noctuidae) in South America and in Europe against the codling moth *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), a pest of apple and walnut.[13,14]

A member of the genus *Betabaculovirus (lepidopteran granuloviruses)*, Phthorimaea operculella granulovirus (PhopGV) has been considered for the management of different lepidopteran pest species of the Gelechiidae family such as *Phthorimaea operculella* (Zeller), *Tecia solanivora* (Povolny) and *T. absoluta*.[15–19] Recently, a microbial insecticide based on PhopGV was commercially developed against *T. absoluta* (Tutavir©, Andermatt Biocontrol, Switzerland). The product is register for use in Brazil and has been granted emergency approval in different European countries such as Greece, Cyprus, Germany and Switzerland (Andermatt personal communication). When ingested by *T. absoluta* neonates, the PhopGV induces mortality and sublethal effects such as delayed development, pupation failure, and reduced fecundity. [15–17] Nevertheless, very high doses (weekly application of the highest recommended concentration) are required to lethally infect the most damaging older larvae, so that complementary control measures are required.

The use of baculoviruses for pest control takes place within a wider agricultural system, which may include biological control agents that are present naturally or mass-released. Due to the high target specificity of baculoviruses,[20] natural enemies such as hymenopteran parasitoids are not susceptible to infection. Yet, negative fitness consequences have been observed, such as reduced size or weight in parasitoid offspring that emerge from virus-infected hosts.[21,22] These are indirect consequences of an altered quality of the infected hosts. Deleterious effects of viruses on parasitoids can also result from the viral infection killing the host larva before the parasitoid has completed its development or,[23] more generally, from overlapping resources requirements of immature parasitoid stages and the replicating virus.[11]

Many larval parasitoids have been found attacking *T. absoluta* in the Mediterranean region, among them *Necremnus tutae* (Reuter) (Hymenoptera: Eulophidae), which is particularly abundant and efficient.[24–29] While several companies have attempted to mass-produce this species, the utilization of *N. tutae* is now restricted to conservation biological control due to an ineffective cost-benefit balance.[9] More recently, *Dolichogenidea gelechiidivoris* (Marsh) (Hymenoptera: Braconidae), which originates from the Neotropics, has established in Spain and Algeria.[30,31] In

South America, it is considered an essential agent against *T. absoluta,*[32] and in 2017 it was imported to Kenya from Peru to contribute to the control of *T. absoluta* in Africa.[33] These parasitoid species have very distinct life history traits. *D. gelechiidivoris* is a solitary koinobiont larval endoparasitoid that prefers to parasitize early larval instars of *T. absoluta,*[33] whereas *N. tutae* is an idiobiont ectoparasitoid, which preferably parasitizes later instars.[34] As a koinobiont parasitoid, *D. gelechiidivoris* develops alongside its host, whereas the idiobiont *N. tutae* paralyzes the host and stops its development. Whether virus applications might have adverse effects on the parasitoids attacking *T. absoluta* and how these effects relate to the parasitoid's life strategy is currently unknown. Understanding these interspecific interactions will better predict the compatibility of PhopGV applications and larval parasitoids to control *T. absoluta*.

We conducted laboratory experiments to investigate interactions between PhopGV and the parasitoids *N. tutae* and *D. gelechiidivoris*. In a first step, we assessed the parasitoids' capacity to discriminate between virus-treated and healthy host larvae. In a second step, we evaluated parasitoid development in larvae that had been treated with the virus before and after parasitization.

Material and methods

Insect and plant material

The study was conducted jointly at the biosafety laboratory of Agroscope in Switzerland (*N. tutae*) and the sustainable plant protection laboratory of IRTA in Spain (*D. gelechiidivoris*). Tomato plant varieties used in Switzerland and Spain were *Solanum lycopersicon* cv Rentita and Simona, respectively. Plants were grown in greenhouses.

All insect rearing and experiments in both countries took place in climate chambers at 25 ± 1°C, 70 ± 10% RH, and a 16:8 h L:D photoperiod. In Switzerland, *T. absoluta* was provided by Andermatt Biocontrol; in Spain, it was obtained from an established laboratory colony that originated from field-sampled adults from the Barcelona region. Adults were kept in mesh cages (50 × 50 × 50 cm) (bug dorm, MegaView Science Co., Ltd., Taiwan) and provided cotton soaked in honey-water (10% v/v) placed on the top of the cage and tomato plants for egg-laying. After seven days, plants with eggs and young larvae were moved to another cage to start a new colony.

N. tutae and *D. gelechiidivoris* rearing was initiated with individuals collected in commercial tomato fields in El Maresme county (Barcelona, Spain). Adult parasitoids were kept in mesh cages and provided cotton soaked in honey-water and tomato plants with *T. absoluta* second to third larval instar for parasitization. After emergence, adult parasitoids were collected and stored at 12°C with honey and water. Every ten days, a new parasitoid generation was started. All female parasitoids used in the experiments were naive, mated (stored with males for at least two days), and less than a week old.

Virus application

The virus suspension Tutavir© (Andermatt Biocontrol, Switzerland) containing > $2*10^{13}$ occlusion bodies (OB)/l of PhopGV was diluted with tap water to the highest recommended rate for field-application 0.02% v/v (Andermatt Biocontrol, personal communication). Thus, the suspension used in the experiments contained > $4*10^9$ OB/l. The suspension was sprayed on the tomato plants to obtain infected larvae for experiments. A hand sprayer was used, and attention was paid to covering all parts of the leaves. The plants were sprayed with the virus suspension twice at a four day interval, i.e., inoculation occurred in L1 larval instar for all experiments except for experiment 2.4.2 where L2-L4 larval instars were inoculated.

Virus effect on *Tuta absoluta* larvae mortality and weight

Mortality in *T. absoluta* larvae caused by PhopGV infection usually increases after incubation for nine to 11 days.[19] However, after six days, inoculated larvae showed typical baculovirus infection symptoms like loss of mobility, swollen body, decreased feeding rate, and change in color from green to white. In the late stages, larvae with lethal infection become sluggish and flacid before complete lysis of the body, resulting in death.[17,19] Sublethal effects in the late stage were visible when the larvae were characteristically swollen with a bright white-orange color and did not pupate even after several weeks.

To assess mortality, experiments were conducted in plastic containers (10 cm dia.; 15 cm height) covered with a mesh. Five eggs of *T. absoluta* (less than 24 h old) were placed on a tomato leaf using a fine brush (one leaf per container with five fully grown leaflets with each one egg). In each container, the leaf stem was placed in a solution of agar water (8 g:1 l) to provide moisture. At day one and four after egg hatching, leaves in half of the containers (n = 18) were treated with the virus (inoculation of L1 larval instar), leaves in the other half of the containers (n = 18) were left untreated. Fifteen days after the start of the experiment, the number of healthy pupae (natural shape and color) was compared between the control group and the virus group.

To assess the effect of virus treatment on larval weight after different incubation times, another experiment was conducted. On day one and four after egg hatching, the tomato plants in the treatment group were sprayed with the virus suspension to infect larvae (inoculation of L1 larval instar). Then, healthy and virus treated larvae were weighted after different incubation times. Groups of six and 12 days old healthy and treated larvae were randomly selected and killed in ethanol. The ethanol was then evaporated on a paper towel and the fresh-weight was taken subsequently (n = 40 larvae per group).

Parasitoid host-choice

A choice test was set up in the laboratory to determine whether infection with PhopGV affected parasitoid host preference (see Fig. 1). On day one and four after egg hatching, the tomato plants in the treatment group were sprayed with the virus suspension to infect larvae (inoculation of L1 larval instar). Twenty-four h prior to each experiment, larvae were placed on clean leaflets (not sprayed with the virus) to allow time to burrow into a mine. Tomato leaflets (5 ± 2 cm length, freshly cut) with either a healthy or a virus-treated host larva were offered to female parasitoids at equal distances in a Petri dish (15 cm dia.) covered with a mesh.

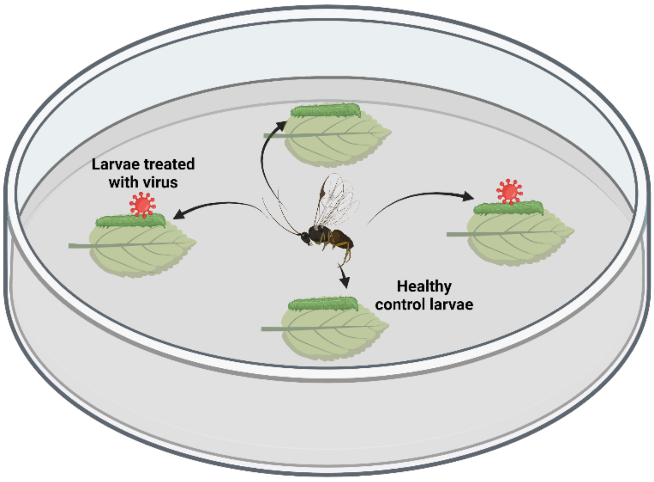

Figure 1 Experimental study set-up for parasitoid host-choice. *Tuta absoluta* larvae were treated with PhopGV on day one and four after hatching, untreated larvae served as control. An equal number of tomato leaflets with healthy or treated larvae were offered to female parasitoids at equal distances in a Petri dish. Leaflets were offered to naive females *Necremnus tutae* and *Dolichogenidae gelechiidivoris* for respectively 24 h and 30 minutes. For *D. gelechiidivoris*, behavioral observations were conducted during the 30 minutes to assess first and second choice (first host that was stung by the wasp) and record all stung larvae. At the end successful parasitisation was assess for both parasitoids.

Necremnus tutae

For the parasitoid *N. tutae*, six leaflets with a healthy or a virus-treated host larva were offered for 24 h to single naive females in a Petri dish (n = 27 replicates). Host larvae were eight days old (L3 larval instar), corresponding to the parasitoids' preferred host instar.[34] Subsequently, females were removed, and each host larva was observed under a stereomicroscope to assess if it was: 1) parasitized (paralyzed and presence of *N. tutae* eggs); 2) host-fed (dead and presence of biting or stinging marks); 3) dead without any attack mark, or 4) alive. An additional experiment was conducted with 12-day-old host larvae (L4 larval instar) to evaluate the host choice with older larvae. For this experiment, only four leaflets (carrying healthy or virus-treated host larvae) were offered to single *N. tutae* females (n = 27).

Dolichogenidae gelechiidivoris

The same experimental set-up was used for *D. gelechiidivoris*. Since preliminary experiments showed that female *D. gelechiidivoris* are much more active than *N. tutae*, they were only allowed to parasitize for 30 minutes (n = 15 replicates). Host larvae were six days old (L2 larval instar), corresponding to the preferred host instar of *D. gelechiidivoris*.[33] Behavioral observations were conducted during the 30 minutes to assess first and second choice (first host that was stung by the wasp) and record all stung larvae. Subsequently, the females were removed, and each larva was individualized in a Petri dish with fresh tomato leaflets. Since *D. gelechiidivoris* is an endoparasitoid, parasitization is only recognizable when larvae leave the host for pupation (approximately 12 days after parasitization, J. Gonthier, personal observation). After 14 days, each larva was observed under a stereomicroscope and compared with the behavioral data to assess if it was: 1) stung and parasitized with the presence of an external cocoon; 2) stung but rejected (host larvae emerged as an adult); 3) stung but host larvae died; 4) not stung and host larvae emerged; 5) not stung but host larvae died. An additional experiment was conducted with 12-day-old host larvae to evaluate the host choice with older larvae. For this experiment, only four leaflets per Petri dish (carrying healthy or virus-treated host larvae) were offered to the *D. gelechiidivoris* females (n = 19 replicates).

Host quality - Virus treatment before parasitization

Healthy and virus-treated host larvae were parasitized to assess the effect of host-virus infection on parasitoid offspring. Treated larvae were parasitized at different time intervals after the virus treatment, considering the long incubation time. The plants were sprayed with the virus suspension twice at a four-day interval, i.e., on day one and four after egg hatching (inoculation of L1 larval instar). Two treatments, each with a control were compared: 1) parasitized host larvae six days after virus treatment (6V); 2) parasitized host larvae 12 days after virus treatment (12V). Parasitized six and 12-day-old host larvae served as control (6C and 12C) (Fig. 2A).

Necremnus tutae

Six days after *T. absoluta* larva emergence, a few leaflets with about 20 larvae (treated or healthy as control) were placed in a Petri dish for parasitization by individual females for 24 h. Leaflets were kept fresh throughout the experiments by placing the stem in a piece of wet cotton, and a droplet of honey was available to the parasitoid females during parasitization. The experiments were conducted in three runs resulting in a total of n = 31 replicates for 6V and n = 38 for 6C. Each female was considered as one replicate. Five days after parasitization, the leaflets were dissected under a stereomicroscope.

Per female, the first five parasitoid pupae that were found were individualized in small tubes (1 cm dia.; 7 cm height) covered with a mesh. Development time, emergence rate, sex ratio, adult size, fertility, and survival of parasitoid offspring were monitored. Survival was measured daily from emergence until death or up to 35 days, and parasitoids were fed every other day with a drop of honey and water. The tibia length of individuals was measured under a stereomicroscope as a proxy for body size. When possible (more than one female emerged in a replicate), one female was killed in ethanol on the third day after emergence to measure its egg load as a proxy for fecundity. The mean of the male and female offspring for each replicate was compiled for the measured parameters to avoid pseudo-replication (from one to four individuals). The experiment was repeated with 12-day-old host larvae (healthy control: 12C n = 34; treated: 12V n = 21)

Dolichogenidae gelechiidivoris

The same experiment (Fig. 2A) was conducted for *D. gelechiidivoris* over two runs (6C n = 24; 6V n = 23; 12C n = 18; 12V n = 9). Since it is a koinobiont parasitoid, new leaflets were placed on top of the older ones as an additional food source for further host larvae development after removing the female parasitoids. Fourteen days after parasitization, the leaflets were dissected under a stereomicroscope. The first five parasitoid pupae that were found were individualized in small tubes, as described above.

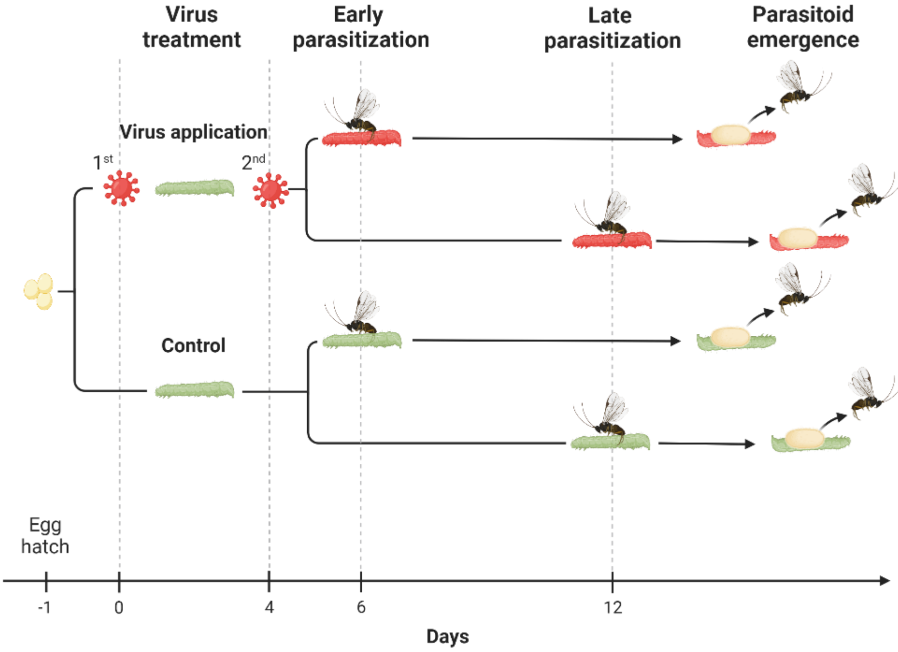

Figure 2A Experimental study set-up for virus treatment before parasitization. The experiment was conducted separately with the parasitoids *Necremnus tutae* and *Dolichogenidae gelechiidivoris*. Half of the *Tuta absoluta* larvae were treated with PhopGV one day after emergence. Half of the treated larvae were subsequently parasitized six days after virus treatment (6V) and the other half 12 days after infection (12V). The untreated larvae served as control and were similarly parasitized after six days (6C) and 12 days (12C).

Host quality - Virus treatment after parasitization

As host larvae parasitized by *D. gelechiidivoris* keep feeding and can become infected by the virus after parasitisation, this experiment was set-up to evaluate the virus's impact on the parasitoid offspring when infection occurs after parasitisation (Fig. 2B). Two treatments were compared with healthy larvae. For the first group, virus treatment occurred one day after parasitization (V1). For the second group, virus treatment occurred five days after parasitization (V5). The leaflets were sprayed with the virus suspension twice at a four-day interval, i.e., for V1 starting on day one after parasitization (inoculation of L2-L3 larval instars) and for V5 starting on day five (inoculation of L3-L4 larval instars). Parasitized larvae without virus treatment served as control.

For each group (control and treatment), the following procedure was performed. Five days after the emergence of host larvae, a few leaflets with about 20 untreated *T. absoluta* larvae were placed in Petri dishes and offered to individual females for parasitization as described in the previous experiment. After removing the females, new leaflets were placed on the older leaflet as additional food for the host larvae (ad libitum). Fourteen days after parasitization, the leaflets were dissected under a stereomicroscope, and the first ten parasitoid pupae that were found were individualized in small tubes covered with a mesh. For each replicate and each measured parameter, the means of male and female offspring were compiled to avoid pseudo-replication (ranging from one to ten individuals). Survival and the different fitness traits in the offspring were monitored as described above. Sixty-two replicates (Control = 20; V1 = 21, V5 = 21) were conducted over two experimental runs.

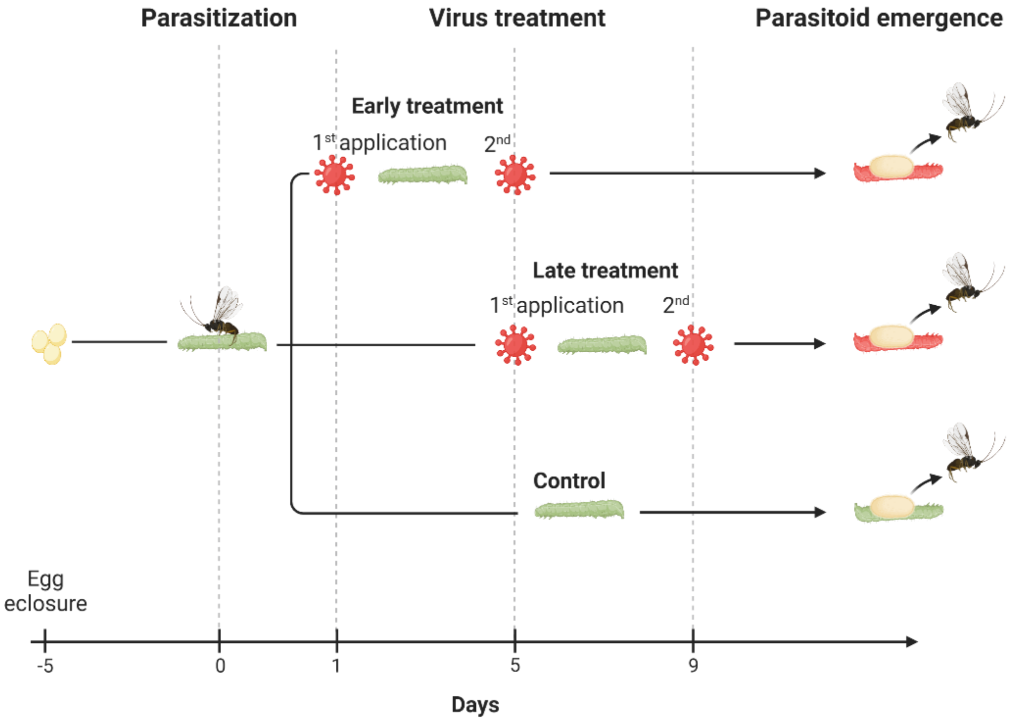

Figure 2B Experimental study set-up for virus treatment after parasitization. Larvae of *Tuta absoluta* were parasitized by the parasitoid *Dolichogenidae gelechiidivoris* five days after emergence. One-third of the larvae were treated with the virus PhopGV one day after parasitization, one-third were treated five days after parasitization, and one-third remained untreated (control).

Data analysis

The software NCSS (2020) (NCSS, LLC, US) was used for statistical analysis. Data were tested for normal distribution using the Shapiro–Wilk's test, and visual inspections of the data were made using Q–Q–plots. The data were mostly not normally distributed (Shapiro–Wilk's test, $p < 0.05$). The first and second host-choice (healthy *vs.* treated) in the behavioral observation with *D. gelechiidivoris* were compared to the unbiased proportion of 50% with a binomial test. A chi-square test on contingency tables (2x4 for *N. tutae* and 2x5 for *D. gelechiidivoris*) was used to compare the category ratio (alive, dead, parasitized, host-fed, etc.) between healthy and treated larvae. A Wilcoxon signed-rank test was used as a post-hoc test. The effect of virus treatment on weight and mortality of *T. absoluta* larvae was assessed with a Mann-Whitney U test. The same test was used for the host quality experiments to compare the emergence rate, development time, sex ratio, egg load, and tibia length between healthy and treated larvae at the two different time intervals with the respective control. Survival analyses were conducted using Kaplan–Meier survival curves and Log rank tests (Mantel–Haenszel test) with pairwise comparison. P-values lower than 0.05 were considered statistically significant.

Results

Effect of the virus on weight and mortality of *Tuta absoluta* larvae

Virus treatment significantly decreased the larval survival of *T. absoluta* from 76 ± 6.2% (mean ± SE; n = 18) in the control to 47 ± 6.0% in the treated group (n = 18; U = 70,5; p = 0.003; Mann-Whitney U test).

The weight of *T. absoluta* larvae was affected by the virus six days but not 12 days after virus treatment. Six days after virus treatment, the treated larvae were lighter (1046 ± 117.2 µg; mean ± SE; n = 42) than the control larvae (1420 ± 130.8 µg; n = 41; U = 541; p = 0.003). Twelve days after virus treatment, no significant differences were found between the control larvae (4106 ± 189 µg; n = 43) and the treated larvae (4508 ± 291.5 µg; n = 41; U = 795; p = 0.439).

Parasitoid host-choice

Necremnus tutae

Eight days after virus treatment, the proportion of larvae parasitized, host-fed, alive and dead was not significantly different from the healthy larvae ($\chi2$ = 0.015, df = 3, p = 0.999; Fig. 3A; chi-square test). Each female parasitized 11 ± 5% *vs.* 16 ± 6% and host-fed on 36 ± 5% *vs.* 31 ± 5% of healthy or virus-treated larvae, respectively (mean ± SE; Fig. 3A; all p-values for paired comparisons > 0.870; Wilcoxon signed-rank test). Twelve days after virus treatment, the proportion of larvae parasitized, host-fed, alive and dead did not differ significantly between treated and healthy larvae ($\chi2$ = 0.031, df = 3, p = 0.998; Fig. 3B; Chi-square test). Females parasitized 19 ± 5.0% *vs.* 11 ± 4.0% and host-fed on 26 ± 5.0% *vs.* 31 ± 7.0% of healthy and virus-treated larvae, respectively (Fig. 3B; all p-values for paired comparisons > 0.206; Wilcoxon signed-rank test).

Dolichogenidae gelechiidivoris

Behavioral observations revealed that female *D. gelechiidivoris* differentiated between healthy and treated host larvae. Six days after virus treatment, significantly more healthy larvae were stung as first choice, 66%, 38.3-88.1% (percentage of healthy larvae stung, 95% CI; n = 15; p = 0.031; Binomial test). However, no preference was detected in the second choice, 40%, 12.1-73.7%

(n = 10; p = 0.612). Twelve days after virus treatment, significantly fewer healthy larvae were stung as first choice, 31%, 12.5-56.5% (n = 19; p = 0.001), but, no preference was observed in the second-choice 43%, 17.6-71.1% (n = 14; p = 0.513).

Six days after virus treatment, the proportion of larvae parasitized, rejected (stung but host larvae emerged), dead (stung and dead), alive (not stung), and dead (not stung) did not differ significantly between treated and healthy larvae ($\chi 2 = 0.096$, df = 4, p = 0.998; Fig. 3C; Chi-square test). Females parasitized 24 ± 8% *vs.* 31 ± 9% and rejected 12 ± 5% *vs.* 13 ± 6 of healthy and virus-treated larvae, respectively (mean ± SE; Fig. 3C; all p-values for paired comparisons > 0.263; Wilcoxon signed-rank test). Twelve days after virus treatment, the proportion of larvae parasitized, rejected, dead (stung), alive (not stung), and dead (not stung) was also not significantly different from that of the healthy larvae ($\chi 2 = 0.038$; df = 4; p = 0.998; Fig. 3D; Chi-square test). However, females rejected significantly more larvae treated with the virus (34 ± 9.0%) than healthy larvae (3 ± 3.0%) (mean ± SE; Z = 2.737; p = 0.006; Fig. 3D; Wilcoxon signed-rank test). Nevertheless, they successfully parasitized (progeny successfully developed and emerged) 24 ± 8.0% *vs.* 26 ± 9.0%, of healthy and virus-treated larvae, respectively (Fig. 3D; all p-values for paired comparisons > 0.146).

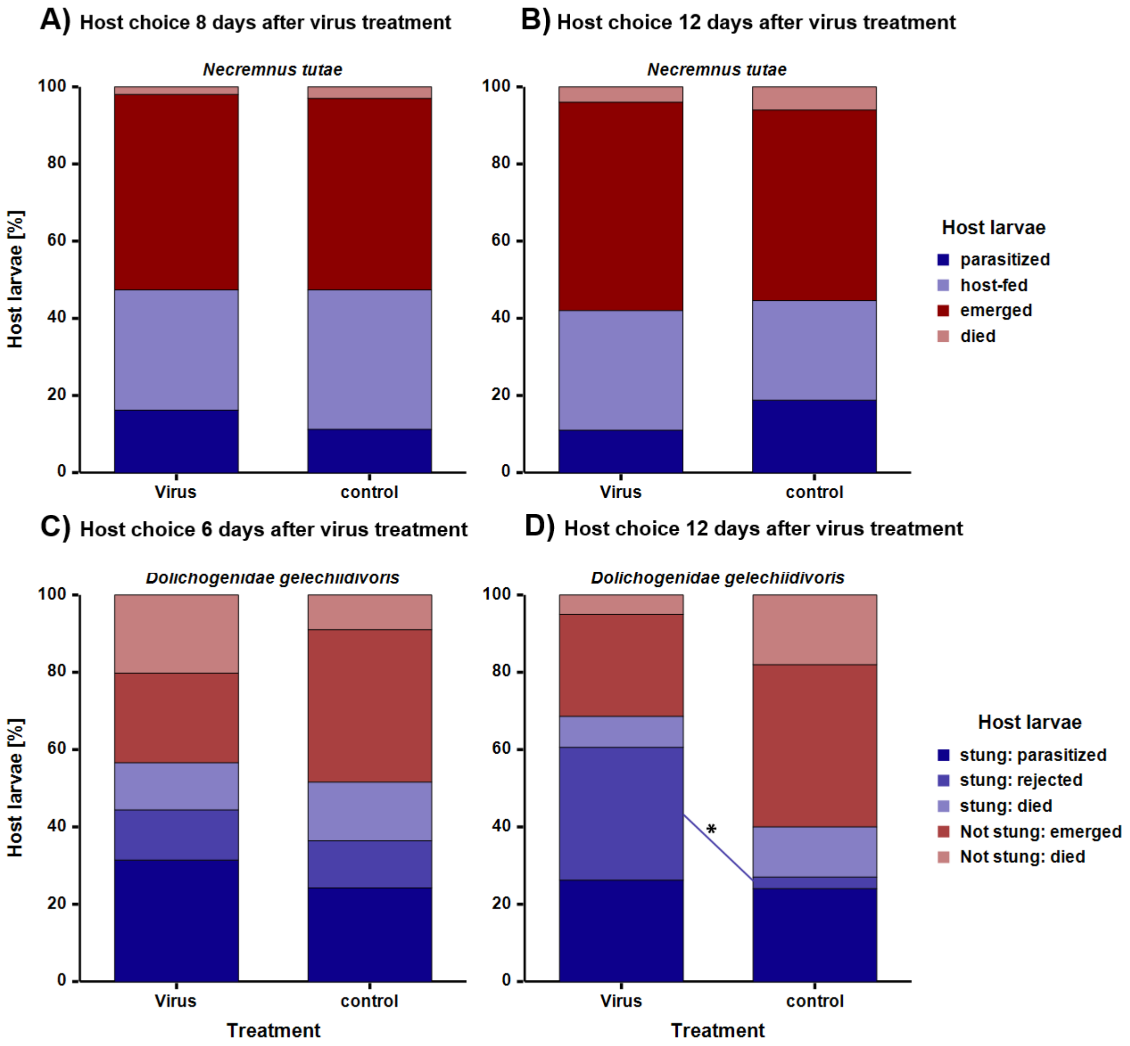

Figure 3 Host preference of female *Necremnus tutae* and *Dolichogenidae gelechiidivoris* (% of host larvae). Single females were offered the same number of healthy and virus-treated hosts. **A** Female *N. tutae* were offered for 24 h three healthy and three larvae treated with the virus eight days before the experiment (n = 27). **B** Female *N. tutae* were offered for 24 h two healthy and two larvae treated with the virus 12 days before (n = 27). **C** Female *D. gelechiidivoris* were offered for 30 minutes three healthy and three larvae treated with the virus six days before (n = 15). **C** Female *D. gelechiidivoris* were offered for 30 minutes two healthy and two larvae treated with the virus 12 days before (n = 19). (*P < 0.05; Wilcoxon signed-rank test).

Virus treatment before parasitization

Necremnus tutae

Three of the fitness traits assessed for *N. tutae* offspring developing in virus-treated (6V) larvae were affected, but effects differed for males and females. The development time of immature males was extended compared to the control group (U = 172.5; p = 0.030; Table 1; Mann-Whitney U test), whereas no difference was observed in the immature female development time. In female offspring, the tibia length was reduced compared to the control group (U = 18.5; p = 0.010), whereas male tibia length was similar in treated and control hosts (U = 41; p = 0.384). The male offspring's survival was reduced compared to the control group (p = 0.031; Fig. 4A), although no difference was observed in female survival (p = 0.882; Fig. 4A; Log-rank test). Offspring were otherwise not affected when developing in 6V larvae and had a similar emergence rate, egg load, and sex ratio that of offspring emerging from 6C larvae (all p-values > 0.195; Table 1; Mann-Whitney U test). No significant differences in the fitness traits of offspring developing in 12C and 12V larvae were detected (all p-values > 0.126; Table 1 & Fig. 4B).

Table 1 Measures of fitness traits of *Necremnus tutae* offspring developing in healthy or virus-treated hosts. Two different treatments were compared to the respective controls: 6V = host larvae parasitized six days after virus treatment; 6C = control group with healthy host larvae of the same age; 12V = host larvae parasitized 12 days after virus treatment; 12C = control group with healthy host larvae of the same age. All data are shown as means ± SE (sample size). F = females, M = males (ns. not significant; *P < 0.05; **P < 0.01; Mann-Whitney U test).

		6 days			**12 days**		
		Control (6C)	**Virus (6V)**		**Control (12C)**	**Virus (12V)**	
Emergence [%]		83.6 ± 5.7 (30)	90.3 ± 4.5 (38)	ns	65.1 ± 9.8 (34)	80.6 ± 6.2 (21)	ns
Development [d]	F	12.0 ± 0.2 (13)	12.0 ± 0.2 (15)	ns	13.0 ± 0.3 (27)	13.2 ± 0.2 (12)	ns
	M	11.9 ± 0.1 (20)	12.3 ± 0.2 (27)	*	12.3 ± 0.3 (9)	12.3 ± 0.1 (4)	ns
Sex ratio [male%]		67.4 ± 7.0 (29)	64.4 ± 8.2 (33)	ns	23.3 ± 10.8 (29)	18.7 ± 6.0 (15)	ns
Egg load		5.1 ± 0.3 (14)	5.8 ± 0.5 (13)	ns	5.0 ± 0.4 (15)	5.6 ± 0.3 (15)	ns
Tibia length [µm]	F	672.5 ± 23.7 (10)	525.3 ± 40.9 (11)	**	714.7 ± 36.4 (15)	635.1 ± 32.7 (11)	ns
	M	451.0 ± 13.5 (9)	475.1 ± 23.9 (13)	ns	435.0 ± 46.1 (12)	447.3 ± 21.2 (15)	ns

Dolichogenidae gelechiidivoris

Two of the fitness traits assessed for *D. gelechiidivoris* offspring developing in virus-treated (6V) larvae were increased, but the effects depended on the sex. The tibia length of female offspring from 6V larvae was increased compared to 6C larvae offspring (U = 74; p = 0.009; Table 2; Mann-Whitney U test), while male tibia length was similar in treatment and control insects (U = 41; p = 0.296). The males' survival in the virus group was increased compared to the control group (p = 0.001; Fig. 4C; Log-rank test), whereas no difference was observed in female survival (p = 0.431; Fig. 4C). The offspring of 6V larvae were otherwise unaffected and had a similar development time, emergence rate, egg load, and sex ratio compared to the offspring of 6C larvae (all p-values > 0.086; Table 2; Mann-Whitney U test). Twelve days after virus treatment, the tibia length of female offspring of 12V larvae was also increased compared to offspring of 12C larvae (U = 5.5; p = 0.031). No other significant differences in the fitness traits were found between offspring developing in 12C and 12V larvae (all p-values > 0.109; Table 2 & Fig. 4D).

Table 2 Measures of fitness traits of *Dolichogenidae gelechiidivoris* offspring developing in healthy or virus-treated hosts. Two different treatments were compared to the respective controls: 6V = host larvae parasitized six days after virus treatment; 6C = control group with healthy host larvae of the same age; 12V = host larvae parasitized 12 days after virus treatment; 12C = control group with healthy host larvae of the same age. All data are shown as means ± SE (sample size). F = females, M = males (ns. not significant; *P < 0.05; **P < 0.01; Mann-Whitney U test).

		6 days			**12 days**		
		Control (6C)	**Virus (6V)**		**Control (12C)**	**Virus (12V)**	
Emergence [%]		57.8 ± 5.2 (23)	50.4 ± 5.7 (24)	ns	52.5 ± 7.4 (9)	38.9 ± 11.5 (18)	ns
Development [d]	F	20.9 ± 0.2 (21)	21.6 ± 0.5 (19)	ns	19.9 ± 0.2 (6)	19.3 ± 0.4 (13)	ns
	M	20.8 ± 0.4 (10)	22.1 ± 0.6 (12)	ns	19.9 ± 0.5 (3)	19.3 ± 0.3 (8)	ns
Sex ratio [male%]		31.1 ± 8.1 (22)	23.1 ± 6.4 (24)	ns	34.7 ± 10.3 (7)	28.6 ± 15.3 (16)	ns
Egg load		107.3 ± 3.3 (9)	102.1 ± 2.6 (11)	ns	111.2 ± 3.6 (4)	118.3 ± 4.9 (9)	ns
Tibia length [µm]	F	752.8 ± 11.5 (10)	811.3 ± 15.6 (11)	**	721.2 ± 17.2 (11)	837.5 ± 38.9 (15)	*
	M	743.2 ± 13.1 (9)	766.3 ± 13.7 (13)	ns	780.0 ± 9.4 (4)	750.0 ± 75 (12)	ns

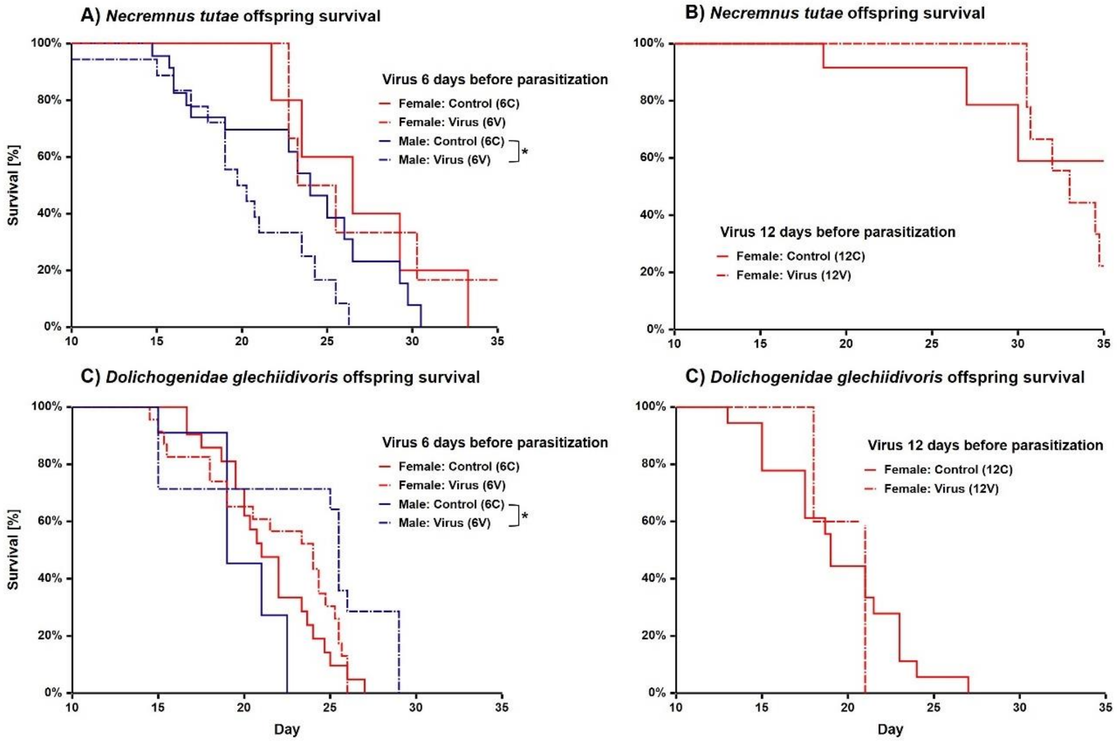

Figure 4 Kaplan–Meier survival curves of *Necremnus tutae* and *Dolichogenidae gelechiidivoris* offspring developing in healthy (control) or virus-treated host larvae of *Tuta absoluta*. **A** Male and female *N. tutae* offspring developing on larvae six days after virus treatment. **B** Female *N. tutae* offspring developing on larvae 12 days after virus treatment. **C** Male and female *D. gelechiidivoris* offspring developing in larvae six days after virus treatment. **D** Female *D. gelechiidivoris* offspring developing in larvae 12 days after virus treatment. Significant differences between groups are indicated (*P < 0.05; Log-rank test: Mantel–Haenszel).

Virus treatment after parasitization

Two out of the six fitness traits assessed for *D. gelechiidivoris* developing in host larvae treated by the virus after parasitization were adversely affected compared to the control. When the treatment occurred one day after parasitization (V1), the emergence rate of offspring (male and female mixed) was reduced (U = 92; p = 0.001; Table 3; Mann-Whitney U test) as well as the survival of the males (p = 0.001; Fig. 5A; Log-rank test). None of the other parameters (i.e., development time, sex ratio, egg load, tibia length, and female survival) were significantly affected, and no differences were observed when host larvae were treated five days after parasitization (V5) (Table 3 & Fig. 5B).

Table 3 Measures of fitness traits of *Dolichogenidae gelechiidivoris* offspring developing in healthy and virus-treated hosts. Comparisons were made between two treatments and a control group: V1 = host larvae treated by the virus one day after parasitization, V5 = host larvae treated by the virus five days after parasitization, C = healthy host larvae. All data are shown as means ± SE (sample size). F = females, M = males (ns. not significant; **P < 0.01; Mann-Whitney U test).

		Control (C)	**Virus (V1)**	**Virus (V5)**	**C *vs* V1**	**C *vs* V5**
Emergence [%]		59.3 ± 3.7 (20)	41.8 ± 3.6 (21)	49.1 ± 4.1 (21)	**	ns
Development [d]	F	22.0 ± 0.3 (14)	22.7 ± 0.5 (17)	22.7 ± 0.5 (12)	ns	ns
	M	21.7 ± 0.5 (12)	21.0 ± 0.6 (12)	22.1 ± 0.6 (15)	ns	ns
Sex ratio [male%]		48.5 ± 10.6 (20)	41.2 ± 9 (20)	60.9 ± 10.2 (21)	ns	ns
Egg load		101.8 ± 4.7 (8)	110.0 ± 4.3 (10)	107.5 ± 7.1 (6)	ns	ns
Tibia length [µm]	F	735.3 ± 10.4 (12)	756.0 ± 18.7 (11)	774.0 ± 19.6 (14)	ns	ns
	M	749.5 ± 25.3 (12)	741.8 ± 10.4 (14)	739.9 ± 10.9 (9)	ns	ns

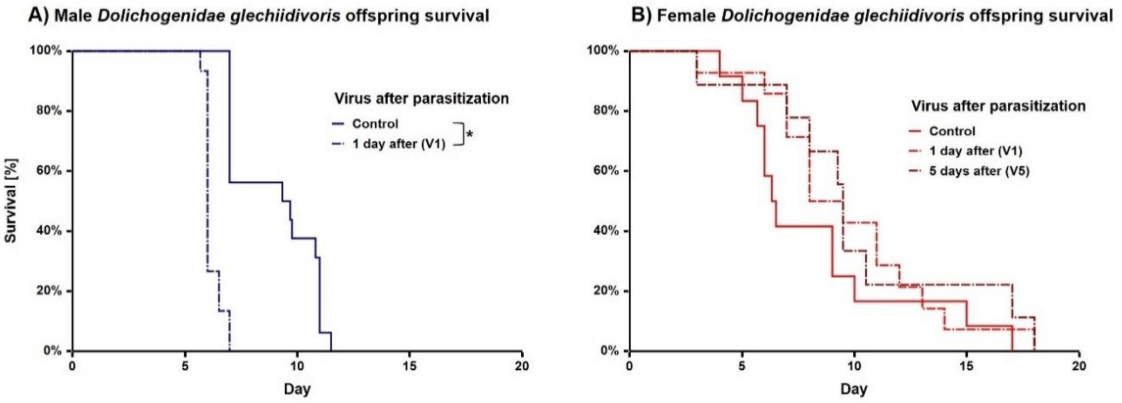

Figure 5 Kaplan–Meier survival curves of *Dolichogenidae gelechiidivoris* offspring developing in healthy (control) or virus-treated host larvae of *Tuta absoluta*. **A** Male offspring of *D. gelechiidivoris* from hosts treated by the virus one day after parasitization. **B** Female offspring of *D. gelechiidivoris* from hosts treated by the virus 1 and 5 days after parasitization. Significant differences between groups are indicated (*P < 0.05; Log-rank test: Mantel–Haenszel).

Discussion

The research presented here is among the first to examine the effects of a baculovirus on two parasitoids with very distinct life histories. The results of these laboratory experiments suggest that the baculovirus would pose negligible risks to the fitness of both the ectoparasitoid and the endoparasitoid of *T. absoluta*. The few observed effects on some fitness parameters were ambivalent (positive and negative) and species- and sex-specific. They depended on the virus incubation time (day since treatment) and whether treatment occurred before or after parasitization.

The observed virus effects on the parasitoids were likely mediated by the virus effects on the host larvae. Virus treatment significantly increased mortality and decreased the weight gain of *T. absoluta* larvae over time. Six days after virus treatment, the treated larvae were lighter than the control larvae confirming earlier studies reporting that first larval instar are highly susceptible to virus infection, which typically slows down the growth of the host.[35] Nonetheless, no differences between healthy and treated larvae were found at twelve days after treatment. Probably, the most susceptible or most heavily infected larvae had already died at that time, dissolving in a pool of virus, and therefore indiscernible at the second sample time on day 12. Usually, the susceptibility of larvae is a function of the virus's virulence, the developmental status of the insect, the stress imposed by the environment, and the dose of the viral inoculum.[11] The prevalence of infection can therefore greatly vary in the same population. Moreover, frequent sublethal symptoms were observed in the older larvae, which points to covert infections of the remaining larvae.[12]

The result of the host choice experiment showed that *N. tutae* could not discriminate between host larvae irrespective of the day since their treatment (eight or 12 days). These results are consistent with those of a previous study,[36] which demonstrated the inability of the hymenopteran parasitoids *Chelonus insularis* Cresson (Hymenoptera: Braconidae) and *Campoletis sonorensis* Cameron (Hymenoptera: Ichneumonidae) to discriminate between nucleopolyhedrovirus-infected and healthy hosts. Host discrimination in parasitic wasps can be mediated by cues from antennal contact with hosts.[37] Since the larvae of *T. absoluta* feed inside mines, no direct antennal contact with the host is possible, which might explain why this parasitoid species could not discriminate. Nevertheless, parasitoids may also detect infected hosts upon insertion of the ovipositor, the so-called probing behaviour.[38] Unfortunately, due to the slow parasitization behavior of this species, direct observations were not feasible. Nonetheless, the female wasps killed the same number of treated and healthy larvae at the end of the experiment. Since *N. tutae* is known for host-killing *T. absoluta* during probing,[39] this supports the conclusion that the females were not probing more on treated larvae.

Interestingly, behavioral observations during the host choice experiment with the parasitoid *D. gelechiidivoris* showed contrasting results. When virus treatment occurred six days before parasitization, females preferred as first choice to sting healthy larvae. In contrast, when virus treatment occurred 12 days before parasitization, females preferred to sting treated larvae as first choice. Conversely, no preferences were observed in the second choice. Therefore, discrimination for stinging does not seem to be consistent over time in this species. We can, however, not rule out the possibility that the apparent inconsistency in response to six and 12 day-old treated larvae is due to the relative small sample size (n=15) of females responding.

Comparing the larvae stung and successfully parasitized by *D. gelechiidivoris* showed that treated larvae were rejected more often. Even more interesting, rejection was found only with older larvae after an incubation period of 12 days; thus, the ability to discriminate likely increases with the severity of the infection. Similar results were found in a previous study,[40] in which female

Meteorus gyrator (Thunberg) (Hymenoptera: Braconidae) reduced the number of eggs inserted into granulovirus infected Indian meal moth, *Lacanobia oleracea* (L) (Lepidoptera: Noctuidae) larvae, based on the level of host infection. In fact, host discrimination is a well-known phenomenon and numerous hymenopteran parasitoids can discriminate against infected larvae to varying degrees.[41] Consequently, by discriminating between healthy and treated hosts, *D. gelechiidivoris* is less susceptible than *N. tutae* to being adversely affected by granuloviruses. In terms of pest control efficacy, the combination could be even synergetic since the parasitoid can minimize harmful interference and control those hosts that have escaped the virus. Moreover, they also can spread infection by transmitting budded virions when stinging the larvae.[11]

Unexpectedly, even though female *D. gelechiidivoris* rejected more treated than healthy larvae 12 days after virus treatment, the same number were successfully parasitized. There are several possible explanations. First, treated host larvae could be more attractive at first (as shown by the first choice preference), with more volatile cues being emitted by treated larvae, but considered less suitable after closer examination, i.e. through probing. Secondly, female *D. gelechiidivoris* are very efficient in host finding and handling (J. Gonthier, personal observation). Even though they rejected many larvae, they could have managed to find enough hosts with a low infection level to parasitize during the time allowed (30 min period). It is important to bear in mind that we cannot exclude that hosts categorized as rejected (stung but *T. absoluta* emerged) could also be hosts in which the parasitoids egg did not survive (e.g., egg encapsulation by the host). However, this is unlikely since the same number of parasitoids successfully pupated in treated and healthy larvae.

The host quality experiment showed ambivalent results. When virus treatment occurred before parasitization, offspring of the parasitoid *N. tutae* developing in larvae six days after treatment (6V) were negatively affected in comparison to offspring from healthy control larvae (6C). Specifically, males had a longer development time and shorter lifespan, and females were smaller. In parasitoid wasps, fitness is often correlated with body size;[42] thus, the smaller body size might negatively affect the fitness of the females. However, the prolongation of development by less than a day is unlikely to affect male’s fitness. Similarly, the reduced male lifespan is unlikely to affect reproduction since more than 90% of males developing in virus treated larvae survived until day 20. In fact the mating occurs in the first few days since oviposition by females *N. tutae* start the latest four days after emergence.[34] A possible explanation for these negative effects might be that by paralyzing the host larvae, the wasp weakened the host's immune response against the virus, which could have favored virus replication and reduced host quality. It’ is important to keep in mind that the reduce lifespan might be exacerbate under field conditions due to harsh conditions and other stress factors and it’s not impossible that a compound effect occurs in the field.

Increased parasitoid development time due to virus infection of the host has been documented previously. For instance, the development of *Ascogaster reticulata* Watanabe (Hymenoptera: Braconidae) was lengthened in infected hosts.[43] Virus infection also reduced the weight of the offspring of the parasitoid *Apanteles glomeratus*.[21] The seeming differences between sexes in our study might be explained by the marked sexual dimorphism of *N. tutae*, with particularly large females and small males. Females might have chosen the larger, lightly infected hosts for production of female offspring. Smaller hosts, with a high virus infection level, might have been used for male offspring. Males developing on those hosts compensated for the lower host quality by slower development to attain a minimum size necessary for emergence. It is interesting to note that no differences between offspring developing in host larvae 12 days after virus treatment (12V) or healthy control larvae (12C) were found. A possible explanation might be that, as already explained; host larvae had mostly mild and covert or sublethal infections at this infection stage, whereas the most heavily infected larvae had already died. Those infections seem not to have affected the developing parasitoids. An alternative explanation might be that the late instar 12 day-old larvae may be large enough for the parasitoids, which naturally prefer the third instar.[34] Therefore, host resources might not have been a limiting factor.

Koinobiont endoparasitoids such as *D. gelechiidivoris* spend most or all of their larval development inside an actively feeding host insect, which provides the sole source of nutrition for the immature parasitoid and the environment in which parasitoids develop.[44] One could expect endoparasitoids to be more susceptible to virus-related indirect effects than ectoparasitoids. In contrast, the host quality experiment showed that PhopGV even improved some fitness traits of *D. gelechiidivoris* offspring developing in hosts six days after treatment (6V) - females were larger, and males had a longer lifespan. Likewise, female offspring were larger when developing in hosts 12 days after treatment (12V). Because of the evolution history with *D. gelechiidivoris*,[32] *T. absoluta* has likely evolved a solid immune response against this parasitoid, which might have been compromised by the virus infection,[11] benefitting the parasitoid. The granulovirus might act similar to endosymbiotic polydnaviruses of parasitoids, which cause suppression of host cellular immune responses and inhibit feeding or prolong or arrest development, among others.[11,45]

Interestingly, when the treatment occurred after parasitization, the offspring of *D. gelechiidivoris* were negatively affected. Offspring had a lower emergence, and males had a shorter lifespan when treatment occurred one day after parasitization. As discussed, one possible explanation for this result is that infection may cause a general decrease in host quality. In this case, the virus could have the opposite effect by boosting the host's immune response. A previous study reported that infection with a baculovirus expressing a protease decreased the survival of the braconid parasitoid *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) emerging from tobacco budworm larvae *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae) if the host was infected with the virus less than 72 h post parasitisation.[45]

No effects were visible in offspring that developed in treated hosts five days after parasitization. This result corroborates the idea that postponing the exposure of parasitized larvae to a baculovirus increases the percentage of successful parasitoid development.[11] Overall, these results must be interpreted cautiously because it is impossible to detect parasitization by *D. gelechiidivoris* before pupation without dissecting the host. Therefore, assessment of the impact of the virus on the young parasitoid immature stages of *D. gelechiidivoris* (egg to pupae) was not possible.

In this study, we examined the relative importance of indirect effects of a granulovirus infecting *T. absoluta* on two of its parasitoids with very distinct life-history traits. We found that *D. gelechiidivoris* could discriminate against treated larvae after prolonged incubation and is likely less susceptible to baculovirus application. However *N. tutae* could not discriminate and suffered more than *D. gelechiidivoris* from virus treatment of the host after parasitization. Overall, both parasitoids were negligibly affected by the virus treatment, indicating that they can be combined with the baculovirus for control of *T. absoluta* and that the virus is unlikely to impact naturally occurring parasitoid populations. Future research is currently underway to assess the efficiency of such a combination, and further modeling work will be conducted to understand the underlying mechanism at the population level.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data supporting this study's findings can be found at the figshare repository. See (https://doi.org/10.6084/m9.figshare.20557488).

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Article 3: A parasitoid wasp allied with an entomopathogenic virus to control *Tuta absoluta*

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Abstract

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) is a major threat to tomato production worldwide. Sustainable management methods are needed to control the pest while reducing adverse effects due to chemical control. Phthorimaea operculella granulovirus (PhopGV) and the parasitoid wasp *Necremnus tutae* Ribes & Bernardo (Hymenoptera: Eulophidae) were identified as promising biocontrol agents of *T. absoluta*. We conducted a semi-field experiment to evaluate the efficacy and compatibility of *N. tutae* and PhopGV when combined to control *T. absoluta*.

N. tutae used alone and combined with PhopGV significantly reduced the number of adult moths cumulated over the trial by 39% and 40%, respectively. While PhopGV treatment was similar to the control during the first two generations of the pest, it significantly reduced adult numbers by 78% in the third generation. Both agents combined reduced the cumulated number of larvae by 24%, i. e. 11% and 10% more than the parasitoid and the virus alone. However, the differences between the treatments were non-significant. Both agents, alone and combined, caused a significant and similar damage reduction of 24% to 29%. The virus had no negative effect on the parasitoid.

Our results demonstrate the compatibility and the potential of *N. tutae* and PhopGV to reduce population growth and mining damage of *T. absoluta* under semi-field conditions. While more research is needed to fully evaluate the potential of combining *N. tutae* and PhopGV for *T. absoluta* control, our findings provide interesting insight into an innovative combination of biocontrol agents.

Keywords

Eulophidae, Baculovirus, combination, South American tomato pinworm, integrated pest management, biological control

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Introduction

About 190 million tons of tomatoes (*Solanum lycopersicum* L.) are produced yearly on five million hectares worldwide (FAO; https://www.fao.org/faostat/en/#home). The tomato leafminer, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is a major threat to this essential cash crop many people rely on.[1] The pest can cause up to 100% damage in all production systems, ranging from low-tech open-field crops to high-tech greenhouse crops.[2–4] *T. absoluta* is native to South America and has, since its arrival in Spain in 2006, spread to nearly 100 countries outside its endemic region (EPPO; https://gd.eppo.int). Females lay their eggs individually on leaves, stems, and petioles, preferentially on apical and median plant parts.[5, 6] The leaf-mining larvae penetrate the leaves and feed on the mesophyll throughout their four instars development. Larvae mainly damage leaves and attack fruits when the population density is high.[2, 6] Mature larvae usually drop to the soil and pupate on the ground. *T. absoluta* has a high reproduction potential and can complete up to 12 generations per year under favorable environmental conditions.[2, 7]

Chemical control is largely used to control the moth but is unsustainable as *T. absoluta* rapidly develops high levels of resistance to many insecticides.[8] Moreover, chemical insecticides harm key beneficial arthropods, disrupt existing IPM programs and cause environmental and human health concerns.[7] Therefore, alternative sustainable management methods to control the moth are required to ensure the economic viability of tomato production in infested regions. Biological control methods involving egg parasitoids, mirid predators, and microbial agents have been successfully integrated into pest management programs against *T. absoluta* in various regions, including South America, Europe, Africa, and Asia.[9] Using multiple pest control methods, including diverse biological control agents, provides numerous advantages compared to relying solely on a single agent. One benefit is the possibility of enhancing effectiveness, as different control methods have advantages and disadvantages, and combining them can help overcome each method's limitations. For instance, combining various natural enemies may be more effective at controlling a pest population as each species can have distinct foraging behavior or target different pest life stages.[10] Regarding *T. absoluta*, most studies on combining different biological control agents focused on combining *Trichogramma* egg parasitoids with other methods,[9, 11, 12], and the ones testing the efficacy under semi-field conditions are scarce. To address this knowledge gap, we performed a semi-field experiment using a recently developed entomopathogenic virus and a larval parasitoid to control *T. absoluta*.

Phthorimaea operculella granulovirus (PhopGV, Baculoviridae: Betabaculovirus) is a granule-shaped virus highly specific to Lepidoptera and infectious to larvae of different Gelechiidae insect species. It was first developed for the biocontrol of the potato moths *Phthorimaea operculella* (Zeller) and *Tecia solanivora* (Povolný), which are closely related to *T. absoluta*.[13, 14] Transmission and replication of baculoviruses occur exclusively in the larval stage. Larvae become infected when they feed on plants contaminated with occlusion bodies (OBs) containing occlusion-derived viruses (ODVs). After ingestion, OBs are dissolved in the insect midgut, releasing ODVs that infect the midgut epithelial cells. Budded viruses (BVs) then disperse and replicate in other susceptible tissues. The larval tegument ruptures upon death, releasing new OBs and contaminating the plant surface.[15, 16] The PhopGV has a good level of pathogenicity against *T. absoluta* and causes sublethal effects such as retarded larval growth and pupation failure.[13, 14]

Necremnus tutae Ribes & Bernardo (Hymenoptera: Eulophidae), previously known as *Necremnus artynes* (Walker), is native to the Mediterranean region and is the most abundant and widespread larval parasitoid of *T. absoluta* in that area.[17,18] This synovigenic idiobiont ectoparasitoid parasitizes and feeds on second to fourth instars larvae of the moth with a distinct preference for the third instar.[19] *N. tutae* causes extra mortality by host-killing (i. e. host-feeding, host-stinging). It is currently not commercially available but occurs spontaneously and abundantly in many tomato fields.[7, 17, 20, 21]

Augmentative releases of *N. tutae* alone seem insufficient to keep damage caused by *T. absoluta* on tomato plants below the economic threshold.[7, 22, 23] Regarding PhopGV, high doses (weekly application of the highest recommended concentration) are required to infect the most damaging older larvae lethally, so complementary control measures are required.[24] Combining both biocontrol agents could increase pest control against *T. absoluta*. The virus is more effective on young larvae, while the parasitoid targets the later instars. In addition, no adverse effects of the PhopGV were detected on the development of *N. tutae*, indicating that both agents are compatible for combined use.[24]

We conducted a semi-field experiment to assess the efficacy of *N. tutae* and the PhopGV used alone and in combination for the biocontrol of *T. absoluta*. We addressed the following questions: 1) Do *N. tutae* and the PhopGV influence the population growth of *T. absoluta* when used alone or in combination? 2) Do *N. tutae* and the PhopGV influence the level of leaf damage caused by *T. absoluta* when used alone or in combination? 3) Is PhopGV compatible with *N. tutae* under semi-field conditions?

Material and methods

Study area and greenhouse

The semi-field experiment was conducted from June to August 2021 in the facilities of Agroscope in Conthey (Valais, Switzerland). Twenty-eight walk-in cages (hanging cages; Diatex SAS, France) of 2.6 m^2 surface and 2.5 m high with thrips-proof mesh (300 x 300 μm^2) were set up in two identical 90 m^2 glass greenhouses (8 cages in greenhouse 1 and 20 cages in greenhouse 2). The floor below the cages was covered with a white woven ground cloth (100 g/m^2). Temperature, relative humidity, and irrigation were managed using a remote-control system.

Biological material

Tomato plants

Ungrafted single-stemmed tomato plantlets cv. Admiro (De Ruiter) reaching 30 cm height and five fully expanded leaves with leaflets were repotted in 10 liters pots, fertilized with Osmocote (NPK 15/9/12), a long-term fertilizer providing enough nutrients for four months, and moved into the walk-in cages. Pesticides were strictly avoided during the growing phase. The temperature was set at 20°C during the day and 18°C at night. To match the typical plant density for tomato cultivation (2.3 to 2.5 plants/m^2) and leave enough space for sampling, four plants were placed in each of the 2.6 m^2 walk-in cages. The main stem of each plant was trellised on a string attached to a wire above the cage and passed through an insect-proof hole in the ceiling of the latter. Secondary shoots were removed weekly, and plants were watered daily through drip irrigation.

Insects

Pupae of *T. absoluta* were provided by Andermatt Biocontrol Suisse AG. Those were originally collected in Switzerland and reared in the company's facilities, and a sample was checked to ensure a 1:1 sex ratio. The pupae were released in a plate placed on the floor in the center of each cage.

N. tutae parasitoids were provided by IRTA Cabrils. The parasitoids were collected from commercial tomato fields in El Maresme county, Barcelona, Spain, and a colony was established at Agroscope, Switzerland. Adult parasitoids were kept in bug dorm (50 × 50 × 50 cm) (bug dorm; MegaView Science Co. Ltd., Taiwan) and provided with honey-water (10% v/v) soaked cotton and tomato plants with *T. absoluta* larvae in their second or third instar for parasitization. After emerging, adult parasitoids were gathered and kept at 12°C with honey and water. Every ten days, a new generation of parasitoids was started. Only naive, mated (stored with males for at least two days), and less than one-week-old female parasitoids were used in the experiments. Adult parasitoids were briefly cooled in a cold room (4°C) for counting (sex ratio 1:1). They were then placed in an emerging box for 24 h to feed and mate before being released in the middle of each walk-in cage. Honey was supplied twice a week for one month to the parasitoids to ensure their proper establishment.

Baculovirus

Tutavir®, containing a minimum of 2×10^{13} OB/l of PhopGV, was produced and provided by Andermatt Biocontrol. The product was applied with an electrical backpack sprayer following the supplier's instructions specific to the trial. Good coverage of the underside of the leaves was achieved by using a sprayer with the nozzle pointing upwards. A plastic sheeting was hung between the cages before each application of Tutavir® to avoid cross-contamination.

Experimental setup

The four following treatments were compared in a completely randomized block design with seven replicates: **1)** Control with *T. absoluta* only (CO), **2)** *T. absoluta* and *N. tutae* (PA), **3)** *T. absoluta* and Tutavir® (VI), and **4)** *T. absoluta, N. tutae* and Tutavir® (VP). Seven blocks of four adjacent walk-in cages were distributed between greenhouse 1 (2 blocks) and greenhouse 2 (5 blocks). Each of the 28 cages corresponded to a plot. To provide *T. absoluta* with food at libitum, several batches of plants were transplanted in the walk-in cage throughout the trial. A first set of four potted plants was inserted on day one, followed by a second one on day 45 (Table 1). Ten plants cut in a neighbouring greenhouse were placed in each cage on day 48, and two last potted plants were added on day 58. All batches of plants (except the last one) were sprayed with water (CO and PA) or with Tutavir® (VI and VP) at high dosage (0.015%, $\geq 3 \times 10^9$ OB/l) on the day of their introduction (Table 1, Figure 1). Additionally, the first batch of plants was sprayed at a high dosage on days 3 and 10 and at a low dosage (0.0015 %; $\geq 3 \times 10^8$ OB/l) on days 28 and 42. Fifty *T. absoluta* pupae were released in the 28 plots on day one. *N. tutae* neither parasitizes nor feeds on first-instar *T. absoluta* larvae.[19] Thus, the parasitoid was first released on day 16, coinciding with the first availability of the second instar larvae of the moth (Table 1, Figure 1). Thirty couples of adults were released in each plot of treatments PA and VP. A second release of thirty couples was achieved on day 41.

Table 1. Experimental set-up of a three-month semi-field experiment comparing the efficacy of two biocontrol agents against *Tuta absoluta*. Cell numbers indicate the exact day each action occurred (1 = start of the trial). ^{50} = 50 *T. absoluta* pupae, ^{30} = 30 couples of *Necremnus tutae*, ^{H} = application of Phthorimaea operculella granulovirus (PhopGV) at high dosage (0.015%, $\geq 3 \times 10^9$ OB/l), ^{L} = application of PhopGV at low dosage (0.0015 %; $\geq 3 \times 10^8$ OB/l), ^{ad} = monitoring of *T. absoluta* adults only.

Description	**Weeks 2021**										
	23	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**
Tomato plants	**1**						**45**	**48**	**58**		
***T. absoluta* release**	**1^{50}**										
PhopGV application	**1^{H}** **3^{H}**	**10^{H}**			**28^{L}**		**42^{L}** **45^{H}**	**48^{H}**			
***N. tutae* release**			**16^{30}**				**41^{30}**				
Pest monitoring			**16**	**23**	**30**	**37**	**44**	**51**	**55^{ad}** **58**	**62^{ad}** **65**	
Yellow sticky traps										**62** **65**	**72**

Pest sampling and damage monitoring

Weekly, one plant per cage was sampled starting two weeks after *T. absoluta* release (Table 1). Each time, a different plant was sampled. Two leaflets of leaves 6, 8, 10, 12, and 14 (apex =1) were collected systematically on each selected plant, giving ten leaflets per cage. The number of healthy *T. absoluta* larvae in each leaflet was counted using a binocular. After counting, leaflets were placed in a transparent sheet and scanned using a standard printer, and the percentage of surface mined by the larvae of *T. absoluta* was assessed using the software ImageJ (Version 1.53j). Leaflets were put back in their original cage at the latest 36 h after sampling to avoid bias due to the removal of insect individuals.

Adults of *T. absoluta* were counted systematically on three lateral side walls of each cage just after the weekly leaflet collection. Two additional counts were done on days 55 and 62 to get detailed data about the population development in the second generation of adults. On day 62, two yellow traps with a pheromone dispenser were hung in each cage to catch the remaining adults of *T. absoluta* and *N. tutae*. The traps were replaced twice on days 65 and 72 while keeping the same pheromone dispenser. Once removed from the cages, the traps were wrapped in plastic film, scanned on both sides and placed in the refrigerator. All scans were sent to Trapview B.V. in the Netherlands, where the number of *T. absoluta* adults was counted using a specific algorithm. In addition, the number of *N. tutae* males and females was visually assessed on the traps using a binocular.

Statistical analysis

Statistical analysis was performed with the R statistical package, version 4.2.3 (2023) of the R Foundation for Statistical Computing. The cumulated number of healthy pest larvae and the cumulated number of pest adults recorded over the trial, as well as the total number of pest adults caught on the yellow traps at the end of the trial, were considered to assess the treatment effects on the population growth of *T. absoluta*. The average percentage of leaf damage recorded over the trial was examined to evaluate the treatment effects on the level of leaf damage. The total number of parasitoid adults caught on the yellow traps at the end of the trial were sqrt(x) transformed to normalize distribution prior to analyzing the treatment effects on the build-up of the population of *N. tutae*. Linear models with the treatment as fixed factor were fitted using the nlme package (version 3.1-162). For all models, assumptions were checked according to the graphical validation procedures recommended by Zuur et al. (2009).[25] The block factor was neglected in all models as it had no significant effect on the residuals. All the above-mentioned dependent variables were compared among treatments using a two-way ANOVA and Tukey's test for mean separation. The sex ratio of the parasitoids caught on the yellow traps at the end of the trial was assumed to follow a binomial distribution. Thus, a generalized linear model with the treatment as a fixed factor was fitted using the stats package. Comparisons between treatments were conducted using a chi-square test. P values lower than 0.05 were considered statistically significant.

Results

T. absoluta completed almost three generations during the three-month semi-field experiment (Figure 1). The first generation lasted from day 1 to day 30, while the second spanned from day 30 to day 60. The third generation started around day 60. The start and end of each generation were determined based on the adult and larval peak populations. On day 65, little damage was observed compared to the recorded number of larvae, with mostly first-instar larvae present, suggesting that earlier instars caused less damage than later instars. Strong variations were observed between treatments over time. Therefore, the analysis was conducted on the variation of the number of *T. absoluta* larvae and adults and the average percentage of leaf damage cumulated over the trial.

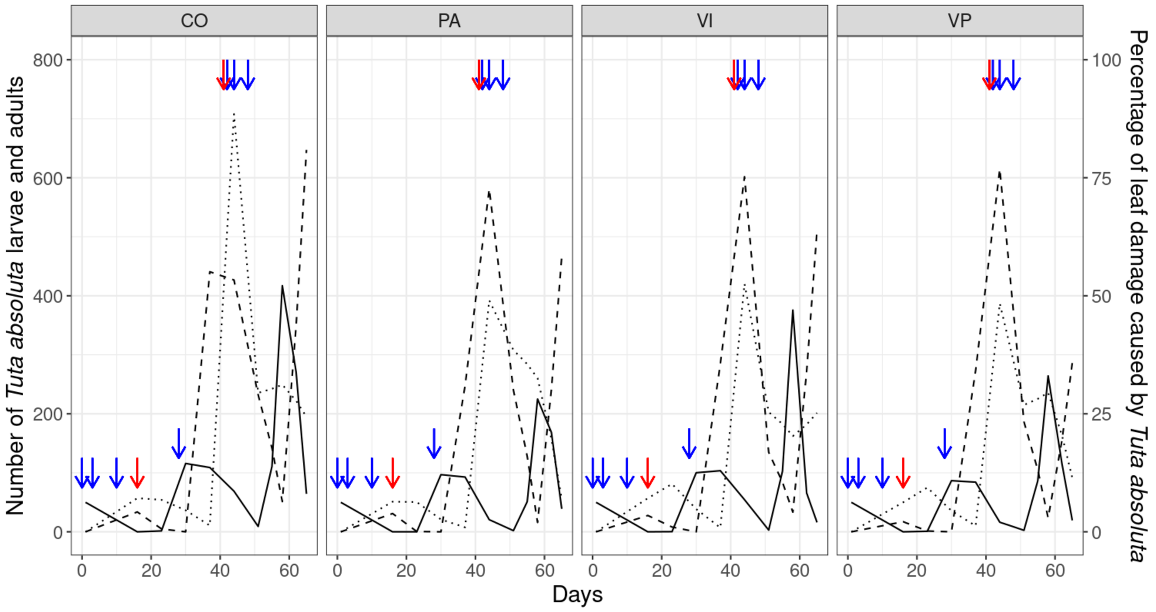

Figure 1. Population dynamics of *Tuta absoluta* and leaf damage over three generations monitored on tomato plants over a three-month semi-field experiment including four biocontrol strategies (CO = control without treatment, PA = parasitoid (*Necremnus tutae*), VI = virus (PhopGV), VP = virus + parasitoid). The solid line represents the density of adults (starting with 50 individuals), and the dashed line represents the density of larvae. These two parameters are connected to the y-axis on the left. The dotted line represents the percentage of leaf damage and is connected to the y-axis on the right. The blue arrows indicate when the baculovirus was applied, and the red arrows indicate when the parasitoids were released.

Effect on the leaf damage

Both agents, alone and combined, caused a significant and similar damage reduction cumulated over the trial (Anova, $p = 0.001$, Tukey's multiple comparisons: PA-CO $p = 0.003$, VI-CO $p = 0.005$, VP-CO $p = 0.001$, Figure 2A). Compared to the control, PhopGV and N. tutae reduced damage by 24% and 26%, and the combined biocontrol agents reduced leaf damage by 29%. No significant differences were found between the biocontrol agents used alone or in combination ($p > 0.894$).

Effect on the population growth of *Tuta absoluta*

Regarding larval reduction, PhopGV and *N. tutae* reduced the cumulated number of *T. absoluta* larvae by 13% and 14%, respectively, compared to the control (Figure 2B). Both biocontrol agents combined decreased the cumulated number of larvae by 24%. However, the differences between the four treatments were non-significant (Anova, p = 0.238).

Compared to the control, the number of *T. absoluta* adults cumulated over the trial was significantly reduced in plots where *N. tutae* was used alone (-39%) and in combination with PhopGV (-40%) (Anova, p = 0.008; Tukey's multiple comparisons: PA-CO p = 0.017, VP-CO p = 0.012, Figure 2C). PhopGV alone reduced the number of *T. absoluta* adults by 28% compared to the control. However, this difference was non-significant (VI-CO p = 0.108). No significant differences were found between the biocontrol agents used alone or in combination (p > 0.750).

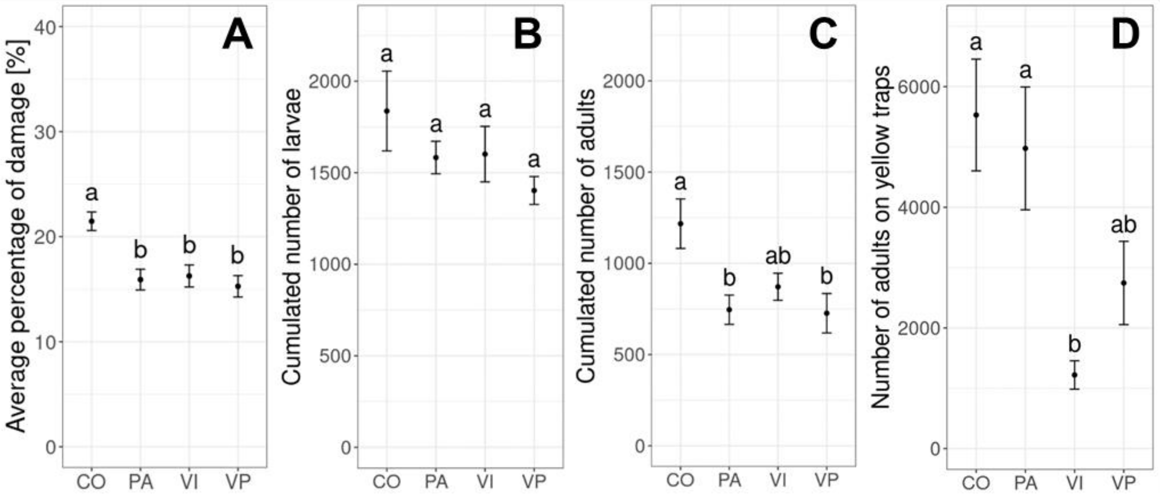

Figure 2. Average percentage of leaf damage monitored on tomato plants (**A**) and cumulated number of *Tuta absoluta* larvae (**B**) and adults (**C**) over the whole trial. Total number of adult on the third generation catched on yellow traps (**D**).The three-month trial included four biocontrol strategies (CO = control without treatment, PA = parasitoid (*Necremnus tutae*), VI = virus (PhopGV), VP = virus + parasitoid). Error bars represent the standard error, and Lowercase letters indicate significant differences between the biocontrol strategies.

The numbers of adult *T. absoluta* caught on the sticky yellow traps at the end of the trial were inconsistent with data from weekly sampling (Figure 3). The virus treatment was the best, with the lowest adult density, followed by the virus-parasitoid combination. Compared to the control and the parasitoid treatment, PhopGV significantly reduced the third generation of adults by 78% and 75%, respectively (Anova, p = 0.002, Tukey's multiple comparisons: VI-CO p = 0.004, VI-PA p = 0.011). The virus-parasitoid combination caused a reduction of 50%, which was almost significant (p = 0.08). With a reduction of 10% only, the parasitoid treatment was similar to the control (p = 0.958). No significant differences were found between the biocontrol agents used alone and in combination (p > 0.207).

Compatibility of the biocontrol agents

N. tutae and PhopGV were found to be compatible under semi-field conditions. Similar numbers of parasitoids were recorded at the end of the trial in the parasitoid and the virus-parasitoid treatments (Anova, p = 0.602). Similar sex ratios were found in both treatments, indicating no adverse effect of PhopGV on *N. tutae* (Chi-square test, LRT_1 = 2.380 p = 0.123).

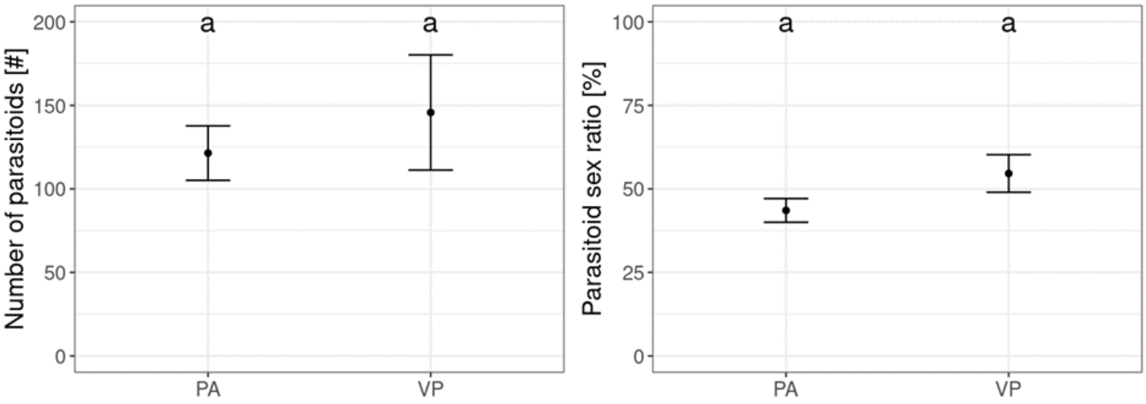

Figure 3. *Necremnus tutae* adults (left) and mean percentage of *N. tutae* females (right) recorded on sticky yellow traps at the end of a three-month semi-field experiment including different experimental treatments (CO = control without treatment, PA = parasitoid (*N tutae*), VI = virus (PhopGV), VP = virus + parasitoid). Error bars represent the standard error. Different lowercase letters indicate significant differences between the experimental treatments according to Tukey's HSD (adults) and chi-square test (sex ratio).

Discussion

This research demonstrates the potential of the baculovirus PhopGV and the parasitoid *N. tutae* when used alone and combined against *T. absoluta* under semi-field conditions in the greenhouse. Both the virus and the parasitoid have previously displayed potential against this pest when used separately in a laboratory and in a semi-field experiment, respectively.[23, 26] We found that using both agents in combination resulted in the lowest pest numbers, and the lowest plant damage, yet the additive reduction in comparison to the use of each agent alone was not statistically significant.

The parasitoid alone significantly reduced adult density and plant damage compared to the control. We further found that the number of *T. absoluta* adults strongly correlated with the number of *T. absoluta* larvae, suggesting that the adult population reduction results from the larval population reduction. Larval density in the presence of *N. tutae* displayed a cumulative reduction of 14% compared to the control, yet this effect was not significant. A similar semi-field study using the same parasitoid found a significant reduction in larval density.[23] However, with 2.2 times more parasitoids than pests per cage, the parasitoid-pest ratio in the referenced study was much higher than the ratio of 1.2 in ours. Compared to adult numbers, larval numbers displayed higher variance within treatments. According to Cocco et al. (2014),[6] counting mines on the median proportion of the tomato canopy is best suited to provide reliable estimates of the total infestation. We monitored specifically this part of the plant. However, it is possible that high infestation levels may have caused an altered distribution of larvae on the plant and thus masked differences that would be visible otherwise. In addition, *N. tutae* preferably parasitizes larvae in the 2nd to 4th larval stage[27]; therefore, a proportion of healthy larvae has been recorded before parasitism but would not appear in the counts of adults.

While PhopGV doubled the natural mortality of *T. absoluta* larvae in the laboratory significantly,[24] no significant effect of the baculovirus on pest larval and adult density was visible during the first eight weeks of the semi-field experiment. It is common to find less pronounced effects when moving from the laboratory to the less controlled conditions of semi-field and field experiments. Environmental factors such as UV radiation that leads to faster degradation of virus particles and inferior leaf coverage due to spraying larger surfaces may have reduced the effectiveness of the virus treatment. Further, baculoviruses can have a long-term impact on the population that may be observable only later or in the plant damage.[28] In accordance with this, we found a significant reduction in leaf damage in the virus-only treatment. Sublethally affected larvae may have reduced their feeding activity[29] and thus produced fewer and smaller mines than healthy ones. In addition, *T. absoluta* adults were reduced by 50-78% in the third generation in the cages where the virus was applied, suggesting a later effect of the virus.

Our semi-field trial showed that combining both agents tended to result in the strongest pest and damage reduction; however, compared to using both agents alone, the effect was insufficient to be significant. So far, few studies have addressed the combination of entomopathogenic viruses with parasitoids beyond the laboratory scale. In a greenhouse trial, damage to tomato plants by *Lacanobia oleracea* (Lepidoptera: Noctuidae) was reduced by the Lacanobia oleracea baculovirus (LoGV) and the parasitoid *Meteorus gyrator* (Hymenoptera: Braconidae). Similar to our study, the combination of the two agents further reduced damage, but the difference was not significant.[30] When Spodoptera exigua nucleopolyhedrovirus (SeNPV) and *Microplitis pallidipes* (Hymenoptera: Braconidae) were used on cabbage grown in commercial greenhouses, the reduction in *Spodoptera exigua* (Lepidoptera: Noctuidae) population was greater by *M. pallidipes* carrying SeNPV than by parasitoids without virus.[31] In this case, the parasitoid was able to transmit the virus, for example, when it emerged from an infected host.

Timing and sequence have been identified as crucial factors for successfully combining entomopathogens with parasitoids (Koller et al., submitted). Parasitization after virus application was detrimental for *Euplectrus plathypenae* (Hymenoptera: Eulophidae), whereas infesting *Spodoptera exigua* with Spodoptera exigua multiple nucleopolyhedrovirus (SeMNPV) two days post parasitism had no significant effects on the parasitoid.[32] Likewise, *Campoletis sonorensis* (Hymenoptera: Ichneumonidae) survival improved with increasing intervals between parasitism and the infection with the Spodoptera frugiperda multiple-enveloped nucleopolyhedrovirus (SfMNPV) of the host.[33] Since the PhopGV becomes less effective with increasing age of *T. absoluta* larvae[29] and *N. tutae* targets older larvae,[27] both agents would complement each other for pest control best when the virus is applied several days prior to the parasitoid. Previous research has shown that prior parasitism can reduce the pathogenicity of baculoviruses in hosts.[20,34] We ignore whether this aspect has played a role in the limited positive effect of the combination in our study, and further investigation is needed to explore this aspect of the interaction. However, since the number and sex ratio of parasitoids found in the PhopGV-treated plots was similar to that in the plots without the virus at the end of the trial, we can confirm that infection with PhopGV after parasitization has no negative impact on the population of *N. tutae*.[24] Consequently, PhopGV should also be compatible with naturally occurring populations of *N. tutae*, which is a significant advantage for its use in conservation biocontrol due to the wide distribution of this parasitoid.

The semi-field setup of our study allowed for precise application, dosage, and release timing. However, such a controlled environment cannot accurately represent field conditions. Factors such as high temperature, low aeration, and lack of shading screens can significantly impact the growth of plants and the biological cycle of the pest and the parasitoid. Additionally, exposure to UV light, heat, and desiccation may increase the degradation of PhopGV, further affecting the interaction outcome. The infestation was artificially set very high in our experiment and conducted in a single day. Real-world populations of *T. absoluta* are more complex, with individuals immigrating and emigrating, aggregated spatial distribution, and multiple developmental stages present simultaneously.[6] Under those conditions, where precise control may be challenging, combining the two biocontrol agents can offer greater flexibility in the timing and dosage of application. Further research should be conducted to understand better the effectiveness of biocontrol agent combinations in real-world settings and determine whether and how parasitoids can assist in spreading or accelerating the spread of the virus within the pest population.[31]

The difficulties linked with the mass rearing of *N. tutae*[7] may challenge the widespread implementation of this strategy. Combining PhopGV with the larval parasitoid *Dolichogenidea gelechiidivoris* (Marsh) (Hymenoptera: Braconidae), which is also compatible with PhopGV,[24] could be an interesting alternative. The parasitoid has the potential to reach 86% parasitism rate on *T. absoluta* and is easy to mass rear.[35] Moreover, its combined use with the entomopathogenic *Metarhizium anisopliae* in the laboratory resulted in additive control effects despite the reduction of parasitoid fitness by the fungus.[36]

Conclusion

Our results demonstrate the compatibility and the potential of *N. tutae* and PhopGV to reduce population growth and mining damage of *T. absoluta* under semi-field conditions. Further investigations are warranted for optimizing the timing of application and dosage, such as with a modeling framework. The system should be studied under various environmental conditions to advance this research area further, fully understand its potential, and estimate possible benefits under real-world field or greenhouse conditions. Additionally, a cost-benefit assessment of this control strategy could provide valuable information for smallholders, organic growers, and large-scale productions. It would also be essential to study the seasonal and infestation level variations in the efficacy of this control strategy. In summary, while more research is needed to fully evaluate the potential of combining PhopGV and *N. tutae* for *T. absoluta* control, our findings provide interesting insight into an innovative combination of biological control agents.

Authors contributions

JK, JG, JC, LS conceived the study. JK, JG collected the data. JK analysed the data and led the writing of the manuscript. JK, JG, JC wrote the manuscript. LN, JA, JG, JC, LS critically revised the study concept and manuscript. All authors contributed to the writing of the manuscript and gave final approval for its publication.

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Conflict of Interest Declaration

The authors have no conflicts of interest to declare.

Data availability

The data supporting this study's findings can be found at the figshare repository. See https://doi.org/10.5061/dryad.cfxpnvx9x.

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Article 4: Models for the practitioner: Predicting the efficient biocontrol of *Tuta absoluta* under different management scenarios

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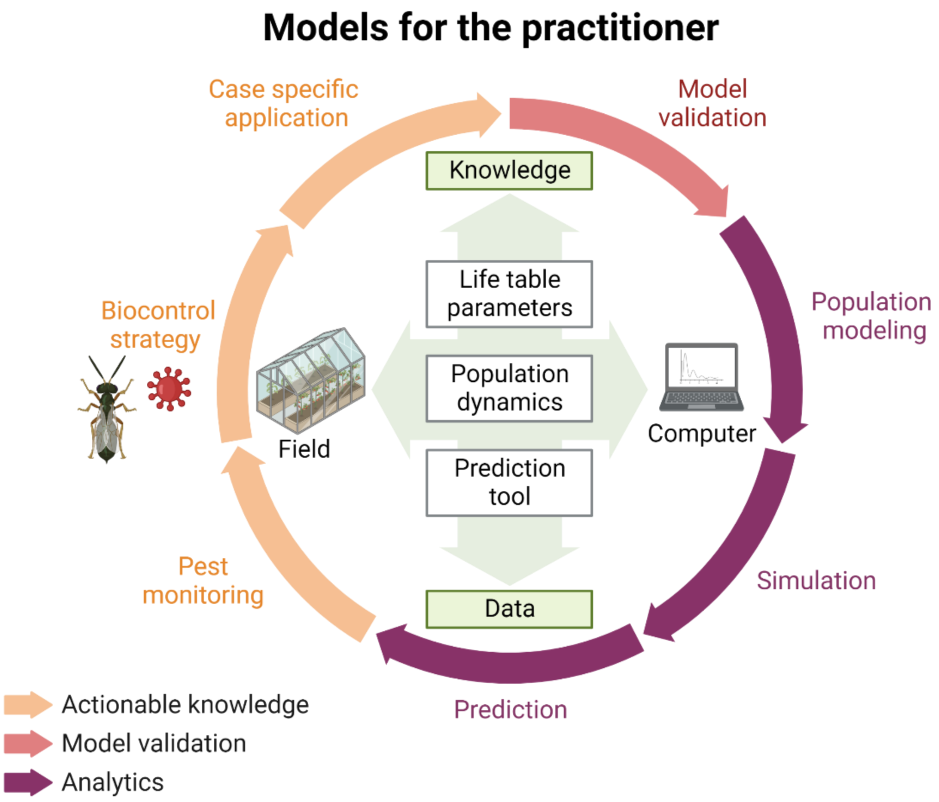

Keywords

Augmentation biocontrol, biocontrol efficiency, population dynamics, release parameters, stage-structured modeling, baculovirus parasitoid combination

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Abstract

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) is one of the most destructive pests of solanaceous crops. One possible biological control strategy is the augmentative release of parasitoids. However, parasitoid intervention efficacy is highly sensitive to the release timing and intensity. Virus-based biopesticides are additional control means since they are highly selective and can be combined with natural enemies. We developed a stage-structured population model, validated with results from a semi-field experiment, to investigate and predict the population dynamics of the pest *T. absoluta* and one of its parasitoids, *Necremnus tutae* Ribes & Bernardo (Hymenoptera: Eulophidae). The aim of the study was to assess the best release parameters of *N. tutae* to control the pest in four different management scenarios: without any other control method, with a biopesticide (PhopGV, Baculoviridae), with the natural presence of the parasitoid, and when combining the natural presence of the parasitoid with a biopesticide. Moreover, in each scenario, two growing seasons of different lengths were compared. To achieve the same control level, 3 to 45% fewer parasitoids are necessary in a long growing season than in a shorter one. Biopesticide applications reduce the number of required parasitoids by 66% and 78%, whereas the natural presence of parasitoids reduces it by 11% and 17% for short and long growing seasons, respectively. On average, with biopesticide application, the parasitoid intervention can be delayed by a month and remains efficient. These findings highlight the importance of mathematical models in applied pest management drawing precise predictions crucial for efficient control.

Introduction

To prevent harmful effects on the environment and human health, as well as the rise of pesticide resistance, agricultural systems must reduce their reliance on synthetic pesticides (Gould et al. 2018; Mahmood et al. 2016; Yadav & Devi, 2017). Moreover, to comply with the European pesticide regulation and the goal to reduce the use and risk of pesticides by 50% by 2030 (EC 2020), alternative management options are required, which have to be reliable and efficient. An alternative to synthetic pesticides is the use of natural enemies to regulate pest populations. From microorganisms to insects, natural enemies are able to control pest outbreaks in natural equilibrium and as commercial solutions (van Lenteren et al. 2018).

Nevertheless, one single agent is frequently unable to control a pest sufficiently. Due to complementary resource use, multiple natural enemies instead of a single species often result in more efficient pest suppression (Cardinale et al., 2003; Mody et al., 2017; Stiling & Cornelissen, 2005). As a viable agronomic solution, biocontrol must be efficient and reliable in many different scenarios. Hence, the main challenges for ecologists, agronomists and farmers are to predict the influence of natural enemies on the pest dynamics, to predict the effectiveness of the combined use of different biocontrol agents, and to optimize their effectiveness via precise dose and timing.

In order to provide better guidelines for biological control practitioners, the system of a worldwide, highly relevant invasive species was used to develop a framework for an optimal release strategy of natural enemies, i.e., the release of a parasitoid complemented with a biopesticide. *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), recently reclassified as "*Phtorimaea absoluta*" (Chang & Metz, 2021), is native to South America and causes catastrophic damage to tomato production in Europe, Africa, and Asia (Biondi et al. 2018; Aigbedion-Atalor et al. 2019; Han et al. 2019). The pest has a high reproductive potential and can cause complete crop failure if no control method is implemented (Desneux et al. 2010). In the most common European growing season (seven months), planting takes place from January to April (from south to north), and the crop is in production until July-October. The long seasons of continuous tomato crop (11 months) are present in northern countries, where glasshouses with heating systems are used (Arnó et al., 2009).

Numerous parasitoids have been found parasitizing *T. absoluta* in its invasive range, often colonizing the crop spontaneously (Arnó et al., 2021). Among them, the European larval parasitoid *Necremnus tutae* Ribes & Bernardo (Hymenoptera: Eulophidae), previously referred to as *N. artynes*, seems to be particularly efficient, reaching natural parasitism rates depending of the region ranging from 18% (Arnó et al., 2021) in N-E Spain to 73% (Crisol-Martínez & van der Blom, 2019) in S-E Spain. Also in augmentative release, the parasitoid significantly reduced *T. absoluta*'s pest density in greenhouse experiments (Calvo et al., 2016, Campos et al., 2020). At least two European companies have produced the parasitoid for augmentative biological control, but its utilization is now shifting to conservation biological control due to an unfavorable cost–benefit ratio (Desneux et al., 2022). Therefore, reducing the number of parasitoids necessary by optimizing the timing of releases with a selective biopesticide could be a promising strategy. Recently, a microbial insecticide (biopesticide) based on *Phthorimaea operculella* granulovirus (PhopGV, Baculoviridae) was developed and tested to control *T. absoluta* (Tutavir©, Andermatt Biocontrol, Switzerland). The product is available in Brazil and has been granted emergency approval in different European countries. Tutavir© is recommended to be applied weekly upon the first detection of the pest. When ingested by neonate larvae of *T. absoluta*, PhopGV induces mortality and, in larger larvae, sublethal effects such as delayed development and pupation failure. Yet, very high doses (weekly application of the highest recommended concentration) are required to lethally infect the larger and most damaging larvae (Larem, 2019) thus complementary measures, such as *N. tutae* targeting the later larval instars of the pest (Calvo et al., 2013), are needed.

In the past, baculoviruses have been used successfully (Beas-Catena et al., 2014) and may replace broad-spectrum synthetic insecticides in the future. Baculoviruses are selective and pose no direct threat to parasitoids (Cory, 2003). Recent laboratory experiments have shown that PhopGV poses no risk for *N. tutae* (Gonthier et al., 2023). Although complementarity and intraguild predation in biocontrol application has been well-studied between parasitoids and predators in tomato (Chailleux et al., 2013; Malo et al., 2012; Moreno-Ripoll et al., 2014; Müller & Brodeur, 2002), few studies have evaluated the efficiency of combining parasitoids with biopesticides such as baculoviruses (Cossentine, 2009). Moreover, no study has ever assessed the impact of parasitoids with biopesticide application on pest population dynamics over an entire growing season. In this study, to understand the range of possible ecological interactions between the pest and the two antagonists, an exploration of the theoretical dynamics of the parasitoid-pest-baculovirus system was conducted.

A stage-structured dynamic population model has been developed to simulate the interaction between antagonists and the pest. The model was calibrated with life table parameters from the published literature and later corrected and validated with data from a large-scale semi-field trial (Koller et al. 2023). The model was developed to respond to the following research objectives: To assess the best timing and intensity of parasitoid intervention for successful augmentative biocontrol of *T. absoluta* with *N. tutae*: **A** as a single control agent (reference scenario, *T. absoluta* + augmentative release); **B** in combination with natural parasitism, the situation in Southern Europe where *N. tutae* is naturally present (*T. absoluta* + augmentative release + natural parasitism); **C** in combination with a biopesticide (*T. absoluta* + augmentative release + PhopGV); and **D** where the biopesticide application is made in areas with *N. tutae* naturally present (*T. absoluta* + augmentative release + natural parasitism + PhopGV). The release parameters and control effectiveness for a short (seven months) and a long growing season (11 months) were compared for all four scenarios.

Material and methods

The stage-structured population model

The stage-structured population model used to describe the population dynamics of *T. absoluta* and its parasitoid *N. tutae* is based on Nisbet & Gurney formulations of delay-differential equations for a continuous-time population dynamics model (Nisbet & Gurney, 1983). These equations are implemented in the R package StagePop (Kettle & Nutter, 2015). The model assumes that an organism begins its life as an egg and progresses through different stages (larvae, pupae, and adult) if it survives long enough. The assumption is that individuals within each stage have identical vital rates, such as death rates and stage duration. Two equations can describe the model:

One equation tracks the change in the number of individuals in each stage over time, accounting for recruitment, development (maturation), and per capita loss rate where the change of the stage variable, i.e., the number of individuals Ni in stage i over time t, is described as:

1) $$dNi(t)/dt=Ri(t)-Di(t)-\delta i(t)Ni(t)$$

Where Ri(t) corresponds to the recruitment into stage i, Di(t) describes the development from stage i, and δi(t) is the per capita loss rate of individuals in stage i.

The second equation accounts for the stage development or maturation, which is determined by recruitment, survival rate, and a time delay factor. Recruitment into the first stage is based on the reproduction rate in the adult stage. The development rate, Di(t), is given by:

$$2) \quad \mathbf{Di(t)=Ri(t-\tau i(t))Pi(t)(1-d\tau i(t)/dt)}$$

where Ri(t) corresponds to the recruitment into stage i, Pi(t) denotes at time t the fraction of individuals entering stage $i-\tau i(t)$ that has survived to time t. Development from one stage corresponds to recruitment into the next stage.

Assigning life history parameters

The life stage durations of *T. absoluta* eggs, larvae, and pupae were based on Campos et al. (2020). The mortality parameters were then adapted to field-realistic values. To do so, the initial model of unconstrained *T. absoluta* population growth was fitted with step-wise increasing the natural mortality from Campos et al. (2020) until the modeled dynamic matched with data from semi-field trials (Koller et al. 2023, see SI first section and Fig S1). For the adults, the reproductive stage duration from Krechemer & Foerster (2015) was used instead of the total lifespan since only reproductive adults should be considered in the model dynamic. The time unit of the model is the day; therefore, the published rate (such as oviposition or mortality), usually determined for an entire stage, had to be adapted to a daily rate. The entire adult lifespan was used for the *N. tutae* adult stage duration since female wasps almost do not cease oviposition during their lifetime (Calvo et al., 2013). *N. tutae* lays more than one egg onto a host larva, on average 1.41 per host (Chailleux et al. 2014). Therefore, to model the number of offspring emerging from a parasitized host larvae, each host parasitized was multiplied by the average number of eggs laid per host so that a single host parasitized produces more than one parasitoid offspring. Previous laboratory experiments at 25°C showed that two PhopGV applications after the emergence of the neonate increased the natural mortality of *T. absoluta* larvae from 24% in the control to 53% in the treated group (Gonthier et al. 2023). Therefore, to simulate the *T. absoluta* population with PhopGV, the natural mortality of *T. absoluta* larvae was increased two-fold. All other parameters were taken at 25°C without modification from the references and are summarized in Table 1.

Table 1. Life table parameters used in the model for the pest *Tuta absoluta* (Lepidoptera: Gelechiidae) and the parasitoid *Necremnus tutae* (Hymenoptera: Eulophidae), with a description of each parameter, the time unit, the absolute value, and the literature reference.

Parameter	Unit	Value	Reference
Stage duration of *T. absoluta* (egg, larvae, pupae, adult)	d	4.8, 12.3, 7.4, 13.9	Campos et al. 2020; Krechemer & Foerster, 2015
Stage duration of *N. tutae* (egg, larvae pupae, adult)	d	1.6, 4, 5.3, 16	Calvo et al. 2013
Number of eggs laid by female *T. absoluta*	eggs/d	10.71	Krechemer & Foerster, 2015
Number of eggs laid by female *N. tutae*	egg/d	2.68	Chailleux et al. 2014

Number of eggs laid by female *N. tutae* per host larvae	egg/host larvae	1.41	Chailleux et al. 2014
Number of host larvae parasitized by female *N. tutae*	host larvae/d	1.9	Chailleux, et al. 2014
Number of host larvae killed by parasitism and host feeding by female *N. tutae*	host larvae/d	3.6	Chailleux, et al. 2014
Death rate of *T. absoluta* per stage (egg, larvae, pupae, adult)	individual rate/d	0.078, 0.036, 0.06, 0.021	Campos et al. 2020; Krechemer & Foerster, 2015; Mohamed et al. 2022
Death rate of *N. tutae* per stage (egg, larvae, pupae, adult)	individual rate/d	0.15, 0.05, 0.007, 0.06	Calvo et al. 2013
Mortality of *T. absoluta* caused by PhopGV biopesticide	individual rate/d	0.072	Gonthier et al. 2023

Modeling parasitoid population and host-interaction

The host species *T. absoluta* (T) has four life stages (eggs, Te; larvae, Tl ; pupae, Tp; adult, Ta). Reproduction of *T. absoluta* is given by Ta(t)* the daily number of eggs laid by a single female. *T. absoluta*, the host, is attacked by the parasitoid *N. tutae* (N). *N. tutae* also has four life stages (eggs, Ne; larvae, Nl; pupae Np; adult, Na).

The life cycle of the parasitoid summarizes as follows: the female lays its eggs on the larvae of *T. absoluta*, then the eggs emerge, and the larvae feed on the host until pupation. Therefore, the reproduction into the parasitoid egg class (parasitism rate) was Na(t)* the daily egg rate of *N. tutae* female. The daily parasitoid reproduction was limited to the number of available host larvae in the system. Only females lay eggs, and since the sex ratio of *T. absoluta* is about 0.5 (Leite et al., 1999) and that of *N. tutae* ranges between 0.2 and 0.7 (depending on the host larval instar, Gonthier et al. 2023), the outcome of the reproduction function for the pest and the parasitoid was divided by two. Not each killed host larvae results in a parasitoid offspring because the parasitoid also performs nonreproductive host killing, mainly via host feeding (Chailleux et al. 2014). Therefore, the daily death rates of *T. absoluta* larvae due to parasitoids were (host-feeding rate + parasitism rate)*Na(t) for each host larvae divided by the number of host larvae present at that moment in the system (Nl(t)).

Simulating management scenarios

Four different management scenarios against *T. absoluta* were considered (Table 2). Scenario A (reference scenario) involves using only one control agent, which is the augmentative release of *N. tutae*. Scenario B combines augmentative release with the natural presence of the parasitoid. Scenario C combines augmentative release with the application of the biopesticide PhopGV. Lastly, scenario D involves applying the biopesticide PhopGV in areas where *N. tutae* is naturally present, while also conducting augmentative release.

In all management scenarios, the colonization of ten adult *T. absoluta* was simulated on day one to mimic the natural colonization of a closed environment (greenhouse) by the pest. The food resource for *T. absoluta* (tomato plants) was assumed to be unlimited. The natural parasitism rate of 18% found by Arnó et al. (2021) was simulated by releasing 1.5 individuals simultaneously with the pest on day one (see Fig. S2 for estimation). All initial scenario parameters can be found in Table 2. To calculate the best timing and number of parasitoids necessary for the effective control of *T. absoluta* in each scenario, all possible combinations for the four different scenarios were run for the following release parameters: a) day of release of *N. tutae* (0-90) and b) an increasing number of released parasitoids (0 to 500 by groups of 10). Each scenario was then compared between a short and a long growing season.

Short growing seasons for tomatoes typically last six or seven months, from spring to fall, as seen in Europe's most common growing regions. In contrast, long growing seasons can last up to 11 months and are mostly present in France and Germany. These longer growing seasons are made possible through glasshouses with heating systems, allowing for transplants in fall or winter and a harvest that lasts until October or November (Arnò et al. 2009).

Table 2 Parameters for each scenario. Four scenarios were modeled to address the outlined objectives and assess the optimal parameters of parasitoid augmentative release: **A** reference scenario; **B** natural parasitism level of about 18%; **C** Biopesticide application at the recommended dose and **D** combination of natural parasitism and biopesticide application. Each scenario was compared between a short growing season of 7 months and a long growing season of 11 months.

	Length of growing season	
Scenarios (management strategies)	**Short (7 months)**	**Long (11 months)**
A) Reference **(*T. absoluta* + augmentative release)**	210 days 10 adult *T. absoluta* on day 1 No natural parasitoids on day 1 Mortality of *T. absoluta* larvae = 0.036	330 days 10 adult *T. absoluta* on day 1 No natural parasitoids on day 1 Mortality of *T. absoluta* larvae = 0.036
B) Natural parasitism **(*T. absoluta* + augmentative release + natural parasitism)**	210 days 10 adult *T. absoluta* on day 1 Ratio of 0.15 parasitoid/pest on day 1 Mortality of *T. absoluta* larvae = 0.036	330 days 10 adult *T. absoluta* on day 1 Ratio of 0.15 parasitoid/pest on day 1 Mortality of *T. absoluta* larvae = 0.036
C) Biopesticide application **(*T. absoluta* + augmentative release + PhopGV)**	210 days 10 adult *T. absoluta* on day 1 No natural parasitoids on day 1 Mortality of *T. absoluta* larvae = 0.072	330 days 10 adult *T. absoluta* on day 1 No natural parasitoids on day 1 Mortality of *T. absoluta* larvae = 0.072
D) Natural parasitism & biopesticide **(*T. absoluta* + augmentative release + natural parasitism + PhopGV)**	210 days 10 adult *T. absoluta* on day 1 Ratio of 0.15 parasitoid/pest on day 1 Mortality of *T. absoluta* larvae = 0.072	330 days 10 adult *T. absoluta* on day 1 Ratio of 0.15 parasitoid/pest on day 1 Mortality of *T. absoluta* larvae = 0.072

The mean runtime of the models was below 3 s. Models with extreme parameter values did not converge within a reasonable time (model running for longer than 300 s); those were interrupted automatically. The code can be found in the Supplementary Information, including all functions to reproduce the presented results. The Supplementary Information also describes the large-scale semi-field assay from Koller et al. (2023) and the model validation (Fig. S3).

Determining control effectiveness

The density over time of *T. absoluta* adults was used as an indicator for control success. Adult density over time is a good proxy for the pest pressure because the simulations with positive slopes resulted in a high density of larvae far beyond any economic threshold. For each model output, the slope of the *T. absoluta* adult density over the entire period was calculated. When the net slope of the adult population (over the total observation time) was negative or neutral, the pest population was defined as under control, and the augmentative release was considered effective (Fig. 1a+b). Conversely, the pest was not under control when the slope was positive, and thus the augmentative release was insufficient (Fig. 1c). All the simulations simplified into binary positive-negative results were plotted for the parasitoid release day and the number of parasitoids released.

Results

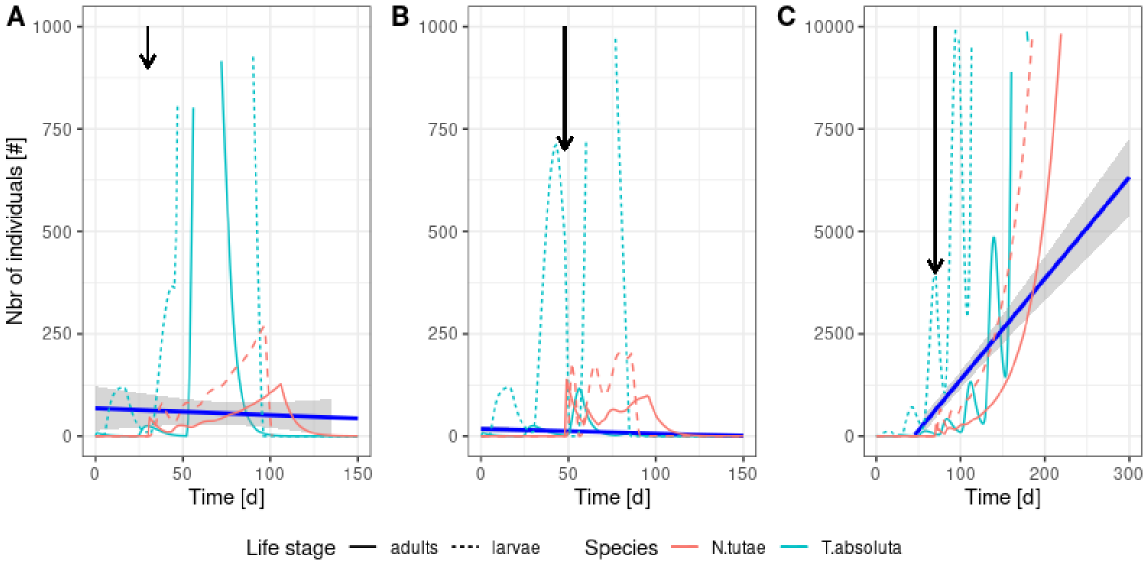

Figure 1 Population dynamic of *Tuta absoluta* (light blue) and its parasitoid *Necremnus tutae* (red) under different release parameters. The black arrows show the parasitoid release timing (arrow length is proportional to intervention intensity), and the dark blue line represents the linear slope of the adult pest density over time. **A** Release of 50 parasitoids on day 30 - the pest is under control (neutral or negative slope for the solid blue line); **B** Release of 150 parasitoids on day 50 - the pest is under control; **C** Release of 300 parasitoids on day 70 - the pest is not under control (positive slope for the solid blue line). NB axis limit of **A** and **B** = 150 d and 1'000 individuals whereas **C** runs up to 300 d and 10'000 individuals.

Running the model for the different scenarios with the different release parameters provided 18'564 model outputs. Three are shown in Fig. 1 as examples of situations in which control is achieved (Fig. 1A & B) or not (Fig. 1C) for the reference scenario when the only control method is the release of the parasitoid. When the parasitoids are released into the system early on day 30 (Fig. 1A), a small intervention of 50 *N. tutae* (five times more than the initial inoculum of *T. absoluta*) is sufficient to control the pest in the third generation. If the parasitoid is released later on day 50 (Fig.

1B), the intervention with the parasitoids must be tripled (150 *N. tutae*) to be successful. However, if released later on day 70 (Fig. 1C), even 300 *N. tutae* cannot control the pest, and the number of *T. absoluta* grows exponentially. For a better understanding, the arbitrarily chosen examples from Fig. 1 are also marked in Fig. 2A, where all model outputs are represented.

Optimizing the parasitoid release

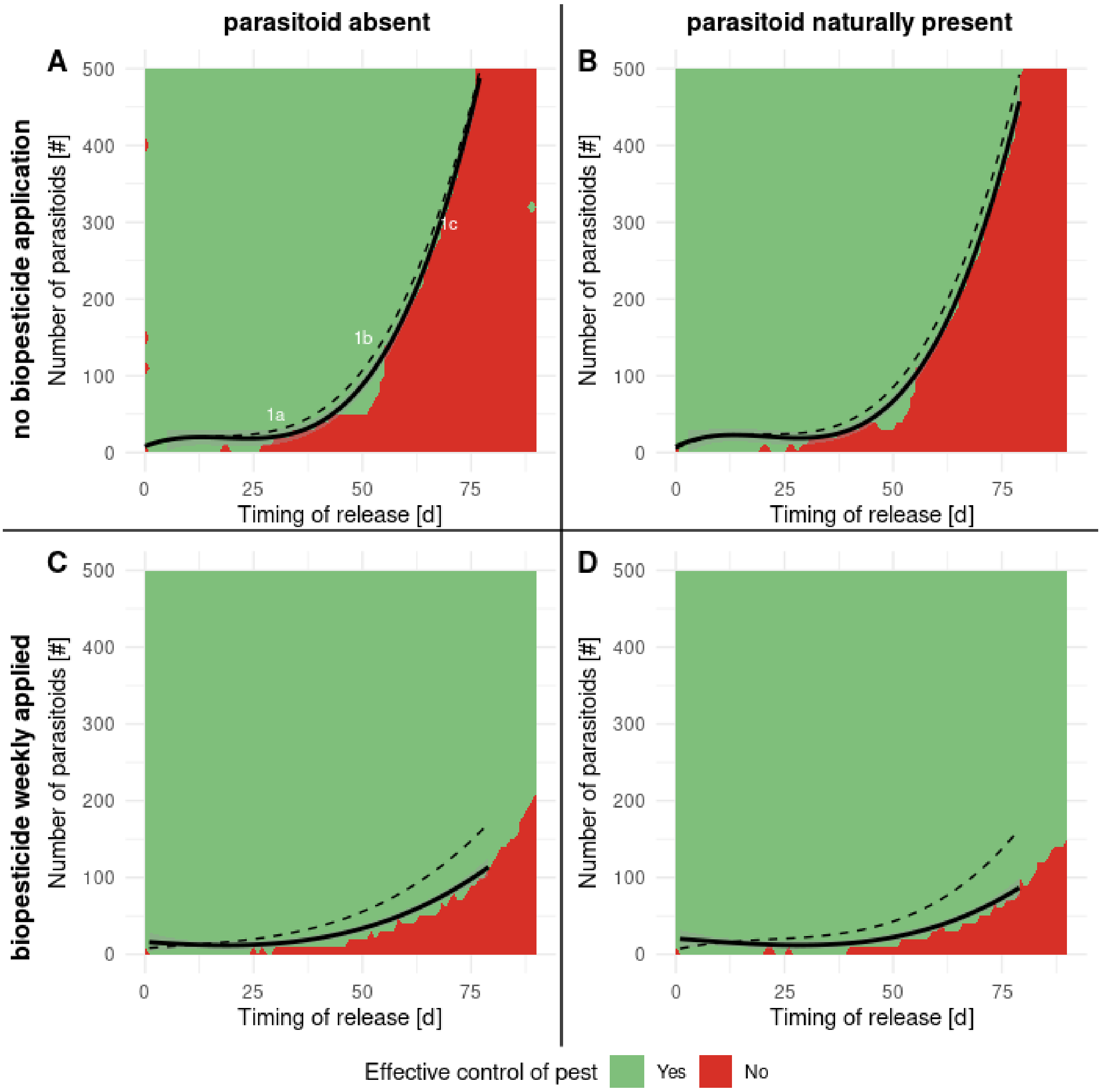

Figure 2 The outcome of pest control: successful (green: negative or neutral slope of *Tuta absoluta* adults over the growing season) or pest outbreak (red: positive slope of adults over the growing season, the exponential growth of the pest) in relationship with the intervention parameters of the parasitoid (intensity, i.e., number on the Y-axis) and timing of the intervention (ranging from 0 to 90 days on the X-axis). The solid line shows the polynomial regression of the threshold line (separation of green and red) for the long growing season and the dashed line for the short growing season. **A** Reference scenario with no intervention other than the parasitoid release. 1a, 1b, and 1c represent the three examples plotted in Figure 1. The dynamic of the pest outbreak, when no parasitoids are released (no control method), is displayed at 0 on the Y-axes. **B** Situation with the parasitoid being naturally present (southern Europe). **C** Combination of the intervention with PhopGV biopesticide and **D** PhopGV biopesticide applications in locations where the parasitoid is naturally present.

Polynomial regression analysis estimated well the border relationships between areas with effective control (green, Fig. 2) and areas where the pest is out of control (red, Fig. 2). The best release parameters for parasitoid intervention vary sensibly depending on the simulated scenarios. Overall, few parasitoids (less than 25) are sufficient to keep the pest population under control until the beginning of the second pest generation around day 25 (Table 3). Later, the curves become steeper, and on day 50, when the third generation starts in the reference (scenario A), more than 400 parasitoids must be released for effective control of the initial pest infestation of ten individuals (Table 3). After day 75, the polynomial function reaches a steep slope with no control possible anymore (Fig. 2).

Table 3. The minimum number of parasitoids necessary for efficient augmentative release in each scenario at day 25 (start of the second pest generation) and 75 (start of the fourth pest generation). Polynomial functions are shown with the correlation coefficient (r). Intervention strength shows, on day 75, the percentage of parasitoids necessary for efficient control in each scenario relative to the reference scenario. Reduction long *vs.* short shows for each scenario how much fewer parasitoids are necessary for the long growing season on day 75.

Scenarios	**Growing season**	**Function**	**r**	**Minimum number**		**Intervention strength***	**Reduction long *vs.* short**
				Day 25	**Day 75**		
A) reference	**Short**	$8.33 + 1.88*x - 0.102*x^2 + 0.00208*x^3$	0.98	24.1	**453.1**	100%	3%
	Long	$8.22 + 2.21*x - 0.132*x^2 + 0.00239*x^3$	0.99	18.3	**439.7**	100%	
B) natural parasitism	**Short**	$5.07 + 2.80*x - 0.139*x^2 + 0.00230*x^3$	0.98	24.1	**403.5**	89%	9%
	Long	$7.77 + 2.67*x - 0.147*x^2 + 0.00234*x^3$	0.99	19.2	**368.3**	83%	
C) PhopGV biopesticide	**Short**	$8.78 + 0.650*x - 0.0129*x^2 + 0.000394*x^3$	0.90	23.1	**151.2**	33%	33%
	Long	$15.8 - 0.0922*x - 0.00235*x^2 + 0.00025*x^3$	0.93	15.9	**101.1**	22%	
D) natural parasitism & PhopGV biopesticide	**Short**	$7.46 + 1.42*x - 0.0486*x^2 + 0.000710*x^3$	0.87	23.7	**140.1**	31%	45%
	Long	$19.2 - 0.00894*x - 0.0131*x^2 + 0.000312*x^3$	0.86	15.6	**76.5**	17%	

*Relative percentage of the effort in comparison to the reference scenario on day 75

The length of the growing season matters

A difference in the number of parasitoids necessary between the short and long growing seasons was only visible in the scenarios with the biopesticide **C** and **D**. More parasitoids were necessary to control the pest in the short growing season than in the long one. On day 75 (the start of the fourth pest generation), in scenarios with biopesticide applications **C** and **D**, up to 33% and 45% fewer parasitoids were necessary, respectively, for efficient control in the long than in the short season (Table 3). In scenarios A and B, similar quantities of parasitoids achieve control in the short and long seasons (reduction of 3% and 9%, respectively; Table 3, Fig. 2).

Natural parasitism and biopesticide effectiveness

The presence of natural parasitism in scenario **B** slightly reduces the number of parasitoids necessary for efficient control compared to the reference scenario **A** (Table 3). In the short growing season 89%, and in the long one 83% of parasitoids are necessary for efficient control at day 75. Compared to the reference scenario, scenario C with biopesticide substantially reduces the number of parasitoids necessary to 33% and 22%, respectively. Adding the natural presence of parasitoids to the biopesticide in scenario **D** reduces the number of parasitoids necessary for efficient augmentative release to only 31% and 17%, respectively, compared to the reference scenario.

Discussion

Mathematical models for population dynamics can help to determine the optimal timing and dosage for augmentative releases of natural enemies (Pfab et al. 2018; Rubio et al. 2022; Shea & Possingham, 2000). The work presented here complements this research and compares diverse scenarios such as different lengths of the growing season for the first time and the combination with a virus-based biopesticide. It also considers the parasitoid population naturally present in the field. The findings suggest that the efficiency of a biological control strategy against *T. absoluta* by augmentation of the larval parasitoid *N. tutae* strongly depends on the timing of the release. According to the models, the optimal release time lies during the first pest generation. Applying the PhopGV biopesticide strongly reduces the number of parasitoids necessary for efficient augmentative release and permits to delay the parasitoid intervention. The following sections discuss some fundamental assumptions and their possible implications.

Determining the optimal timing of a biocontrol intervention

A crop protection intervention's timing is crucial, particularly when working with natural enemies. The release should be early enough for parasitoids to build up a considerable population and keep up with the pest but also late enough to ensure the presence of a minimum number of hosts in suitable stages for parasitoid establishment. The models show that during the first pest generation, the number of parasitoids necessary for efficient control was low and relatively constant, making it the best window of opportunity. Early detection is crucial, mainly if no other control method is applied. In the case of late detection, even a strong parasitoid intervention cannot help reducing the pest population. For example, when waiting with an intervention up to the fourth generation, even the highest effort cannot save the crop (Fig. 2A). Yet releasing parasitoids too early when no hosts are present is counterproductive. Studies have shown that preventative parasitoid releases are less effective than curative ones (Lopes et al. 2009). Theoretically, the parasitoid *N. tutae* should be released six days after crop invasion by its host since they do not accept first-instar larvae (Calvo et al. 2013), and the time to develop from an egg into second-instar larvae of *T. absoluta* at 25°C takes approximately six days (Campos et al. 2020). The first small red marks on day one in Fig. 2 show that slightly more parasitoids are necessary in the first days for effective control than a few days later because no host larvae are present. Nevertheless, experience shows that detection in practice is imperfect and that detection one week after the arrival of the first adults is almost impossible. Therefore, upon detection of the first adult pest, the parasitoid will likely find enough host larvae already.

In this context, biopesticide application and natural parasitism allow more time before parasitoid intervention (Fig. 2). For the same release rate still being efficient - the intervention with *N. tutae* can be delayed, strongly with biopesticide application and slightly with natural parasitism. The biopesticide slows down the pest population build-up by killing some of the larvae. Although the biopesticide alone cannot control the pest population completely, it can be combined with natural enemies. Therefore, applying the biopesticide might be a good strategy when the initial detection probability of the pest is low.

Even though it is generally recommended that parasitoids be released multiple times during the crop season for optimal synchronization with the pest, here we show that one single, well-placed intervention with the parasitoid *N. tutae* can effectively control *T. absoluta*. Since the larval stage of *T. absoluta* is long (about 12 days at 25°C, Campos et al. 2020), the egg and pupal stages are short; there are only a few days without hosts available for the parasitoids. The first red peaks of Fig. 2 around day 18 show that slightly more parasitoids are necessary for effective control when the host larvae of the second generation are not present. However, releasing a few more individuals can compensate for this incorrect synchronization with the pest. A single, well-placed release of parasitoids reduces efforts for the growers compared to multiple releases.

The length of the growing season matters

A long growing season generally has a higher pest increase potential than shorter ones because if no control measures are applied, pest populations can have more generations and, thus, eventually reach the economic threshold. Here, the models show that when combined with biopesticide applications, fewer parasitoids could achieve the same control in the long versus short growing season if release is scheduled correctly and controls the first generation. By increasing the natural mortality of the pest larvae, the biopesticide allows the parasitoid population to keep up and eventually control it in the long growing season. In the short growing season, this seems to play out less likely, because the parasitoids do not have enough time to reach a sufficient density for pest control. However, it is important to note that in some cases, control happens late, and even though the pest population eventually comes under control, the damage might already be too severe for the growers.

Model limitations

The models are highly suitable for detecting long-term changes in populations, nevertheless, several important limitations need to be considered. The current model only examines the dynamics at a fixed temperature of 25°C. This temperature was selected because most published life cycle data are available at this temperature and it is very close to the average greenhouse temperature in Koller et al. (2023). In all cases, laboratory growth experiments conducted over different but constant temperatures suggest that higher or lower temperatures would similarly accelerate or slow down the dynamics of the pest and the parasitoid. For instance, to complete its entire life cycle, *N. tutae* needs twice as many days at 20°C than at 30°C (25 vs. 12.75 days, Calvo et al. 2013). Similarly, *T. absoluta* needs about double the time at 20°C compared to 30°C (34.8 vs. 18.3 days, Krechemer & Foerster, 2015). Fluctuating temperatures could be integrated into future studies, which would imply defining a function involving time-varying stage durations.

The model simulated colonization by a small pest inoculum early in the season. Yet, it did not include immigration and the emigration of pests or parasitoids. Nevertheless, as both species' populations increase rapidly after colonization, immigration of a few individuals later in the season will likely have a weak effect on the already large population. Moreover, greenhouses limit the number of individuals migrating and, thus, are less sensitive to dispersion than open fields. As a consequence, it is possible that the findings would be slightly different in open-field production systems. The model was based on and validated with data from protected crops, and future work should assess if the findings also apply to field conditions.

One goal of the study was to estimate the density that the pest can reach when not under control. Therefore, the model did not consider a carrying capacity for the pest. Greenhouses can span up to several hectares with two to three plants per m^2. Thus, the enormous quantity of food can be considered ad libitum, to some extent.

For reasons of simplicity, the environment was assumed to be homogeneous and affect all individuals in the same way. The model neglected the complex ecological web in which both species are embedded. Often, tomato greenhouses harbour polyphagous predators and other pests, such as whiteflies (Moreno-Ripoll et al. 2014). Such a multi-species system can have numerous effects on the populations and possible control efforts. A natural progression of this work would be the integration of mirid predators into the equation since they play a significant role in controlling *T. absoluta* (Desneux et al. 2022).

Extension of the model framework to other pests and biocontrol agents

Besides *N. tutae*, other parasitoids are known to attack *T. absoluta*, including *Dolichogenidae gelechiidivoris* Marsh (Hymenoptera: Braconidae), which is newly present in the invaded areas and originates from the same area as *T. absoluta* (Denis et al. 2022; Krache et al. 2021; Salas Gervassio et al. 2019). Traditional approaches such as life table assessment remain fundamental for model developments. If the life table parameters are known, the modeling framework could be easily adapted to other larval parasitoids, as long as they have similar environmental needs. Host searching behavior varies greatly between species and would be a key parameter to integrate into future parasitoid-host interaction models via the functional response. For instance, the *N. tutae* host searching capacity is higher at low host density (type two functional response; Bodino et al. 2019) than the capacity of *D. gelechiidivoris* (type three functional response; Mama Sambo et al. 2022).

Likewise, the findings can be transferred directly to other microbial insecticides, such as *Bacillus thuringiensis* (Bt), as long as they do not affect parasitoid fitness, or if they do, their effect could be included in the model, for example, as higher parasitoid mortality or lower fertility.

Conclusions

This applied study can promote sustainable agriculture and reduce pesticide use by improving the efficiency of a biocontrol strategy. Models can help to predict the outcomes of new biocontrol combinations precisely. We showed here that the timing of intervention is crucial; therefore, investing in monitoring to find the right moment for intervention is key to combining both agents efficiently. Based on the model predictions, a tool could be developed to offer practical advice and assist greenhouse tomato producers, such as proposed by Rincon et al. (2023). Finally, the models could be easily adapted to other pest and parasitoid species - for example, to tackle new challenges like the brown marmorated stink bug *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) and *Trissolcus japonicus* (Ashmead) (Hymenoptera, Scelionidae) in greenhouse vegetable production or well-known pest problems like whiteflies and *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae). Including the relative costs of each strategy to choose the best alternative in terms of cost-efficacy would be the next logical step.

Authors' contributions

J.G., J.C. and L.S. designed the research. J.G. and L.S. developed the model and interpreted the data. J.G. and L.S. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

All parameters, codes, and functions to reproduce the results not contained in the manuscript can be found in the Supplementary Information.

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Supplementary information

Parameterizing life history parameters

When parametrizing exploratory models, the mortality rate appeared too low compared to data from the field, which is the consequence of such laboratory assays presenting optimal conditions. Therefore, all mortality rates of *Tuta absoluta* were adapted to field-realistic parameters. The initial model of unconstrained *T. absoluta* population growth was fitted with step-wise increasing mortality parameters. The adult's density over time was correlated with data from a field experiment (Koller et al. 2023), and the correction factor from the model with the highest correlation coefficient (0.83) was applied to all the stages of *T. absoluta* (correction factor = 3.0 Fig. 1 SI). With this correction, the dynamic of adult *T. absoluta* abundance was highly correlated with the adult dynamic found in the greenhouse trials (Koller et al. 2023). The complete raw data from this trial can be found at the Dryad repository. See (DOI: https://doi.org/10.5061/dryad.cfxpnvx9x.)

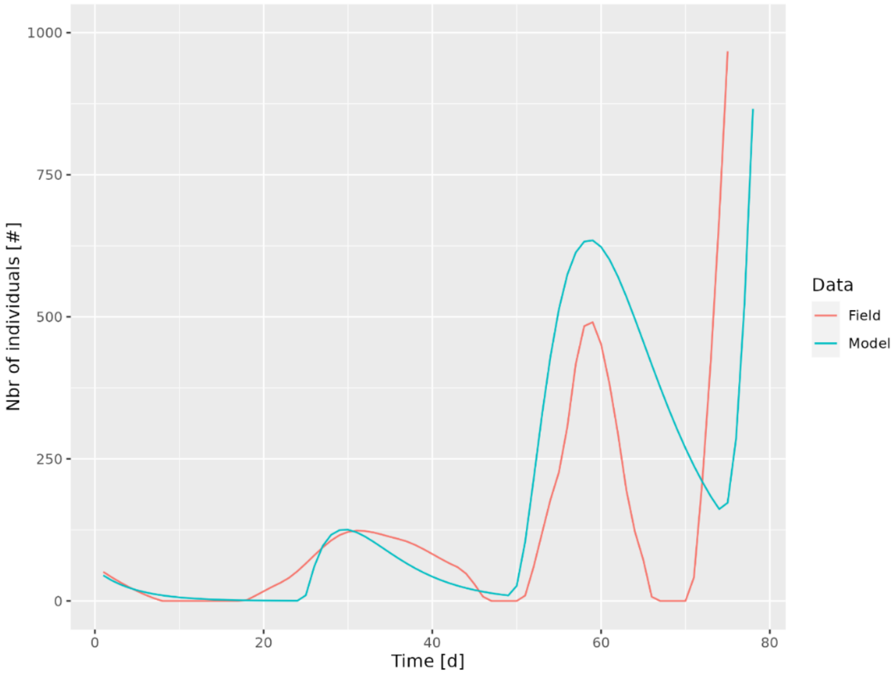

Figure S1 Dynamic of adult *Tuta absoluta* over three generations. The red kinetic show the mean of adult density in different cages collected during weekly monitoring in semi-field experiments conducted by Koller et al. (2023). The blue line shows the model prediction for the same initial conditions, i.e., 50 adults release and food ad libitum. The correlation coefficient between both is 0.83. The mortality rate for adult *T. absoluta* from published laboratory experiment was increased threefold.

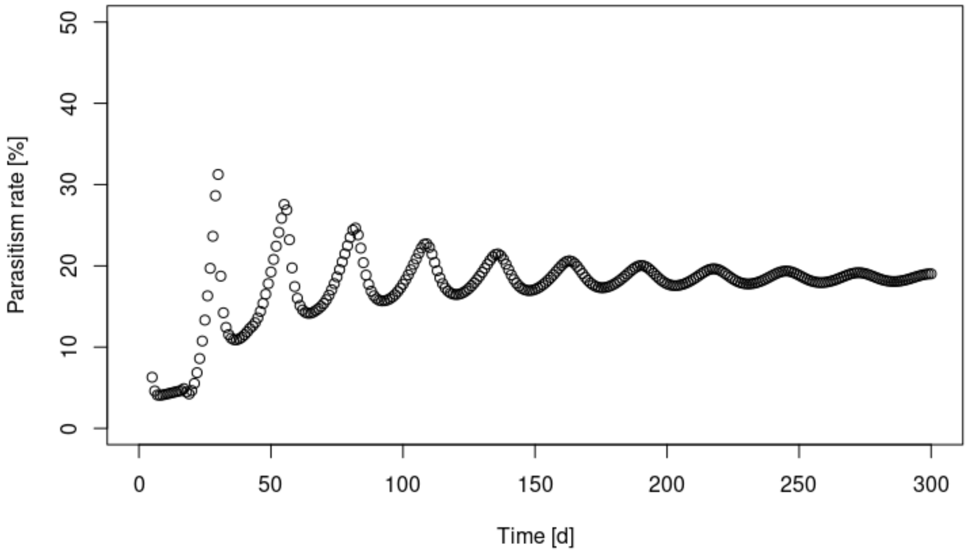

Figure S2 Simulation of 18% natural parasitism with the release of 1.5 adult parasitoids on day 1 for ten adults *Tuta absoluta*. The dots show the natural parasitism over 300 days; X-axis represent the day; Y-axis represent the ratio of *T. absoluta* parasitized.

Semi-field experiment and model validation

The data in Koller et al. (unpublished results) were collected from a semi-field study in Switzerland during the summer growing seasons 2021. Four treatments with seven replicates were set up in a randomized block design in two greenhouses using walk-in (2 x 2 x 2 m), thrips-proof cages: 1) *T. absoluta* only as control; 2) *T. absoluta* and parasitoids (*N. tutae*); 3) *T. absoluta* and baculovirus (PhopGV); 4) *T. absoluta*, parasitoids, and baculovirus. Adult *T. absoluta* were released in each cage, which contained tomato plants. In the parasitoid and the combined treatment, parasitoids were released. In the virus and the combined treatment, the virus was sprayed on the plant in a water solution. Every week for three generations, *T. absoluta* larvae and adults were monitored.

Comparing the observation of this semi-field experiment with the model predictions gives confidence that the model is highly realistic (Fig. S3). The models precisely predict the dynamic of *T. absoluta* in a closed environment, such as a tomato production greenhouse, with and without biopesticide application (PhopGV, baculovirus). On an absolute scale, the predicted abundance is slightly underestimated. However, the increase rate of the population dynamic is similar, which is the most important for predictions. For instance, when the measured abundance is double, the predicted abundance doubles too. Moreover, the model is realistic enough since it reproduces well at the start of the yearly T. absoluta outbreak (Fig. S1), which is, according to our results, the most critical event for timing the parasitoid intervention.

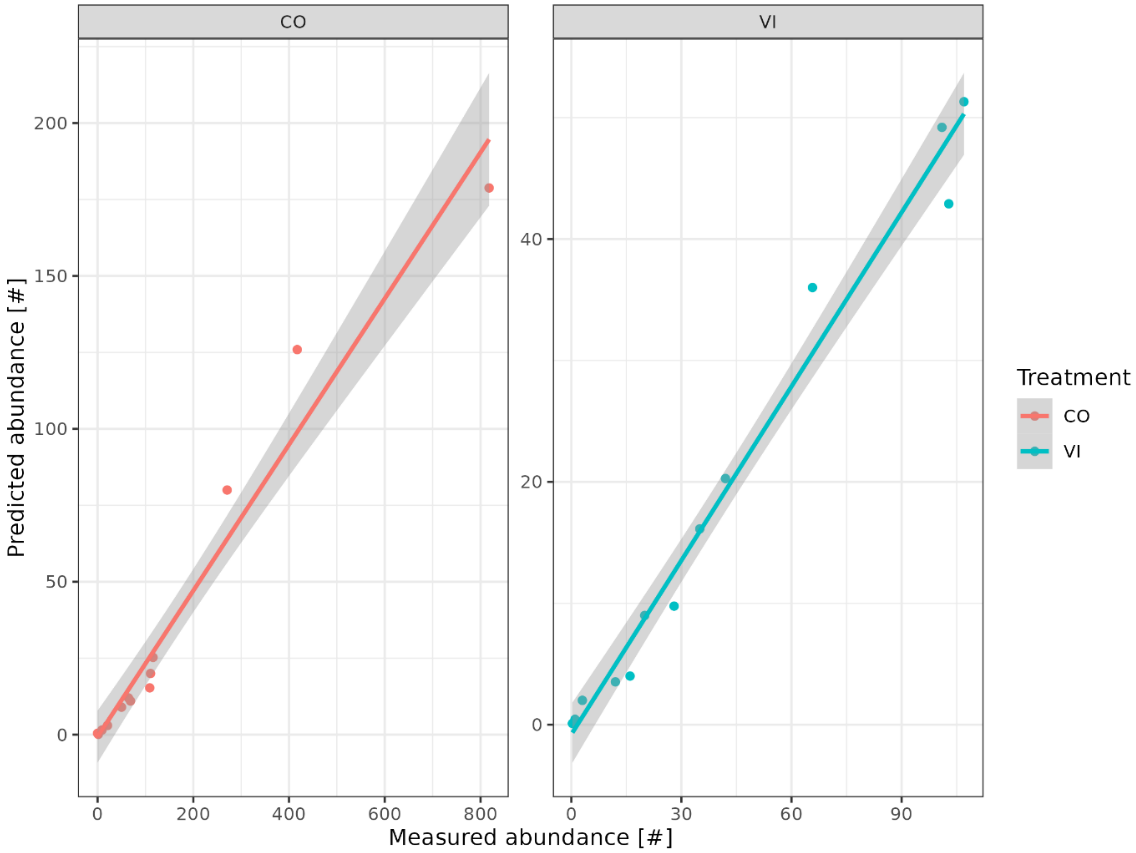

Figure S3 Measured Tuta absoluta adult abundance in the semi-field experiment and predicted abundance by the model under similar initial conditions (no release of parasitoid). Each dot represents the mean *T. absoluta* adult abundance of cages on a weekly measurement from Koller et al. (2023). The linear regression lines and the 95% confidence interval in grey are represented. In red is the reference scenario without biopesticide application application (CO = control); in blue the scenario with biopesticide application (VI = baculovirus, PhopGV). Adjusted R-squared: 0.9619.

R code and functions

```
# stagePop Function ----------------------------------------
rm(list = ls())
stagePop_all_var <- function(LarvDeat= 0.15/12.3*3, # 0.036
                             TutaArri = 0,
                             TutaNumb=10,
                             NecrArri=0,
                             NecrNumb=0,
                             timelength=10,
                             NecrArriNat = 0,
                             NecrNumbNat =0) {
  require(stagePop)
  require(tidyverse)

  solver.options=list(DDEsolver='deSolve',atol=1e-3,rtol=1e-3,hbsize=1e7)

  # Define rate functions----------------------------------------
  qT = 10.71 # Daily number of eggs laid by female T. absoluta. lifetime fecundity = 149 / (reproductive stage duration
= 11.6 + Pre-oviposition period = 2.6) (Krechemer & Foerster, 2015).
  ovipositionRateN = 1.9*1.41 # Daily number of eggs laid by female N. tutae    (Chailleux, Desneux, et al., 2014).
  ParazitizedRateN = 1.9 # Daily number of host larvae parasitized by female N. tutae  (Chailleux, Desneux, et al., 2014
).
  HostCarryCapacity = 1.41 # Daily Number of eggs laid by female N. tutae per host larvae (Chailleux, Desneux, et al.,
2014).
  HostKillingRateN =3.6 # Daily number of host larvae killed by parasitism and host feeding by female N. tutae (Chaill
eux, Desneux, et al., 2014).
```

```
 #All the vectors are specified in the order of the life cycle, e.g. start with eggs and finish with reproducing adults
 # Daily death rate of T. absoluta per stage (egg, larvae, pupae, adult). Mortality rate / stage duration.
 deathE = 0.125/4.8*3.0 # (Campos et al., 2020 / Mohamed et al., 2022)*Mortality correction
 deathL = LarvDeat # (Campos et al., 2020 / Mohamed et al., 2022)*Mortality correction
 deathP = 0.15/7.4*3.0 # (Campos et al., 2020 / Mohamed et al., 2022)*Mortality correction
 deathA = 1/13.9*3.0 # (Krechemer & Foerster, 2015 / Mohamed et al., 2022)*Mortality correction

 # Daily death rate of N. tutae per stage (egg, larvae, pupae, adult). Mortality rate / stage duration.
 deathEN = 0.243/1.6 # (Calvo et al., 2013)
 deathLN = 0.198/4 # (Calvo et al., 2013)
 deathPN = 0.041/5.3 # (Calvo et al., 2013)
 deathAN = 1/16 # (Calvo et al., 2013)

 TutaNecremnusFunctions <- list(

   reproFunc=function(x,time,species,strain){
     if (species==1){reprod=qT*(0.5*x$Tuta['adults',1])}
     if(species==2){reprod=
       ifelse(ParazitizedRateN*0.5*x$Necremnus['adults',1] > x$Tuta['larvae',1],
              (ParazitizedRateN*0.5*x$Necremnus['adults',1]-(ParazitizedRateN*0.5*x$Necremnus['adults',1]-x$Tuta['larv
ae',1]))*HostCarryCapacity, # Carrying capacity of parasitoid, the number of parasitized larvae is limited by the max n
umber of T.absoluta larvae present in the system = Parazitized Rate* number of parasitoid present - ( parazitized rate -
number of larvae present) * number of egg laid per larvae
              (ParazitizedRateN*0.5*x$Necremnus['adults',1])*HostCarryCapacity)}
     return(max(0,reprod))},

   deathFunc=function(stage,x,time,species,strain){
     if (species==1){
       if (species==1){a=c(deathE,deathL,deathP,deathA);v=a[stage]
       if (stage==2){v=a[stage]+HostKillingRateN*0.5*x$Necremnus['adults',1]/(x$Tuta['larvae',1]+1)}}} #0.5 = hostfe
eding only by females
     if (species==2){v=c(deathEN, deathLN, deathPN, deathAN)}
     return(max(0,v))},

   durationFunc=function(stage,x,time,species,strain){
     if (species==1){a=c(4.8,12.3,7.37,13.9)}
     if (species==2){a=c(1.6,4,5.3,16)}
     return(a[stage])},

   immigrationFunc=function(stage,x,time,species,strain){v=0
   if(species==1){if(time>=TutaArri & time<=TutaArri+1) if(stage==4){v=TutaNumb}}
   if(species==2){if(time>=NecrArri & time<=NecrArri+1) if(stage==4){v=NecrNumb}}
   if(species==2){if(time>=NecrArriNat & time<=NecrArriNat+1) if(stage==4){v=NecrNumbNat}}
   return(v)},

   emigrationFunc=function(stage,x,time,species,strain){return(0)})

 # Run model----------------------------------------

 modelOutput = popModel(
   numSpecies=2,
   numStages=c(4,4),
   ICs=list(matrix(0,nrow=4,ncol=1),matrix(0,nrow=4,ncol=1)),
   timeVec=seq(0,timelength,1),
   timeDependLoss=c(TRUE,FALSE),
   timeDependDuration=c(FALSE,FALSE),
   ntol = 0.001,
   rateFunctions=TutaNecremnusFunctions,
   solverOptions=solver.options,
   stageNames=list(c('eggs','larvae','pupae','adults'),c('eggs','larvae','pupae','adults')),
   speciesNames=c('Tuta','Necremnus'),
   saveFig=FALSE,
   figType='png',
   plotFigs=F,)


 modelOutput<- as.data.frame(modelOutput) %>%
```

```
    dplyr::select(1:9) # only select abundance columns

  return(modelOutput)}

# Function to interrupt automatically models running for longer than customisable time period.

interruptor = function(x1=1,x2=1,x3=1,x4=1,x5=1,x6=1,x7=1,x8=1,time.limit=10) {
  results<- NULL
  results<- withTimeout ({stagePop_all_var(x1,x2,x3,x4,x5,x6,x7,x8) } , timeout=time.limit ,onTimeout="silent" )
  if(is.null(results) ) {
    results<- rep(NA,8)}
  return(results)}

# Cluster computation with time limitation ----------------------------------------
library(tidyverse)
library(R.utils)
library(parallel)

LarvDeat=   c(0.03658537,0.07317073)# natural mortality = 0.15/12.3*3, virus mortality doubles natural mortality = (
0.15*2)/12.3*3)
TutaArri =  0 # colonization of T. absoluta  simulated on day zero
TutaNumb=   10 # colonization of ten adult T. absoluta simulated
NecrArri=   seq(0,90,by=1) # Timing of the parasitoid intervention (ranging from 0 to 90 d              ays)
NecrNumb=   seq(0,500,by=10) # Number of parasitoids released (intensity ranging from 0 to 500 by group of 10 indiv
iduals)
timelength= 330 # Duration of long growing season of 11 months (11*30).
NecrArriNat=0 # colonization of natural parasitoid simulated on day zero
NecrNumbNat=c(1.5,0) # to achieve natural parasitism rate

ex.df<-expand.grid(LarvDeat=LarvDeat,
                   TutaArri=TutaArri,
                   TutaNumb=TutaNumb,
                   NecrArri= NecrArri,
                   NecrNumb=NecrNumb,
                   timelength=timelength,
                   NecrArriNat=NecrArriNat,
                   NecrNumbNat= NecrNumbNat)

clus <- makeCluster(14)
clusterExport(clus,list( "interruptor" , "stagePop_all_var" , "withTimeout"))

modelOut<- parRapply(clus,
                     ex.df,
                     function(x) interruptor(x[1],x[2],x[3],x[4],x[5],x[6],x[7],x[8],time.limit = 300))
stopCluster(clus)

modelOut<- [[length(rownames(ex.df))+1]]<- ex.df
save(asd, file = "data/modelOut/scenario_330_300_18564_lowD.RData")
```

Chapter III: Trophic connections in a new parasitoid community

Dolichogenidae gelechiidivoris, picture M. Waldburger

Article 5: Insight into the host-specificity of a native and a newly introduced parasitoid of *Tuta absoluta* and prospect for biological control

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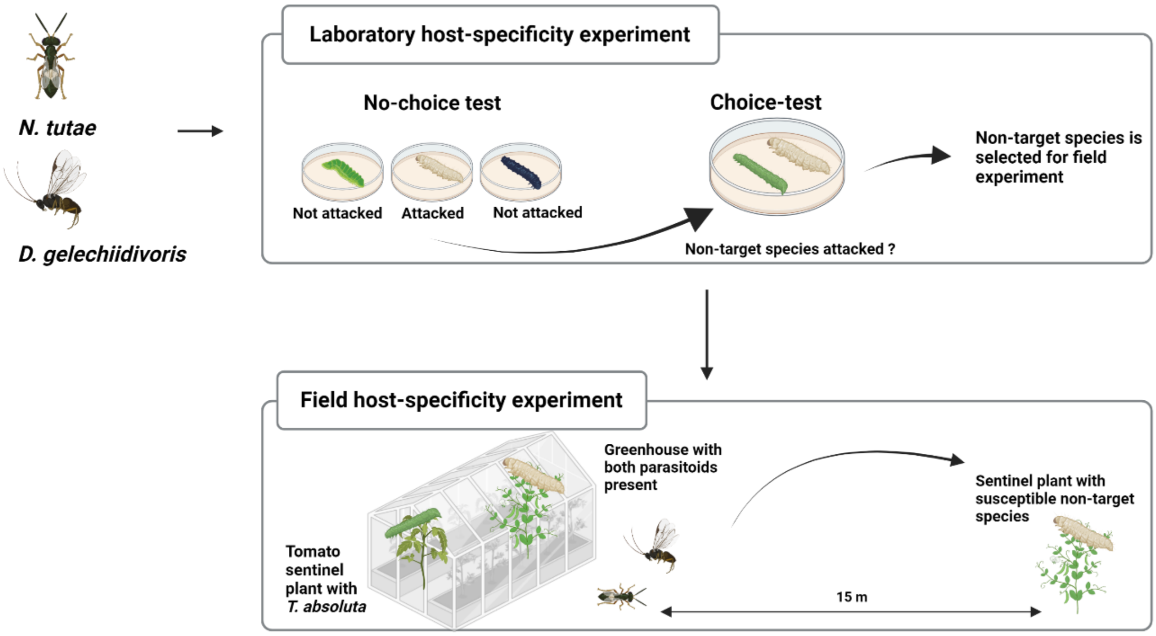

Keywords

Host-range, accidental introduction, sentinel plant, classical biological control; host-parasitoid community; environmental risk assessment

Based on: Gonthier J, Arnó J, Romeis J, Collatz J. Insight into the host-specificity of a native and a newly introduced parasitoid of *Tuta absoluta* and prospect for biological control. *In prep*.

Abstract

New strategies are urgently needed to control *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), one of the most destructive pests of tomato crops. Originating from South America, it is spreading rapidly and has evolved resistance to most common insecticides making it highly challenging to control. Regarding biological control, the parasitoid *Necremnus tutae* Ribes & Bernardo (Hymenoptera: Eulophidae) is widely present around the Mediterranean basin and has adapted to the invader playing a substantial role in its control. A classical biological control program with the neotropical parasitoid *Dolichogenidea gelechiidivoris* Marsh (Hymenoptera: Braconidae) is ongoing in Africa. In addition, this parasitoid has established accidentally following its host in Spain and Algeria. Understanding the trophic connections between *D. gelechiidivoris, N. tutae*, the targeted host *T. absoluta* and potential non-target hosts is essential to assess their value as augmentative or classical biological control agents and to promote their establishment in conservation biocontrol. We conducted host-specificity testing in the laboratory using four European leafminer species to evaluate the specificity of these two parasitoids to *T. absoluta*. We then assessed their affinity for *T. absoluta* by exposing sentinel plants with the alternative host *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) inside and around greenhouses and measuring each parasitoid species' field parasitism rate. Our results show that of the four non-target species tested in the laboratory, *N. tutae* attacked three, while *D. gelechiidivoris* attacked only *P. operculella*. In the greenhouse, *N. tutae* did not prefer *P. operculella* or *T. absoluta,* whereas *D. gelechiidivoris* preferred the latter. The mean parasitism rate of *T. absoluta* over the three months monitoring period in the greenhouses reached 27% for *N. tutae* and 35% for *D. gelechiidivoris*. We discuss the importance of host affinity for understanding potential non-target effects and the value a natural enemy can have as biological control.

Introduction

Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae), recently reinstated as "*Phtorimaea absoluta*" (Chang and Metz 2021), is a serious threat to worldwide tomato production (Campos et al. 2017; Desneux et al. 2011, 2010; Han et al. 2019; Mansour et al. 2018). *Tuta absoluta* is very difficult to control due to the cryptic behavior of its larvae, its increasing resistance to synthetic insecticides, and its high reproduction rate (Guedes et al. 2019; Sylla et al. 2019). Outside of South America, its endemic region (EPPO; https://gd.eppo.int), the pest has already spread to more than 100 countries, including all Mediterranean countries. In 2017, *T. absoluta* reached China, the worldwide biggest tomato producer causing severe damage (Zhang et al. 2020, 2021). North America and the Oceanian continent are at high risk of being invaded.

In the Mediterranean region, *Necremnus tutae* Ribes & Bernardo (Hymenoptera: Eulophidae) is among the most abundant larval parasitoids found to attack *T. absoluta* (Gabarra et al. 2014; Zappalà et al. 2013). It shows an average parasitism rate of about 18% in tomato crops (Arnó et al. 2021) that can reach 75–80% in some cases (Giorgini et al. 2019 and references within). To date, *N. tutae* is promoted in Europe only through conservation biocontrol methods (Desneux et al. 2022). The native hosts of *N. tutae* are unknown because this species (formerly reported as *N.* sp. nr. *artynes*) has only recently been described (Gebiola et al. 2015). The species appears to be polyphagous, targeting lepidopteran leafminer species and even some coleopteran species, although the species is reported to parasitize only host larvae encased in mines (Chailleux et al. 2014). In 2015, *N. tutae* was found parasitizing the almond bark beetle *Scolytus amygdali* Geurin-Meneville (Coleoptera: Scolytinae) in Tunisia (Zeiri et al. 2015). *Cosmopterix pulchrimella* Chambers (Lepidoptera: Cosmopterigidae), a leafminer of upright pellitory, is the third host reported to be parasitized by *N. tutae* (Bodino et al. 2016). Moreover, the closely related *N. artynes*, often mistaken for *N. tutae* in previous surveys on *T. absoluta* parasitoids (Gebiola et al. 2015), has a wide host range. Besides *T. absoluta*, *N. artynes* is also reported on *Aproaerema anthyllidella* Hübner and *Vulcaniella pomposella* (Zeller) (Lepidoptera: Cosmopterigidae).

Recently, another larval parasitoid of *T. absoluta*, the braconid *Dolichogenidea gelechiidivoris* Marsh (Hymenoptera: Braconidae) (Syn.: *Apanteles gelechiidivoris* Marsh), has been reported from the Mediterranean, i.e., Spain and Algeria (Denis et al. 2022; Krache et al. 2021). Native to Colombia, Chile, and Peru (Fernandez-Triana et al. 2020; Yu et al. 2016), it is considered the most important parasitoid for natural and augmentative biological control in Colombian tomato crops (Bajonero & Parra 2017). The host range of *D. gelechiidivoris* seems confined to lepidopteran hosts belonging to the Gelechiidae family and related to solanaceous plants (Salas Gervassio et al. 2019). Although no risk assessment study has been published to date, *D. gelechiidivoris* has been widely used in classical biocontrol programs against *T. absoluta* in Easter Island, Kenya, Uganda, and Ethiopia, as well as against *Phthorimaea operculella* (Zeller) in North America and *Keiferia lycopersicella* (both Lepidoptera: Gelechiidae) in Hawaii, (Ripa et al. 1995, Desneux et al. 2010; Aigbedion-Atalor et al. 2020; Aigbedion-Atalor et al. 2021, Nakao and Funasaki 1979).

With the ongoing spread of *T. absoluta*, both parasitoids have entered the focus of attention. They could be fostered for their control function in areas where they are already present or introduced as classical biological control agents in countries where they are not present. Assessing the host-specificity of these parasitoids is the first step to evaluating potential environmental risks resulting from their introduction. High host-specificity enhances the parasitoid's efficiency in finding and attacking the target pest species (Kimberling 2004; Rossinelli and Bacher 2015) and reduces the risk of ecological disturbance, such as reducing populations of non-target species (McEvoy 1996; Collatz et al. 2021). Moreover, studying the network ecology in the field could help understand trophic interactions in both the native and the invaded ranges, complement laboratory-based host-specificity tests, and provide insights into potential interactions of biological control agents. Therefore, we evaluated the specificity for *T. absoluta* of both parasitoids, first in the laboratory and then in the field. We **(1)** assessed whether parasitism behavior is restricted to leafminers; (**2)** assessed host-specificity in the laboratory with different leafminer species; **(3)** evaluated the natural parasitism rate in different Spanish greenhouses, on *T. absoluta* on commercially grown tomato and on tomato and potato sentinel plants infested with *T. absoluta* and *P. operculella*, respectively; and **(4)** surveyed the parasitoid complex/community on other leafminer species around the greenhouses.

Material and methods

Insect and plant material

The laboratory experiments were conducted at Agroscope in Switzerland. All experiments and insect rearing were conducted in climate chambers at 25 ± 1°C, 70 ± 10% RH, and a 16:8 h L:D photoperiod.

The rearing of *T. absoluta* was established with individuals provided by Andermatt Biocontrol, Switzerland. Adult moths were kept in mesh cages and provided cotton soaked in honey water (10% v/v) placed on the top of the cage and tomato plants (*Solanum lycopersicum* cv. Rentita) for egg-laying. After seven days, plants with eggs and young larvae were moved to another cage to start a new generation. Additional plant material was added regularly.

Colonies of the parasitoids *N. tutae* and *D. gelechiidivoris* were initiated with individuals collected in commercial tomato fields in El Maresme county (Barcelona, Spain). Adults were kept in mesh cages (50 × 50 × 50 cm) (bug dorm, MegaView Science Co., Ltd., Taiwan) and provided cotton soaked in honey-water (10% v/v) and tomato plants with *T. absoluta* second to third instar larvae for parasitism. After emergence, adult parasitoids were collected and stored at 12°C with honey water. Every ten days, a new parasitoid generation was started. All female parasitoids used in the experiments were naive, mated (stored with males for at least two days), and less than a week old.

Four species of leafminers were selected as non-target species: i) The potato tuber moth *P. operculella* as a closely related species from the same clade as *T. absoluta* (Chang and Metz 2021), ii) the horse chestnut leafminer, *Cameraria ohridella* Deschka & Dimic (Lepidoptera: Gracillariidae) and iii) the pear blister leaf miner *Leucoptera malifoliella* Costa (Lepidoptera: Lyonetiidae) as lepidopteran leafminers from two different families than *T. absoluta*, and iii) *Liriomyza bryoniae* (Kaltenbach) (Diptera: Agromyzidae) as a leafminer from another order but feeding on tomato.

Rearing of *P. operculella* was established with individuals provided by Andermatt Biocontrol, rearing of *L. malifoliella* and *L. bryoniae* with individuals collected in a commercial apple orchard and a tomato field, respectively, in the Zurich region. After emergence, adults were kept in mesh cages and provided cotton soaked in honey water placed on the top of the cage. The species were provided potato tubers (cv. Red pontiac), apple branches (cv. Jonagold), and tomato plants (cv. Rentita), respectively, for egg-laying. After seven days, plants with mines were moved to another cage to start a new colony. Because of the short life of cut horse chestnut branches, it was impossible to rear *C. ohridella*; therefore, leaves with mines were collected in the Berne region in Switzerland and used directly for the experiment after checking that those larvae were alive.

Preliminary experiments

Experiments were conducted to evaluate if *D. gelechiidivoris* restricts its parasitism behavior to hosts inside mines and to confirm this observation for *N. tutae,* which has previously been reported (Chailleux et al. 2014). For each parasitoid species, four treatments with only the target host *T. absoluta* were conducted: **A** Two larvae of *T. absoluta* within their mine in the presence of a female parasitoid (positive control); **B** Two larvae outside of the mine in the presence of a female parasitoid (test); **C** Two larvae within their mine without parasitoid (mortality control inside the mines); **D** Two larvae outside the mine without parasitoid (mortality control outside the mines).

The setup consisted of a Petri dish (10 cm dia.) covered with a mesh with a wet cotton disc on the bottom to keep the leaf humid and a droplet of honey as a food source for the parasitoid. Per Petri-dish, two larvae were offered to single female parasitoids for 36 hours. Third instar larvae were used for *N. tutae* and second instar larvae for *D. gelechiidivoris*, corresponding to the parasitoid's preferred host instar (Aigbedion-Atalor et al. 2020; Calvo et al. 2013). For treatments **A** and **C**, larvae collected with a fine brush were placed on tomato leaflets (5 ± 2 cm in length, freshly cut) to allow time to burrow into a mine 24 h prior to each experiment; for treatments **B** and **D,** larvae were collected just prior to the experiment.

Every mine was opened under a stereomicroscope three days later, and each larva was categorized as dead or alive. For *N. tutae*, ectoparasitism was assessed three days later, and for *D. gelechiidivoris*, parasitism was assessed 12 days later, when the parasitoid larvae left the host for pupation. An approximation of the number of larvae host-killed, described as nonreproductive host mortality induced by host feeding and host killing (Zhang et al. 2022), was calculated by subtracting the natural mortality found without parasitoid (treatment **C** and **D)** from the total dead larvae found with parasitoid (treatment **A** and **B)**.

Host-specificity testing in laboratory

Necremnus tutae

In a no-choice test, single female parasitoids were offered non-target host larvae of *L. bryniae, L. malifoliella*, *C. ohridella,* and *P. operculella* or larvae of *T. absoluta* as control. As in the preliminary experiment, both treatments were repeated without parasitoids to control for natural mortality, resulting in four treatments: **A** Target host *T. absoluta* with the addition of a female parasitoid (positive control); **B** Non-target host with the addition of a parasitoid (test); **C** Target host *T. absoluta* without parasitoid (mortality control with target host); **D** Non-target host without parasitoid (mortality control with the non-target host). The same process and methods were used as in the preliminary experiment. Per Petr-dish, two larvae (third instar *T. absoluta* larvae or larvae of the alternative host corresponding in size) were offered to single females for 36 hours. Leaves with mines containing larvae were selected and cut (1-2 cm^2).

Dolichogenidae gelechiidivoris

Because *D. gelechiidivoris* is an endoparasitoid, the parasitism can be assessed only after 12 days when the parasitoid immature leaves the host larva for pupation. Since it was impossible to keep the pieces of cut leaves containing the larvae in a good state for longer than a few days, behavioral observations were conducted for this parasitoid. Single parasitoid females were offered two mines of the alternative host in a no-choice setup. Leaves with mines were collected and cut (1-2 cm^2) to select mines containing larvae similar in size to the second instar *T. absoluta* larvae. Per Petri-dish, two pieces of leaf with second instar *T. absoluta* larvae or alternative hosts were offered at an equal distance to single females. Subsequently, each female was offered a leaf with about five *T. absoluta* mines for one minute to assess its responsiveness. Observations of females not responsive during this time were discarded.

When females stung the alternative host, behavioral observations were repeated in a choice setup. Per Petri dish, two larvae of each host were offered, totaling four larvae per dish. Observations in no-choice and choice tests were noted using Observer XT11 (Noldus, Netherlands). Behaviors were recorded for seven minutes and categorized as cleaning, resting, walking on the Petri dish, antennating/searching the leaf (all: continuous behaviors), and stinging (event). Due to the noticeable attraction of female *D. gelechiidivoris* reared on *T. absoluta* on tomato plants for *P. operculella* in the choice setup, observations were repeated with females reared on *P. operculella* on potato plants.

Field experiment

The field experiments were conducted in and around three commercial tomato greenhouses in El Maresme and La Selva counties (Northeast of Spain), and samples were evaluated at IRTA's Research Center (Barcelona, Spain). The greenhouses were located within a 10 km radius distance and less than 3 km from each other (see Fig. S1). Location A (41°40'16" N, 2°46'00" E) was a 1'600 m^2 low-cost tunnel, completely open at the sides (up to 3 m height) surrounded by arable land (see Fig. S2). Location B (41°41'48" N, 2°46'30' E) was a 600 m^2 semi-closed tunnel with meshes on the side wall (allowing insects to move in and out) surrounded by a forest on one side and other greenhouses on the other side (see Fig. S3). Location C (41°37'22" N, 2°39'12" E) was a 1'136 m^2 greenhouse completely closed beside the roof opening and surrounded by similar greenhouses (see

Fig. S4). Wild herbaceous vegetation occurred around each greenhouse. The greenhouses were selected based on the presence of *T. absoluta* and both parasitoids.

Host preference

Infested tomato and potato sentinel plants were placed inside and around the three greenhouses to assess the preference of both parasitoids for *T. absoluta* and *P. operculella*. Sentinel plants were planted in pots (15 cm dia., 20 cm height) and were used when they were about 30 cm high with at least five fully developed leaves. Before the experiment, plants were offered for ten days to newly emerged adult moths (10 per plant, sex ratio 0.5) to obtain infestation with L1 to L3 instar larvae at the beginning of the experiment. *T. absoluta* and *P. operculella* were obtained from an established laboratory colony that originated from field-sampled adults from El Maresme County and were reared as described above. Adult moths were fed with honey water during the entire infestation period. Sentinel plants were then covered with a mesh (mesh size 0.4 mm) to avoid predation by adult mirids and larger animals, and the pots were sprayed with insect glue (Soveurode®, Plantin SARL) to avoid predation by soil-dwelling insects (see Fig. S5 of sentinel plant).

Three consecutive runs were conducted at two weeks intervals between September and November 2022 (starting on 29.09, 20.10, or 10.11), during which the plants stayed for four days in each location. Twenty-one sentinel plants were placed in each location: seven tomato and eight potato plants inside and nine potato plants outside the greenhouses. Sentinel plants inside were placed 15 m or more from each other and were distributed equally. The potato plants outside were placed to assess the preference when the target host, *T. absoluta*, was not close. They were placed at five to 15 m distance to the greenhouse wall and 15 m or more from each other (see Fig. S3, S4, S5).

After each run, each sentinel plant was individualized in a plastic container (20 cm dia., 30 cm height) covered with a mesh and incubated at 22°C in the lab until moths or parasitoids emerged. Additional plant material was added when needed (sentinel plant completely consumed). After emergence, all insects were aspirated for counting. The parasitism rate for each parasitoid was assessed as follows:

$$\text{For } D.\ gelechiidivoris = \frac{\text{Total number of } D.gelechiidivoris \text{ emerging}}{\text{Total number of insects emerging}}$$

$$\text{For } N.tutae = \left(\frac{\text{Total number of } N.tutae \text{ emerging}}{1.41^{1}}\right) / \text{ Total number of insects emerging}$$

In addition, mines of *T. absoluta* were collected during each run to evaluate the natural parasitism rate inside the greenhouse. Three persons collected mines for 20 minutes or up to 150 mines.

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[1] Mean number of egg laid per host larvae (Chailleux et al. 2014)

Survey of non-target species in the surroundings

Three persons searched for 20 minutes the surrounding (up to 100 m) of the greenhouse to collect any leafminer species. All samples were individualized and incubated at 22°C in the lab until the leafminers or parasitoids emerged.

Statistical analysis

The software NCSS (2023) (NCSS, LLC, US) was used for statistical analysis of the laboratory experiments. Data were tested for normal distribution using the Shapiro–Wilk's test, and visual inspections were made using Q–Q plots. The data were mostly not normally distributed (Shapiro–Wilk's test, $P < 0.05$). To compare the ratio of the four categories (Parasitized, Host-killed, Dead, and Alive) between hosts inside and outside of mines (experiment 2.1.1) and target and non-target hosts (2.1.2), a $\chi 2$ test on contingency tables (2×4) was used. A Mann- Whitney U-test was used to compare the time spent searching on mines of different non-target hosts under no-choice conditions, whereas a Wilcoxon signed-rank test was used to compare the searching time between target and target non-target host in the choice setup. The proportion of females stinging the mines was compared with a $\chi 2$ test (experiment 2.2).

For experiment 2.3.1, all computations were carried out with the R 4.2.2 software (R Development Core Team 2022). Firstly, scatter plots and Pearson's correlation coefficients were used to assess possible relationships between pest density in the greenhouses and parasitism rate on sentinel plants. Differences in parasitism rate between sentinel plants were then assessed with a generalized linear mixed-effects model (glmm) with binominal data distribution (parasitism yes/no) and Month*Greenhouse as a random effect in all models to take into account pseudoreplication (3 greenhouses x 3 months = 9 replicates). We tested all models for evidence of overdispersion (based on the ratio of residual deviance to residual degrees of freedom being close to 1, as in (Bolker et al. 2009). Since we had relatively large counts (>5) in all groups, it was justified to use glmmPQL to account for overdispersion (Bolker et al. 2009). Therefore, we refitted overdispersed models using penalized quasi-likelihood (function glmmPQL from R package MASS). Two different models were run for each parasitoid. **A)** Experiment with potato plants infested with *P. operculella* and tomatoes infested with *T. absoluta* inside the greenhouse. The influence of host type on the parasitism rate was evaluated with the host (*T. absoluta*/*P. operculella*) as a fixed effect. **B)** Experiment with potato plants infested with *P. operculella* inside and outside the greenhouse. The influence of the sentinel plant location on the parasitism rate was evaluated with location (inside/outside) as a fixed effect. Finally, the natural parasitism rate of *T. absoluta* in the greenhouses was evaluated with a generalized linear model where the months were set as a fixed factor.

Results

Preliminary experiments: Are parasitoids attacking larvae outside the mines?

In the control group, where host larvae were offered within the mines, females of *N. tutae* and *D. gelechiidivoris* each parasitized 17.5% of the *T. absoluta* larvae. However, when offered outside of the leaf, no larva was parasitized by either parasitoid species. Nonreproductive host-killing was found for both parasitoids, regardless of whether the larvae were inside or outside the mines. For *N. tutae*, however, a significantly higher percentage of larvae were host-killed when they were in the mines, 47.5% *vs.* outside the mines, 22.5% ($X^2 = 13.2$, $df = 2$, $P = 0.001$). *D. gelechiidivoris* host-killed the same number, 32.5% inside or outside the mines.

Host-specificity testing in laboratory

Necremnus tutae

The proportions of larvae parasitized, host-killed, alive and dead of the non-target hosts *L. bryoniae*, *L. malifoliella,* and *C. ohridella* differed significantly from those of the target host *T. absoluta* (Chi-square test; all: $\chi 2 < 21.6$, $df = 3$, $P < 0.001$; Fig. 1). No significant difference was detected for *P. operculella* ($\chi 2 = 6.71$, $df = 3$, $P = 0.082$). None of the female *N. tutae* parasitized the dipteran leafminer *L. bryoniae*. Conversely, females parasitized all three non-target lepidopteran species, ranging from 5% for *L. malifoliella* to 7% for *P. operculella* and 16% for *C. ohridella* (see Fig S6). The parasitism rate did not significantly differ from the target host *T. absoluta* (6 to 10%) except for *C. ohridella,* where parasitism was significantly higher ($\chi^2 = 30.45$, df = 3, $P = 0.001$). A high host-killing rate ranging from 42.5 to 46% was found in all the control groups with *T. absoluta,* for the non-target host *L. bryoniae* and *P. operculella,* a lower host-killing rate was witnessed at 5% and 35%, respectively. No host-killing was found for *C. ohridella* and *L. malifoliella*.

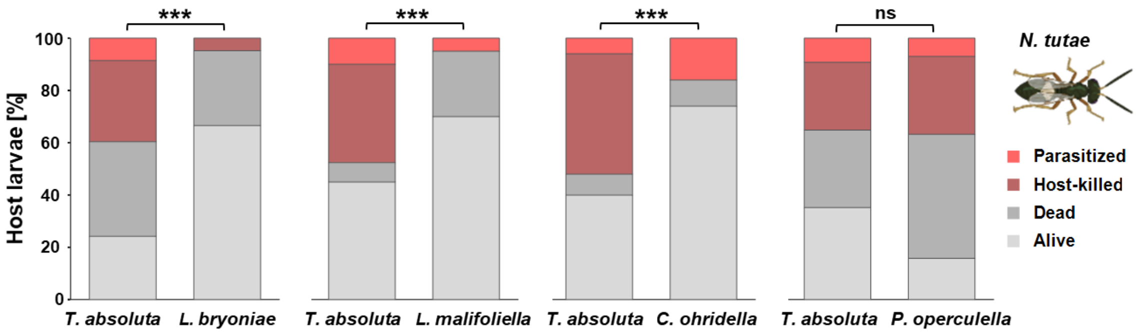


Figure 1. Result of no-choice experiment with the parasitoid *Necremnus tutae* and from left to right the different non-target hosts (right bars) *Liryomiza bryoniae*, *Leucoptera malifoliella*, *Cameraria ohridella*, and *Phtorimaea operculella.* The target host *Tuta absoluta* was used as a control in each no-choice assay (left bars). An approximation of the number of host-killed larvae was calculated by subtracting the calculated natural mortality of the control from the total dead larvae found in the treatment. The ratio of the four categories (Parasitized, Host-killed, Dead, and Alive) between target and non-target hosts was compared with a $\chi 2$ test on contingency tables (2 × 4); ns $P > 0.05$; *** $P < 0.001$.

Dolichogenidae gelechiidivoris

The time spent searching by female *D. gelechiidivoris* in no-choice tests on mines of the non-target hosts was low, ranging from 12.5 ± 12.53 (mean seconds ± SE) for *L. bryoniae* to 65.2 ± 19.13 for *C. ohridella* (Fig. 2A). Females spent significantly more time searching the mines of *C. ohridella* than those of *L. malifoliella* ($U = 206$, $P = 0.001$) and *L. bryoniae* ($U = 121.5$, $P = 0.001$). No differences were found between the times spent searching the mines of *L. bryoniae* and *L. malifoliella* ($U = 293$, $P = 0.602$; Mann–Whitney U-test). None of the females stung larvae of the dipteran leafminer *L. bryoniae*. However, a small proportion of female parasitoids stung *L. malifoliella* (3.3%) and even more *C. ohridella* (12.5%) (see Video S1). Since all females stung *T. absoluta* mines during the observation, no individuals were rejected as non-responsive.

When given a choice, females spent significantly more time searching the mines of the target host *T. absoluta* than the mines of *L. malifoliella* or *C. ohridella* (Wilcoxon signed-rank test; both: $Z > 3.84$, $P < 0.001$; Fig. 2B). Even though significantly more females (≥ 93.3%) stung the mines of *T. absoluta* (Chi-square test; $\chi^2 > 42$, $df = 1$, $P < 0.001$), a proportion of females stung the mines of *L. malifoliella* (3.3%) and *C. ohridella* (15.4%).

Female *D. gelechiidivoris* reared from *T. absoluta* spent similar time searching the mines of *P. operculella* and *T. absoluta* (Wilcoxon signed-rank test; $Z > 1.11$, $P < 0.264$), and those females did not show any stinging preference (Chi-square test; $\chi^2 = 1.06$, $df = 1$, $P > 0.302$). When reared on *P. operculella* for one generation, females spent significantly more time searching the mines of *P. operculella* (Wilcoxon signed-rank test; $Z > 3.81$, $P < 0.001$), but more females (56.6%) stung the larvae of *T. absoluta* (Chi-square test; $\chi^2 = 5.55$, $df = 1$, $P = 0.018$) than *P. operculella* (26.6%).

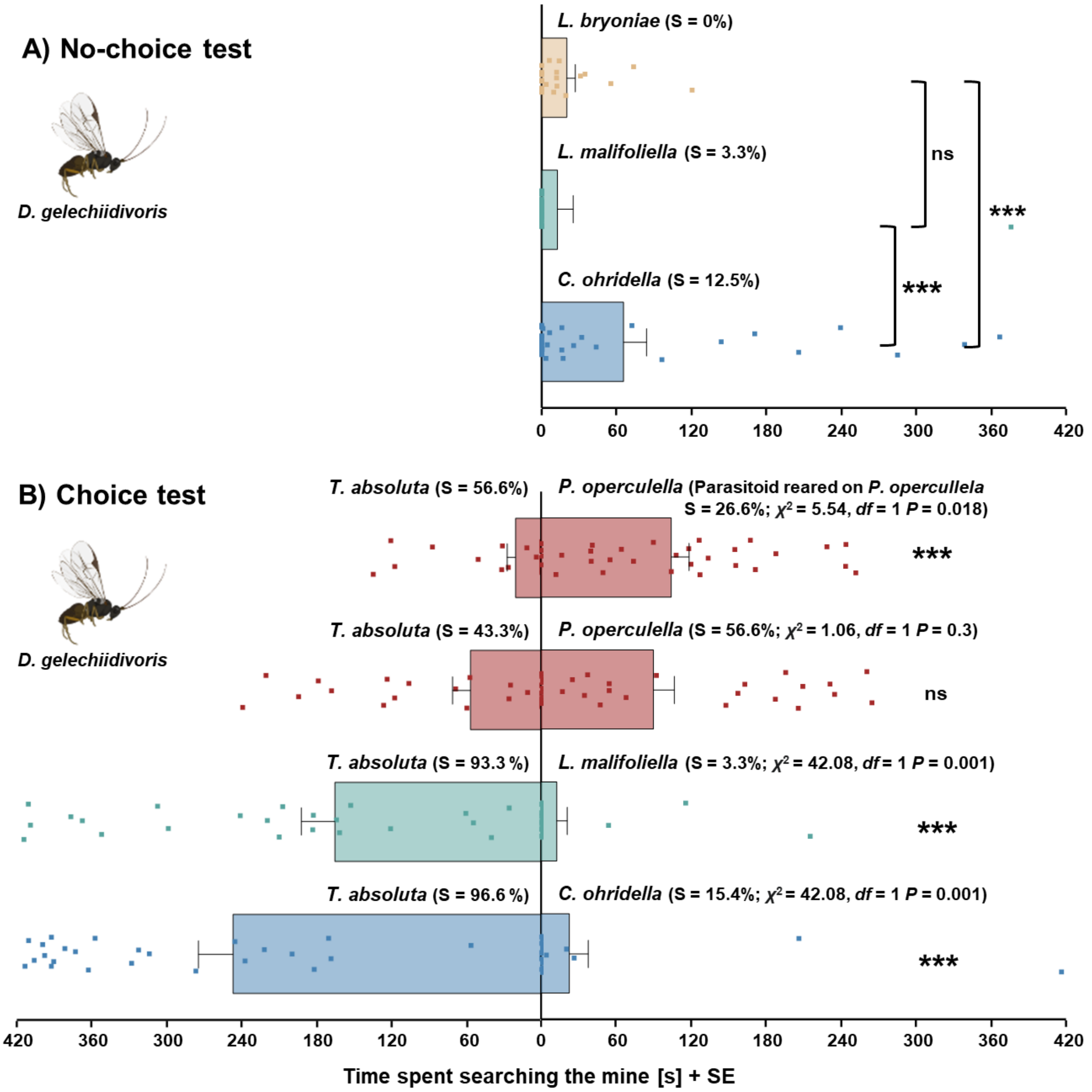


Figure 2. Mean time spent searching the host mine by female *Dolichogenidae gelechiidivoris*. Observations were conducted for 7 minutes. Each female stinging the mine was also recorded (S = proportion of female stinging). The sample size of observed females ranged from 20 to 32. **A**) No-choice experiment with the non-target host *Liriomyza bryoniae*, *Leucoptera malifoliella,* and *Cameraria ohridella.* A Mann-Whitney U-test was used to compare the time spent searching. **B**) Choice experiment with the target host *T. absoluta* and the non-target host *P. operculella, L. malifoliella,* and *C. ohridella.* A Wilcoxon signed-rank test was used to compare the searching time, and a $\chi 2$ test was used to compare the proportion of females stinging a larva. ns $P > 0.05$; *** $P < 0.001$.

Field experiments

Host preference

The infestation levels of the sentinel plants were similar, with a mean of 75.6 and 76.9 individuals (pest + parasitoids) emerging per potato and tomato sentinel plants, respectively. When assessing the relationship between the pest density in the greenhouses and the parasitism rate of hosts on sentinel plants by *N. tutae*, no significant correlation between the pest density in the greenhouses and parasitism rate was found for *N. tutae* (|r| (215) = -0.003, P = 0.963; Fig. 3A, Table 1 SI). Sentinel plant type or location also did not affect the parasitism rate by *N. tutae* ($P > 0.175$, Fig. 3B, Table S1).

For *D. gelechiidivoris*, a significant negative correlation was found between the pest density in the greenhouses and the parasitism rate of hosts on sentinel plants (Pearson's correlation, |r| (215) = -0.306, *P* = 0.001; Fig. 3C). On potato sentinel plants outside the greenhouse, parasitism rate was significantly higher in comparison to potato sentinel plants inside (*P* = 0.001, Fig. 3D, Table S1). Inside the greenhouse, *D. gelechiidivoris* caused significantly higher parasitism levels on tomato sentinel plants with *T. absoluta* when compared to potato sentinel plants with *P. operculella* (*P* = 0.004, Fig. 3B, Table 1 SI).

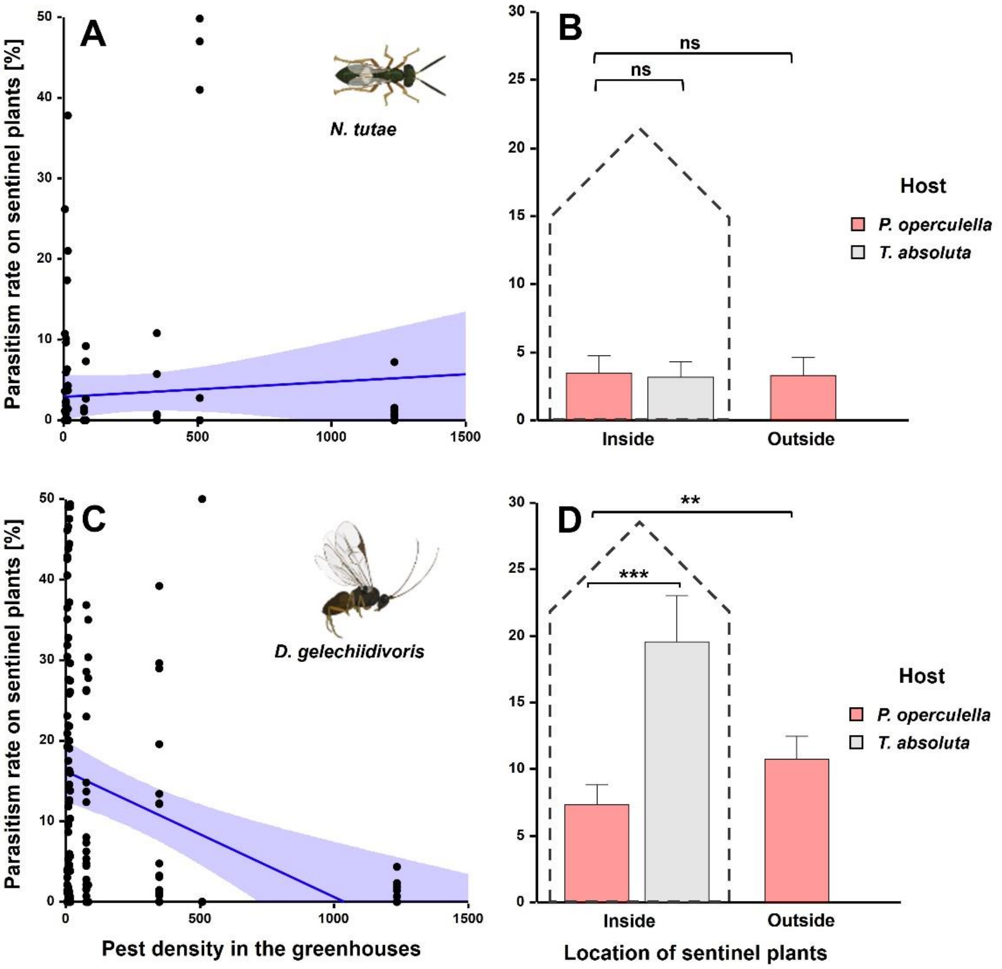


Figure 3. A Correlation between the pest density in the greenhouses and the parasitism rate on the sentinel plants by *Necremnus tutae*. The regression line (blue) and the confidence band (blue cloud) are shown. **B** Influence of host identity (*Phtorimaea operculella* on potato plant or *Tuta absoluta* on tomato plant) and sentinel plant location (inside or outside of the greenhouse) on parasitism rate by *N. tutae*. Parasitism rates on sentinel plants + SE are shown. **C** Correlation between the pest density in the greenhouses and the parasitism rate on the sentinel plants by *Dolichogenidae gelechiidivoris*, and **D** influence of host identity and plant location on parasitism rate by *D. gelechiidivoris*. Single parasitism rates on sentinel plants + SE are shown. (***P* < 0.01, ****P* < 0.001, a summary of the statistics is presented in Table 1 SI). Note that y-axes have different scales.

The highest discovery rate (at least one host parasitized) of sentinel plants was found for *D. gelechiidivoris* at 60.3% (Table 1). Female *D. gelechiidivoris* found more than 49% of the sentinel plants, whereas *N. tutae* discovered fewer sentinel plants, ranging from 15.5 to 33.3% (Table 1).

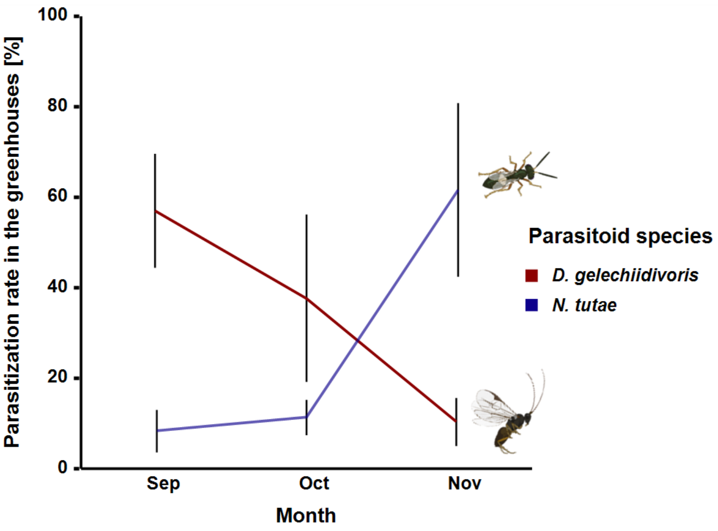
Table 1. Summary of mean discovery rate (at least one host of the sentinel plant parasitized) found over three months on the different sentinel plants placed inside and outside the three greenhouses for *Necremnus tutae* and *Dolichogenidae gelechiidivoris*.

	Discovery rate	
Sentinel plants/greenhouses	***D. gelechiidivoris***	***N. tutae***
T. absoluta on tomato plants inside (n=49)	60.3%	33.3%
P. operculella on potato plants inside (n=56)	53.8%	15.5%
P. operculella on potato plants outside (n=53)	49.4%	32.1%

Over the three months, the mean parasitism rate by *D. gelechiidivoris* in the greenhouses reached 35.1%, whereas *N. tutae* parasitism rate reached 27% (Figure 4). The mean of the added parasitism rate of both parasitoid species reached 65.2% in September, 48.8% in October, and 72.2% in November.

The number of host insects collected to assess parasitism strongly differed between greenhouses due to different pest densities and ranged from 348 to 1234 individuals (pest + parasitoids) for the open greenhouse, 13 to 76 for the semi-open greenhouse, and 5 to 83 for the closed greenhouse. In September and October, a significantly lower parasitism rate of *N. tutae* was found in the greenhouses (*P* <0.002; Table. S2).

Figure 4. Mean parasitism rate + SE of *Tuta absoluta* by the parasitoids *Dolichogenidae gelechiidivoris* and *Necremnus tutae* over time in three greenhouses (n=3). The sample size of hosts collected ranged in September from 9 to 1234, October from 16 to 508, and November from 5 to 348.



Survey of non-target species in the surroundings

Only one leafminer species was found during the sampling around the greenhouses: *Chrysoesthia sexguttella* (Thunberg) (Lepidoptera: Gelechiidae) on *Chenopodium album*. It was present around all three greenhouses during September and October, the plant being in senescence in November. More than 50 mines were sampled during the two first monitorings. No parasitism by *N. tutae* or *D. gelechiidivoris* was recorded. However, 15 parasitoid individuals of *Pnigalio soemius* (Walker)

(Hymenoptera: Eulophidae) emerged from the collected samples, and one individual of the Pteromalidae family.

Discussion

The research presented here is the first to examine the host-specificity in the laboratory and in the field of *N. tutae* and *D. gelechiidivoris*, which play an important role in the biocontrol of *T. absoluta* in the native area of the pest and around the Mediterranean basin (Desneux et al. 2022; Denis et al. 2022). Our study provides clear evidence for the polyphagy of *N. tutae* and the oligophagy of *D. gelechiidivoris*.

The preliminary experiment revealed that both parasitoid species did not parasitize any *T. absoluta* larvae offered outside of the mine; however, both displayed nonreproductive host-killing, regardless if the larvae were inside or outside of the mines. For a parasitoid, the host is a crucial resource with distinctive physical, chemical, and behavioral characteristics, which determine the host's recognition and acceptance (Rehman and Powell 2010). The results here indicate that mine recognition is highly important for successful parasitism by these two species. A previous study highlighted that the leafminer parasitoid *Symplesis serieeicornis* Nees (Hymenoptera, Eulophidae) already detects the mine during flight (Casas 1989). A host within the mine may provide additional protection for the parasitoid offspring, particularly for an ectoparasitoid such as *N. tutae*. It might also be that the host larvae in the mines are less mobile and likely less able to defend themselves through aggressive or evasive behavior (Greeney et al. 2012) than outside the mine, which could explain why a higher host-killing rate for *N. tutae* on *T. absoluta* larvae was found inside the mine. However, in the case of *D. gelechiidivoris*, the host-killing rate was the same for larvae outside and inside the mines. Notably, the host-killing rate for *D. gelechiidivoris* was higher than 30%, which represents a significant contribution to the control of *T. absoluta* by this parasitoid. To our knowledge, this is the first quantification of nonreproductive host-killing by this Braconidae.

N. tutae, as a native species, was present in the region prior to the *T. absoluta* invasion in 2006 and has a pre-existing host range beyond *T. absoluta*. Under no-choice conditions, *N. tutae* attacked all three lepidopteran species tested in the host specificity assays, but not the dipteran species, and even parasitized more than twice as many larvae of the non-target host *C. ohridella* as the target host, confirming a broad host spectrum. Interestingly, the nonreproductive host-killing behavior of *N. tutae* was restricted to *T. absoluta* and the closely related species *P. operculella*. This could be explained by the fact that larvae of these species are relatively mobile and, once stung, sometimes leave the mines and die outside (J. Gonthier personal observation), preventing *N. tutae* from laying eggs. In contrast, the non-target hosts *C. ohridella* and *L. malifoliella* are obligate leafminers (Gross and Price 1988), which cannot leave the mines, allowing more time for *N. tutae* for oviposition. Despite this, *N. tutae* accepts many species for oviposition, raising concerns for non-target lepidopteran leafminer species. In the field, *N. tutae* showed no preference for *T. absoluta* on tomato-sentinel plants compared to *P. operculella* on potato-sentinel plants.

Because of the evolutionary history with *T. absoluta* (Salas Gervassio et al. 2019), *D. gelechiidivoris* has likely evolved a strong affinity for this pest. In the choice experiment, female *D. gelechiidivoris* seemed mainly attracted to the mines of *T. absoluta* on tomato leaves. It is worth mentioning that this species is known to display probing behavior and reject many larvae after "stinging" (Gonthier et al. 2023). The females were possibly only probing the non-target host; unfortunately, it was impossible to rear the stung host to assess parasitism since the larvae suffered high mortality due to the decaying leaves. Finally, time spent searching and stinging seems not to be solely determined by the plant species (tomato) as no female *D. gelechiidivoris* were recorded stinging the mine of *L. bryoniae,* and the time spent searching the tomato leaves containing this host was very short in the no-choice test. Females spent the same time searching the mines of *P. operculella* as the mines of *T. absoluta,* and no difference was found between the proportions of females stinging either host. Interestingly, when reared for one generation on *P. operculella,* females spent significantly more time searching the mines of *P. operculella*. This behavior change confirms previous studies demonstrating the influence of the rearing host on parasitoid preferences (Bodino et al. 2016; Boycheva-Woltering et al. 2019; Gonthier et al. 2023).

There are several ways in which biosafety aspects, i.e., host specificity, can be measured and addressed (Barratt 2011). Monitoring the realized host range post-introduction can help to verify predictions made by host-specificity testing under confined conditions (Louda et al. 2003). Here, no preference for *P. operculella* or *T. absoluta* was observed in the laboratory experiments for *D. gelechiidivoris*, but a preference for the latter was visible in the field. This discrepancy could be explained by the fact that the olfactory cues of a cut leaf with only two larvae are much weaker than a whole plant, as *D. gelechiidivoris* attraction for infested plants increased with increasing infestation rate (Ayelo et al. 2022). This is an encouraging result regarding host specificity and risk assessment since both species belong to the same clade (Chang and Metz 2021).

Another interesting finding is that for *D. gelechiidivoris,* the parasitism rate on the sentinel plants was negatively correlated with the pest density in the greenhouse, meaning that the sentinel plants were less attractive to *D. gelechiidivoris* when the pest densities were high. Interestingly pest density did not affect the parasitism rate of the sentinel plants by *N. tutae*. A logical explanation could be that *N. tutae* host searching capacity is higher at low host density (type two functional response; Bodino et al. 2019) than the capacity of *D. gelechiidivoris* (type three functional response; (Gonthier et al. 2023; Mama Sambo et al. 2022b). The monitoring in the greenhouses also supports this theory, with a higher parasitization rate found for *N. tutae* when the infestation rate was low. Therefore, the use of sentinel plants as a sampling or monitoring method for parasitoids can be strongly influenced by the pest density in the vicinity and should be considered for future monitoring studies with sentinel plants.

Flying natural enemies, such as parasitic wasps, can move quickly and cover a large area (Schellhorn et al. 2014), making them impossible to contain. Both parasitoids intensely parasitized hosts on the potato plants inside and outside the greenhouses, and they are thus moving in and out of the greenhouses and potentially impacting non-target species. In the case of *D. gelechiidivoris*, parasitism levels were higher on sentinel plants outside the greenhouse, likely due to the lower density of the target host outside.

Understanding the seasonal dynamics of both the pest and the natural enemies is critical for promoting natural enemies for biological control. The establishment and success of natural enemies can be heavily influenced by the season due to environmental conditions and host availability (Welch and Harwood 2014). Our goal was to assess the situation towards the end of the tomato season. The parasitism rate of *N. tutae* increased towards the end of the season, whereas the rate of *D. gelechiidivoris* decreased. Interestingly, a previous study in 2020 (Denis et al. 2022) recorded a steadily increased parasitism rate for *D. gelechiidivoris* from May (2.7%) until October (21.8%). Why we found a decrease from September to November 2022 remains to be determined, but it is likely that the population of *D. gelechiidivoris* reaches its peak between September and October.

The neotropical *D. gelechiidivoris* may be less adapted to the colder temperatures at the end of the growing season (mean temperature in Malgrat de Mar in November = 14.2°C, minimum recorded 4.4°C, see Fig. S7) than the native *N. tutae*. Exposing pupae of *D. gelechiidivoris* to 4°C for seven days significantly lowered parasitism abilities (Morales-Perdomo et al. 2018). Recent studies on the performance of *D. gelechiidivoris* at 10°C found that the net reproductive rate decreases drastically (Agboka et al. 2022), displaying even a negative intrinsic rate of increase (Aigbedion-Atalor et al. 2022). Finally, because *D. gelechiidivoris* is a koinobiont parasitoid, the immature stages' survival depends on the host's cold tolerance (Bajonero et al. 2008). Larvae of *T. absoluta* are not very cold-tolerant; 50% of larvae died after 12.4 days at 5°C (Van Damme et al. 2015). Consequently, successful overwintering and establishment of *D. gelechiidivoris* in countries with colder climates appear unlikely, and *N. tutae* is likely mainly responsible for pest reduction during winter in non-heated greenhouses. If *D. gelechiidivoris* can overwinter via an alternative host remains to be determined.

Although both parasitoid species preferably target different host instars of *T. absoluta*, interspecific competition (Feng et al. 2015; Karlsson et al. 2018; Savino et al. 2017) could explain the contrasting population dynamic at the end of the season. Denis et al. (2022) reported 11% of larvae ectoparasitized by *N. tutae,* yielding a *D. gelechiidivoris* adult. Several factors can determine which parasitoid will prevail in a competition targeting the same host. For instance, the effectiveness of the venom of idiobiont ectoparasitoids can affect the development of endoparasitic koinobionts (Harvey et al. 2013). Moreover, when an ectoparasitoid and an endoparasitoid engage in multiple parasitisms, the ectoparasitoid usually emerges as the winner, as noted by Mitsunaga and Yano (2004). For a similar situation, a recent study in Kenya evaluated the interaction between

D. gelechiidivoris and the native ectoparasitoid *Stenomesius japonicus* (Ashmead) (Hymenoptera: Eulophidae), which like *N. tutae* targets the third instar of *T. absoluta* (Mama Sambo et al. 2022a). The authors found that both species coexisted and recorded a low multiparasitism level of 5% or less. Even though *N. tutae* is still present in high density six years after the arrival of *D. gelechiidivoris* in Spain (Denis et al. 2021), the outcome of within-host competition remains to be studied, and the overall interaction deserves further investigation.

When surveying alternative hosts around the greenhouse, the leafminer *C. sexguttella* belonging to the same family as the target host *T. absoluta* was found repeatedly during the experiment. Even though no *N. tutae* or *D. gelechiidivoris* emerged from any of the *C. sexguttella* collected, the leafminer was found to be parasitized by *P. soemius*. This species has been recorded parasitizing *T. absoluta* and might contribute to its natural control (Ferracini et al. 2012; Zappalà et al. 2012). Hence, *C. album* could be a plant to promote the presence of *P. soemius* - although its role in controlling the pest is questionable since no parasitism of *T. absoluta* by *P. soemius* was recorded during our experiments. Another potential alternative host species is the micro-Lepidoptera *C. pulchrimella*, which was reported to be parasitized by *N. tutae* in the laboratory (Bodino et al. 2016). Unfortunately, while its host plant *Parietaria officinalis* (Pellitory-of-the-wall), was recorded repeatedly in all three greenhouses, no mines of the pest were found.

The results presented here show that the release of *D. gelechiidivoris* could help control *P. operculella* as much as *T. absoluta*. This finding is highly relevant for China, for instance, since *T. absoluta* is causing severe damage (Zhang et al. 2021), and *P. operculella* is the major constraint to the commercial production of potatoes in the country (Gao 2018). *D. gelechiidivoris* could also benefit the control of future invasions, such as of the tomato pinworm *K. lycopersicella* (Aigbedion-Atalor et al. 2022), whose distribution is now restricted to South and North America. Similarly, *D. gelechiidivoris* might help controlling the ongoing outbreak of the Guatemalan potato tuber, *Tecia solanivora* Povolny (Lepidoptera: Gelechiidae) in Spain (Jeger et al. 2018).

Finally, based on these results, *P. operculella* could be a suitable alternative host for rearing both parasitoid species. The ease of rearing the *P. operculella* on potato tubers (Furong and Zhengyue, 2003) makes it a more convenient and cheaper option than rearing on *T. absoluta*. Nevertheless, as shown by our result and the study of Bodino et al. (2016), the rearing host strongly influenced the preference of the offspring for the future host. This characteristic should therefore be taken into account.

The high host-specificity and attraction of *D. gelechiidivoris* towards *T. absoluta* and related species such as *P. operculella* make it an ideal candidate for augmentative or classical biological control. In contrast, the polyphagous nature of *N. tutae* poses a potential risk for non-target species. The study suggests that countries interested in classical biocontrol should prioritize *D. gelechiidivoris* and conduct host-specificity tests, focusing on leafminers of the Gelechiidae family. Nevertheless, the Gelechiidae family is incredibly large, consisting of 865 species from 109 genera in Europe alone (Huemer and Karsholt 2020). Therefore, when selecting species for host-specificity testing, it is advisable first to evaluate those belonging to the same genera as *T. absoluta* (Gelechiinae) feeding on plants in the Solanaceae family or other closely related families. Moreover, further research is needed to understand the potential intraguild competition of both parasitoids and the overwintering capacity of *D. gelechiidivoris*, which could predict the establishment, spread, and effectivness and be relevant to assess the risk posed in the case of augmentative biocontrol in the northern countries. Last, countries at high risk of being invaded by *T. absoluta* where *P. operculella* is already present, such as New Zealand, Australia, or North America, could develop preemptive biocontrol programs (Caron et al. 2021) using *P. operculella* as an alternative host to screen for native parasitoids.

Authors' contributions

JG, JR, JA, and JC designed the research. JG conducted laboratory experiments. JG and JA led the field trials. JG conducted the statistical analysis. JG, JR, and JC analyzed the data. JG wrote the manuscript. All authors read, edited, and approved the manuscript.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

The data supporting this study's findings are openly available in figshare at https://doi.org/10.6084/m9.figshare.22821008.

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Supplementary Information

Maps of the greenhouse locations and the sentinel plants

The field experiments were conducted in and around three commercial tomato greenhouses in the Barcelona region. Infested tomato and potato sentinel plants were placed inside and around the three greenhouses. Twenty-one sentinel plants were placed in each location: seven tomato and eight potato plants inside and nine potato plants outside the greenhouses. Sentinel plants inside were placed 15 m or more from each other and were distributed equally.

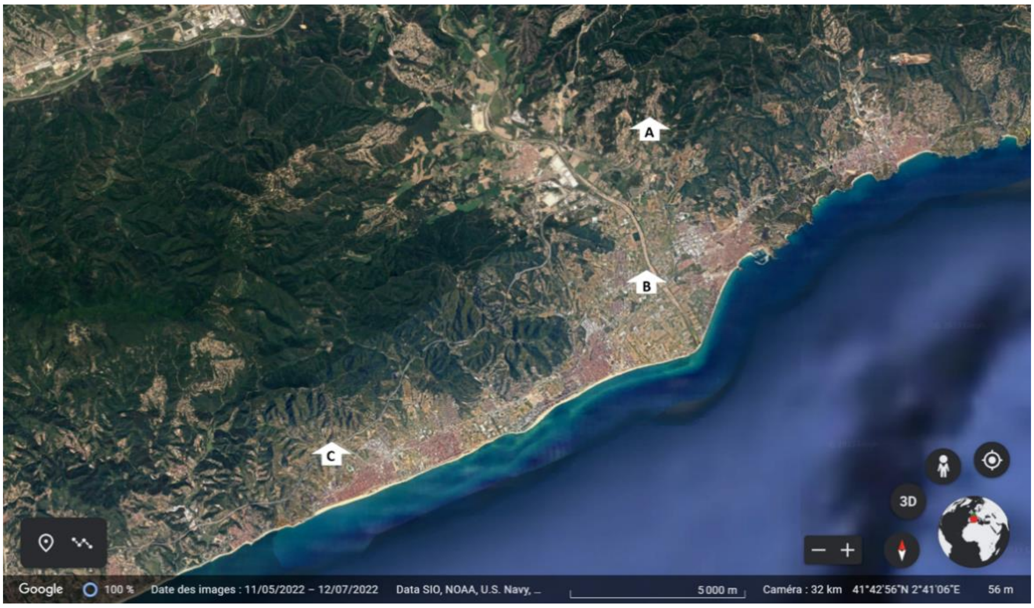

Figure S1. Commercial tomato greenhouses in La Selva (greenhouse **A**) and El Maresme counties (greenhouse **B** and **C**). Greenhouse location A (41°40'16" N, 2°46'00" E); location B (41°41'48" N, 2°46'30' E); location C (41°37'22" N, 2°39'12" E).

Figure S2. Sentinel plant location inside and around greenhouse **A,** consisting of a 1'600 m^2 low-cost tunnel, completely open at the sides (up to 3 m height) and surrounded by arable land. P = potato sentinel plant. T = tomato sentinel plant.

Figure S3. Sentinel plant location inside and around greenhouse B, consisting of a 600 m2 semi-closed tunnel with meshes on the side wall (allowing insects to move in and out) surrounded by a forest on one side and other greenhouses on the other side. P = potato sentinel plant. T = tomato sentinel plant.

Figure S4. Sentinel plant location inside and around greenhouse C consisting of a 1'136 m2 greenhouse completely closed beside the roof opening and surrounded by similar greenhouses. P = potato sentinel plant. T = tomato sentinel plant.

Sentinel plant set-up

Figure S5. Sentinel plants (tomato or potato) were planted in pots (15 cm dia., 20 cm height) and were used when they were about 30 cm high with at least five fully developed leaves. Sentinel plants were covered with a mesh (mesh size 0.4 mm) to avoid predation by adult mirids and larger animals, and the pots were sprayed with insect glue (Soveurode®, Plantin S.A.R.L.) to avoid predation by soil-dwelling insects.

Non-target hosts parasitized by *Necremnus tutae*

Figure S6. On the left, parasitized host larvae of *Leucoptera malifoliella*. On the right, parasitized host larvae of *Cameraria ohridella*. The parasitoid eggs are displayed inside the red circle.

Video of *Dolichogenidae gelechiidivoris* searching and stinging the mine of a non-target host

Video S1. QR code to access the video showing the searching (antennating) and stinging behaviour of a female parasitoid *Dolichogenidae gelechiidivoris* on the mine of the non-target host *Leucoptera malifoliella*. Link to the video: https://youtube.com/shorts/wq8mYgZkKtI?feature=share

Summary of statistics for GLMM

Table S1. Summary of the generalized linear mixed-effects models relating host identity and sentinel plant location to parasitism rates from *Necremnus tutae* and *Dolichogenidae gelechiidivoris*.

Parasitism rate	**Variable**	**Level**	**Value**	**SE**	**t-value**	**p-value**
D. gelechiidivoris	Potato plant location	Location: outside	0.625	0.211	2.966	0.004
	Host type inside	Host: *T. absoluta*	1.109	0.256	4.340	0.000
N. tutae	Potato plant location	Location: outside	0.540	0.396	1.365	0.175
	Host type inside	Host: *T. absoluta*	0.062	0.406	0.154	0.878

Table S2. Summary of the generalized linear models relating months of monitoring to natural parasitism rates from *Necremnus tutae* and *Dolichogenidae gelechiidivoris* found in the greenhouses. For *N. tutae*, the parasitism rate was negatively influenced by the first two months, but months did not influence the parasitism rate of *D. gelechiidivoris*.

Parasitism rate	**Variable**	**Level**	**Value**	**SE**	**t-value**	**p-value**
D. gelechiidivoris	Month	September	1.779	1.209	1.471	0.192
		October	-0.263	1.513	-0.174	0.868
N. tutae	Month	September	-4.033	0.772	-5.227	0.002
		October	-2.185	0.556	-3.928	0.008

Temperature variation during the field experiment

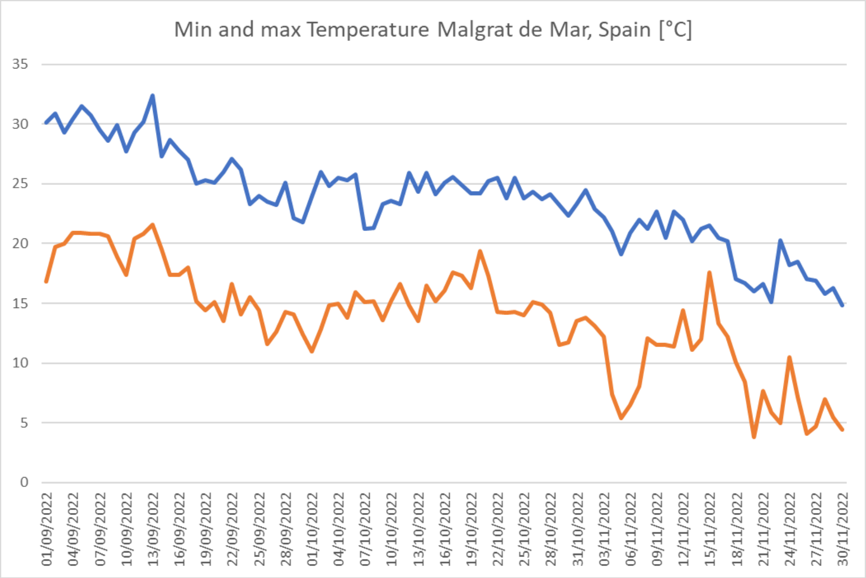

Figure S7. Min (orange) and max (blue) Temperature in Celcius in Malgrat de Mar, Spain, from September to November 2022. Data were obtained from the Malgrat de Mar (Barcelona) station from the agro-meteorological stations (www.ruralcat.cat) of the Generalitat of Catalunya.

Synthesis and Outlook

Over the years, extensive research has been conducted to understand better the biology and behavior of *T. absoluta* and its parasitoids. However, there has been a significant lack of research on improving the efficiency of these parasitoids and better integrating them into different management strategies against *T. absoluta*. As a result, crucial aspects of the interaction between *T. absoluta*, the tomato plant, its parasitoids, and other biocontrol agents such as entomopathogens remain unclear.

To address this gap, the aim of this research was to explore the potential of parasitoids against *T. absoluta* and evaluate various methods to enhance their efficiency and integration in tomato farming systems. This work has identified processes to increase the attraction of egg parasitoids to tomato plants and evaluated a novel combination of larval parasitoids with a baculovirus. Furthermore, considering their host-specificity, the study assessed the value of two promising larval parasitoids as biocontrol agents.

The study's main findings were summarized within the respective chapters: Ch. I, understanding the limiting factors of egg parasitoids; Ch. II, combining larval parasitoids with a Baculovirus; Ch. III, trophic connections in a new parasitoid community. The subsequent section synthesized the findings for each chapter, answering the study's research questions, highlighting the implications of these syntheses, and identifying recommendations for future research and the limitations encountered.

Improving the efficiency of egg parasitoids

Trichogramma egg parasitoids have shown mixed results in controlling *T. absoluta* populations (Giorgini et al. 2019; Milonas et al. 2019). The aim of this chapter was to understand how the attraction of *Trichogramma* to *T. absoluta* tomato plant system plays a role in their efficiency and to find possible ways to improve it. We found that:

a) the odor of tomato leaves innately repelled females of *Trichogramma*;
b) the odor of tomato leaves can become attractive via species-specific learning processes;
c) the increased attraction can translate into higher parasitism.

The innate repellence to the odor of tomato leaves has not been reported before and is a major limiting factor restricting *Trichogramma* use against *T. absoluta*. The fact that parasitoids can be trained to find odor cues attractive through learning is not new (Giunti et al. 2015). Nevertheless, few studies demonstrated a learning process leading to higher parasitism. To our knowledge, it is the first time that three parasitoid species from the same genus are compared for their response to the same plant and such distinct species-specific learning processes are found. Overall, overcoming the wasps' initial repellence to the odor of tomato leaves could improve the cost-benefits currently limiting *Trichogramma* use against *T. absoluta* (Cabello et al. 2009; Chailleux et al. 2012, 2013). Therefore, learning could be integrated into mass-rearing programs, and we recommend that *Trichogramma*'s learning abilities be assessed during the selection process.

As we demonstrated, one of the main challenges to implementing learning to improve *Trichogramma*'s efficiency is the variability in the learning abilities of different species (Chailleux et al. 2012; Hoffmann et al. 2001), which can limit the potential for conditioning and enhancing their efficiency in the field. Even though we found a strain for which associative learning translated into higher parasitism, other strains might react differently.

Further research is needed to determine the potential of learning for the success of *Trichogramma* post-release. Very few studies have directly tested and proved the viability of this concept in the field. Masry et al. (2019) demonstrated the consistency of learning by the fruit fly parasitoid *Diachasmimorpha krausii* (Hymenoptera: Braconidae) from laboratory to the field. To our knowledge, this has not been tested for *Trichogramma.* In this regard, developing an effective conditioning procedure that can be easily implemented in the field is crucial (Kruidhof et al. 2019). Knowing the exact cue responsible for the learning response could help implement this strategy. These cues could be artificially synthesized and integrated into the conditioning procedure during the rearing or in the field. One major limitation of our study method was that we could not identify the various stimuli of the host-plant complex learned by each species. Therefore disentangling the various cues and testing them in the conditioning procedure would be the next logical step to develop this approach further. Moreover, since *Trichogramma* are reared in control conditions, poor behavioral flexibility may prevent them, once released, from adapting to the new environment, which may cause failures in the field due to stressors such as strong temperature fluctuation or different host densities (Li et al. 2010; Roitberg 2004). Addressing these challenges will be critical to harness the full potential of *Trichogramma* as an augmentative biocontrol agent for the sustainable management of *T. absoluta*.

A new combination of biocontrol agents

Naturally occurring parasitoids are getting more exposed to baculoviruses as the popularity of using these microorganisms as a substitute for chemical insecticides in pest management is increasing (Knox et al. 2015; Moscardi et al. 2011; Nikhil Raj et al. 2022), emphasizing the importance of assessing the compatibility of these agents. Moreover, when implementing augmentation biocontrol with parasitoids where baculoviruses are applied simultaneously, it is critical to assess, combine, and optimize the effectiveness of both agents involved to ensure successful pest management (Cossentine 2009). This comprehensive approach could not only maximize pest control effectiveness but also ensure the system's sustainability in the long run. In this chapter, we evaluated the combination of a baculovirus with larval parasitoids of *T. absoluta* in terms of compatibility, efficacy, and population dynamics. We found that:

d) the baculovirus had a low impact on the offspring of the two parasitoid species tested;

e) one parasitoid species could avoid virus-infected larvae;

f) using the parasitoid *N. tutae* alone or together with the baculovirus had an equal effect on reducing adult pest density;

g) using the parasitoid and the virus alone or in combination resulted in the same reduction in plant damage;

h) modeling the pest parasitoids dynamic over a year revealed that fewer parasitoids are necessary to achieve control when the growing season is long, allowing more time for population build-up;

i) the timing of intervention is crucial for efficient augmentative release, and baculovirus application allows more time for parasitoid intervention.

While several studies have addressed the interaction of fungi or bacteria with parasitoids (Koller et al. submitted), few studies have examined the combination of parasitoids with entomopathogenic viruses beyond the laboratory scale, and almost none with baculovirus (reviewed by Cossentine 2009, Abbes et al. 2015). This chapter presents an integrative approach that comprehensively investigated the combination. The research methodology comprises a laboratory study to assess the virus's compatibility and possible indirect effect on the parasitoids, a semi-field experiment to evaluate the efficacy of the combination in more realistic conditions, and a modeling framework to optimize the strategy. Overall, this chapter delivers a robust and in-depth analysis of the performance of this new combination by utilizing multiple methods.

The findings presented in this chapter are highly relevant for planning a management strategy against *T. absoluta,* applying baculoviruses in combination with the augmentation or conservation of parasitoids. Both larval parasitoid species tested can be used with the baculovirus to manage *T. absoluta* without adversely affecting their populations. Our semi-field experiment showed that the most effective reduction of pests and damage was achieved by combining both agents. However, when compared to the use of each agent separately, the difference in effectiveness was not substantial enough to be considered statistically significant. If this is due to an antagonistic effect, too-high pest density or another factor remains to be determined. Parasitoids have been found to reduce the pathogenicity of baculoviruses in hosts (Cossentine 2009), which could explain why we found no positive effect of the combination. Due to practical matters and time constraints, we could not assess how the parasitoid affects the virus pathogenicity, and further study should evaluate this side of the interaction. Finally, our model could be used to test if we released enough parasitoids in our experiment for the initial high pest infestation.

Overall, the semi-field experiment may not fully reflect the complexities of real-world pest populations due to factors such as pest movement, aggregation, and multiple developmental stages. In situations where precise control is difficult, combining two biocontrol agents can provide greater flexibility in timing and dosage. Therefore, the positive effect of combination might be more visible in complex environments. Further research should examine the effectiveness of our combinations in real-world settings. Additionally, it is crucial to investigate the potential role of parasitoids in spreading or accelerating the spread of the virus within the pest population (Jiang et al. 2011).

Trophic connections in a new parasitoid community

To encourage the use of *N. tutae* and *D. gelechiidivoris* as biocontrol agents, it is crucial to comprehend the trophic relationships between them, the target host *T. absoluta*, and other non-target hosts. The goal of this last chapter was to understand these trophic connections and assess their value as biological control agents. We found that:

j) *D. gelechiidivoris* has a high host-specificity and attraction towards *T. absoluta* under laboratory and field conditions;

k) *N. tutae* is polyphagous with a wide host range.

The findings of our study suggest that the introduction or further spread of *D. gelechiidivoris* in Europe would pose a limited risk for non-target species, although its specificity for leafminers of the Gelechiidae family should be further tested. With 865 species belonging to 109 genera only in Europe (Huemer and Karsholt, 2020), the Gelechiidae family is enormous. Therefore leafminer species of the same genera as *T. absoluta* (Gelechiinae) feeding on plants of the Solanaceae or closely related family should be first assessed. This study took place within a year, and it was impossible to evaluate more alternative hosts for practical reasons and time constraints.

While *N. tutae's* polyphagous nature may pose a risk to non-target species outside of its endemic range, this knowledge offers new rearing possibilities. In Europe, cost-effective rearing for *N. tutae* could open the door for implementing this parasitoid within integrated biological control programs (Calvo et al. 2016). Our study discovered three new hosts commonly found in high density and relatively easy to rear (Furong and Zhengyue, 2003; Grabenweger et al. 2009). New mass-rearing using different hosts and integrating learning processes to take advantage of the plastic behavior of this parasitoid (Bodino et al. 2016) could be developed. Much effort has been put into conserving *N. tutae* in Europe, notably with flowering plants (Arnó et al. 2018). Efforts should also be made to promote the establishment of *D. gelechiidivoris* in the new range. It could be done by providing nectar, but another approach with the advantage of supporting the parasitoid population before the pest's arrival would be to set up banker plants with an alternative host. An early establishment of *D. gelechiidivoris* is particularly important due to the high intrinsic rate of increase of *T. absoluta* (Martins et al. 2016). Following this idea, rearing boxes (Niedermayer and Steidle, 2013) could be developed for smallholder farmers with *P. operculella* on potato plants or tuber as an alternative host.

Outlook

The global distribution and ongoing spread of *T. absoluta* pose a major challenge to the sustainable production of tomatoes globally. Using parasitoids to control *T. absoluta* can be part of the sustainable solution with many benefits and challenges, such as availability, release timing, environmental conditions, and compatibility with other control methods. The studies synthesized here have shed light on strategies using parasitoids such as *Trichogramma*, *D. gelechiidivoris*, *N. tutae*, and baculoviruses. While there are still challenges to be overcome, such as developing effective conditioning procedures in the field and understanding how the combination of different agents can be further improved, these findings offer a valuable resource for integrating parasitoids further in sustainable control strategies. Although this research has made significant progress, much must be learned about the interaction between *T. absoluta*, tomato plants, and its biocontrol agents. By continuing to investigate existing management strategies with parasitoids and refining new ones, we can harness their full potential, ensure the sustainable management of *T. absoluta* and guarantee long-term food security.

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Declaration of consent

on the basis of Article 18 of the PromR Phil.-nat. 19

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Bachelor ☐ Master ☐ Dissertation ☑

Title of the thesis:	Improving the efficiency of biological control with parasitoids for Tuta absoluta pest management
Supervisor:	PD Dr. Jörg Romeis

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