Universitat de Lleida

ECOLOGY AND CONTROL OF THE ALFALFA WEEVIL (*Hypera postica* Gyllenhal)

Alexandre Levi Garcia Mourão

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Universitat de Lleida

Departament de Producció Vegetal i Ciència Forestal

ECOLOGY AND CONTROL OF THE ALFALFA WEEVIL (*Hypera postica* Gyllenhal)

PhD Dissertation

Tesi Doctoral

Alexandre Levi Garcia Mourão

Director:
Prof. Dr. Xavier Pons i Domènech

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ECOLOGY AND CONTROL OF THE ALFALFA WEEVIL (*Hypera postica* Gyllenhal)

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SUMMARY

Alfalfa is one of the most valuable forage crops worldwide in temperate climate zones of our globe. The alfalfa weevil (*Hypera postica* Gyllenhal) (Coleoptera: Curculionidae) is a very destructive Eurasian pest of alfalfa. The damage caused reduces forage yield and quality. Despite its origin, very little scientific information is available about its ecology and control in European crop conditions. This thesis contributes to improve this knowledge in Mediterranean conditions of the Ebro Basin, one of the main productive regions of alfalfa in Europe. Specifically, it has been evaluated: (1) the effect of temperature on the insect fitness in order to establish the yearly life cycle in the region; (2) the effectiveness of a winter alfalfa cutting as a preventive cultural and more sustainable control method than insecticide spraying; (3) the occurrence of its main associated natural enemy, the larval parasitoids of the genus *Bathyplectes* spp. (Hymenoptera: Ichneumonidae), and their rates of parasitism; and (4) the influence of the surrounding landscape and the own field characteristics on the abundance of *H. postica* and its natural enemies.

Temperature is determinant for insect activity and can be used to predict their success. The fitness of the alfalfa weevil was determined at eight constant temperatures (8–36 °C) by using models for the description of temperature-dependent development rates and life tables. This study allowed predicting the phenology of this pest taking into account the thermal requirements for development and the day length in the study region. Prediction was validated with collected field data, revealing that no more than two generations are possible within an annual cycle. In a hypothetical case where average temperatures increase due to global warming, the number of generations would not change.

The effectiveness of an alfalfa winter cutting for reducing populations in spring and its interaction with parasitism rate was determined in 42 commercial fields in 2019-2020, which were split in two parts and one was mowed in middle winter. Each part of the field was sampled by net-sweeping before the first alfalfa spring cutting, when damage is concentred, and the larval abundance was recorded. Winter cutting significantly reduced the spring populations and favoured the rate of larval parasitism. Results suggest that winter cutting can be integrated in pest management programs.

Two pairs of primers were designed in order to identify by PCR *Bathyplectes curculionis* and *Bathyplectes anura* inside *H. postica* larvae. The rates of parasitism obtained by the molecular method were compared with those obtained by conventional rearing. The molecular method detected higher rates of parasitism of both parasitoid species. However, both methods should be used in parallel when evaluating host–parasitoid systems, since only rearing methods can confirm the effective parasitism or the presence of unexpected species.

Evidences of the effect of landscape composition on the abundance of the alfalfa weevil and its main natural enemies have been, for the first time, provided by determining the landscape composition in 250, 500 and 1000 m buffer radii, of 65 commercial alfalfa fields sampled during 2018-2020. *H. postica and Bathyplectes spp.* abundances mostly depended on the characteristics of the own alfalfa field and few from the landscape composition. However, the abundance of *C. septempunctata*, an occasional predator of *H. postica*, was positively affected by the area of winter cereal cover.

RESUMEN

La alfalfa es uno de los cultivos forrajeros más valiosos a nivel mundial en las zonas de clima templado. El gusano verde (*Hypera postica* Gyllenhal) (Coleoptera: Curculionidae) es una plaga de la alfalfa de origen euroasiático. El daño causado reduce el rendimiento y la calidad del forraje. A pesar de su origen, se dispone de muy poca información científica sobre su ecología y control en las condiciones de cultivo europeas. Esta tesis contribuye a aumentar este conocimiento en condiciones mediterráneas en la cuenca del Ebro, una de las principales regiones productoras de alfalfa en Europa. Específicamente, se ha evaluado: (1) el efecto de la temperatura en la fitness del insecto para poder establecer su ciclo biológico anual en la región; (2) la eficacia de un corte invernal como método de control cultural preventivo y más sostenible que el uso de insecticidas; (3) la presencia de su principal enemigo natural, los parasitoides larvarios del género *Bathyplectes* spp. (Hymenoptera: Ichneumonidae), y sus tasas de parasitismo; y (4) la influencia del paisaje circundante y las características del propio campo en la abundancia de *H. postica* y de sus enemigos naturales.

La temperatura es un factor determinante para la actividad de los insectos y puede usarse para predecir su éxito. La *fitness* del gusano verde se determinó a ocho temperaturas constantes (8–36 °C) utilizando modelos para la descripción de las tasas de desarrollo y tablas de vida. Este estudio permitió predecir la fenología de la plaga teniendo en cuenta los requerimientos térmicos para su desarrollo y el fotoperiodo en la región de estudio. La predicción se validó con datos de campo, lo que reveló que no son posibles más de dos generaciones en un ciclo anual. En un caso hipotético donde las temperaturas medias aumentaran debido al calentamiento global, el número de generaciones no cambiaría.

Se evaluó la efectividad de un corte invernal para reducir las poblaciones de gusano verde en primavera y su interacción con la tasa de parasitismo en 42 campos comerciales en el período 2019-2020, que fueron divididos en dos partes, una de las cuales fue segada a mediados de invierno. Cada parte del campo fue muestreada con manga entomológica en primavera antes del primer corte comercial de la alfalfa, cuando el daño se concentra, y se registró la abundancia de larvas. El método redujo significativamente las poblaciones primaverales y favoreció la tasa de parasitismo larvario. Los resultados sugieren que este método se puede incorporar en los programas de control integrado de la plaga.

Se diseñaron dos pares de cebadores para identificar por PCR la presencia de los parasitoides larvarios *Bathyplectes curculionis* y *Bathyplectes anura*. Las tasas de parasitismo obtenidas por el método molecular se compararon con las obtenidas por métodos de cría convencional. El método molecular detectó mayores tasas de parasitismo de ambas especies de parasitoides. Sin embargo, ambos métodos deben usarse en paralelo al evaluar el sistema huésped-parasitoide, ya que solo los métodos convencionales pueden confirmar el parasitismo efectivo o la presencia de especies inesperadas.

Por primera vez se ha evaluado, en 65 campos comerciales de alfalfa muestreados durante el período 2018-2020, el efecto de la composición del paisaje en círculos concéntricos de 250, 500 y 1000 m de radio sobre la abundancia de *H. postica* y sus principales enemigos naturales. La abundancia de *H. postica* y *Bathyplectes* spp. dependieron en su mayoría de las características del propio campo de alfalfa y poco de la composición del paisaje. Sin embargo,

la abundancia de *C. septempunctata*, un depredador ocasional del gusano verde, se vio afectada positivamente por el área de cobertura de cereales de invierno.

RESUM

L'alfals és un dels cultius farratgers més valuosos a nivell mundial a les zones de clima temperat. La cuca verda de l'alfals (*Hypera postica* Gyllenhal) (Coleoptera: Curculionidae) és una plaga de l'alfals d'origen euroasiàtic. El dany que causa redueix el rendiment i la qualitat del farratge. Tot i el seu origen, es disposa de molt poca informació científica sobre la seva ecologia i control en les condicions de cultiu europees. Aquesta tesi contribueix a aquest coneixement en condicions mediterrànies a la conca de l'Ebre, una de les principals regions productores d'alfals a Europa. Específicament, es va avaluar: (1) l'efecte de la temperatura en la fitness de l'insecte per poder establir-ne el cicle biològic anual a la regió; (2) l'eficàcia d'un tall hivernal com a mètode de control cultural preventiu i més sostenible que l'ús d'insecticides; (3) la presència del seu principal enemic natural, els parasitoides larvaris del gènere *Bathyplectes* spp. (Hymenoptera: Ichneumonidae), i les seves taxes de parasitisme; i (4) la influència del paisatge circumdant i les característiques del propi camp en l'abundància de *H. postica* i dels seus enemics naturals.

La temperatura és un factor determinant per a l'activitat dels insectes i es pot fer servir per predir el seu èxit. La *fitness* de la cuca verda es va determinar a vuit temperatures constants (8–36 °C) utilitzant taules de vida i models per a la descripció de les taxes de desenvolupament. Aquest estudi va permetre predir la fenologia de la plaga, tenint en compte els requeriments tèrmics per al desenvolupament i el fotoperíode a la regió d'estudi. La predicció es va validar amb dades de camp , cosa que va revelar que no són possibles més de dues generacions en un cicle anual. En un cas hipotètic on les temperatures mitjanes augmentessin a causa de l'escalfament global, el nombre de generacions no canviaria.

Es va determinar l'efectivitat d'un tall hivernal, per reduir les poblacions de la cuca verda a la primavera i la interacció amb les taxes de parasitisme, en 42 camps comercials en el període 2019-2020, els quals van ser dividits en dues parts i una segada a mig hivern. Cada part del camp va ser mostrejat amb mànega entomològica a la primavera abans del primer tall comercial, quan el dany es concentra, i es va enregistrar l'abundància de larves. El mètode va reduir significativament les poblacions primaverals i va afavorir la taxa de parasitisme larvari. Els resultats suggereixen que aquest mètode es pot incloure als programes de control integrat de la plaga.

Es van dissenyar dos parells d'encebadors per identificar per PCR la presència dels parasitoides larvaris *Bathyplectes curculionis* i *Bathyplectes anura*. Les taxes de parasitisme obtingudes pel mètode molecular es van comparar amb les obtingudes per mètodes de cria convencional. El mètode molecular va detectar majors taxes de parasitisme de les dues espècies de parasitoides. Ambdós mètodes, però, s'han d'utilitzar en paral·lel en avaluar el sistema hoste-parasitoide, ja que només els mètodes convencionals poden confirmar el parasitisme efectiu o la presència d'espècies inesperades.

Per primera vegada s'ha avaluat, en 65 camps comercials d'alfals mostrejats durant el període 2018-2020, l'efecte de la composició del paisatge en cercles concèntrics de 250, 500 i 1000 m de radi en l'abundància de *H. postica* i els seus enemics naturals principals. L'abundància de *H. postica* i *Bathyplectes* spp. van dependre majoritàriament de les característiques del propi camp d'alfals i poc de la composició del paisatge. Tot i això, l'abundància de *C.*

septempunctata, un depredador ocasional de la cuca verda, es va veure afectada positivament per l'àrea de cobertura de cereals d'hivern.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

1. History of Alfalfa – alfalfa in the world

Alfalfa is one of the most valuable forage crop and the most productive forage legume in temperate climate zones (Orloff 1997; Frame et al. 1998). Alfalfa was already an important crop for the Babylonian, Persian, Greek, and Roman cultures. Around the 2^{nd} century BC, alfalfa was introduced to Rome, through Greece, and Roman agrarian authors were already commenting on its benefits (Columela, circa 70 AD). In Spain, the beginnings of this crop date back to the period of Roman domination. However, traces of this cultivation were lost until the period of the Muslim invasion, when the Arabs re-introduce plants from the oases, according to Abú Zacaría in the 12th century (Cubero 1999).

It is difficult to know exactly the area cultivated with alfalfa in the world. It seems, however, that around 30 million ha are cultivated worldwide and that the main producing countries are: USA with 6 million ha, China with 4.7 million ha, Argentina with 3.2 million ha, Australia with 3.3 million ha, and Russia with 3.5 million ha (Michaud et al. 1988; Basigalup et al. 2014; Gardner and Putman 2018; Humphries et al. 2018). The European countries with the most important cultivated areas are: Italy with 700,000 ha, France with 300,000 ha, Spain with 266,000 ha and Greece with 137,000 ha (Thiébeau et al. 2003; Nastis et al. 2012; Bortolazzo et al. 2016; MAPA 2019).

2. Alfalfa growth and management

Alfalfa may be sown in spring or autumn, depending on the precedent crop and the soil temperature and may remain several years on the field with high production levels. In Spain stands alfalfa may remain for 3 to 5 years, or sometimes even more (Delgado and Lloveras 2020). Alfalfa plants develop a robust main root with many secondary roots that allow the plant to deep on the soil (Delgado 2020a). As a legume, alfalfa has the capability to fix atmospheric nitrogen in its roots because is provided by symbiotic nitrogen fixing bacteria that live in root nodules. Fertilization with phosphorous and potassium is very important to ensure a good crop establishment and yield maintenance level, tolerance to diseases and cold resistance. Therefore, a rotation with alfalfa increases soil nitrogen levels improves soil structure and drainage and can help in a strategy to improve health for following crops.

Alfalfa crop development may be divided into three stages: plant establishment, vegetative growth and flowering and fruiting. Once the plant has been established, the vegetative growth is the most concern to the alfalfa fodder production. The union of the root with the aerial parts is called the crown and the plant has the capability of produce new stems if it is harvested without damaging the crown.

In Spain, most of the alfalfa is grown under irrigation (around 70% (Lloveras et al. 2020)) being blanket, sprinkler or pivot the irrigation systems. The productive period of alfalfa lasts from the end of winter to autumn and to get fodder the crop is harvested several times during this period. In the Ebro basin, this occurs 5-6 times from the end of April to October in periods of 30-40 days approximately. The period between cuttings corresponds to that of vegetative growth stage. This is the period when plant resources are directed mainly to leaf and stem development. These periods have been named as intercuts in several entomological studies developed in the Ebro basin (Pons et al. 2009, 2011). In relation to this: 1st intercut corresponds to the period from the starting growing season to the 1st cutting, 2nd intercut to the growing period from 1st to the 2nd cutting, and so on. In the Spanish crop system, alfalfa is produced mainly for feed livestock though dehydrated forage, pellets and silages. The pasture of alfalfa stands by sheep or cows is now practically in disuse (Delgado 2020b).

3. Importance of alfalfa in Spain

Nowadays in Spain, alfalfa is considered a traditional component of crop rotations. Alfalfa covers more than 250000 ha in the Spanish territory, accounting for approximately 20% of the cultivation area in Europe (Delgado and Lloveras 2020). The Ebro Basin (western Mediterranean region) is where 60% of the Spanish alfalfa production is concentrated, with 85000 ha approximately in Aragon and, 23000 ha in Catalonia (MAPA 2019).It is one of the main European countries exporting alfalfa (dehydrated or pellets), particularly to the Middle East and China (Capistrós 2020). Dehydrated alfalfa has been exported for four decades and for many years accounted with low percentage of the total weight of alfalfa that was marketed. In recent years, this dynamic has changed and the foreign market has been increasing in importance, reaching 70% of the dehydrated alfalfa produced in Spain. Increased domestic production, with the growing demand for hay from the Middle East and the United Arab Emirates, are the dominant factors, which are driving the market for alfalfa hay in the country. In 2019 the total amount exported was from 342,270 tons and accounted for the export value 341.774€ (MAPA 2019).

4. Potential pests of alfalfa in Spain, relative importance and role of alfalfa as arthropod reservoir

The plurianuality of the crop, in spite of the crop management, make alfalfa fields a much more stable habitat than other extensive field crops such as winter cereals, maize, sunflower or ryegrass. This allows many arthropods to use alfalfa as a habitat for feeding, development, reproduction, and shelter (Summers 1998; Núñez 2002; Pons and Nuñez 2020). Some studies carried out in the Ebro basin, the main cultivation area in Spain, reported the presence of more than 300 species (Núñez 2002; Pons and Eizaguirre 2008; Núñez et al. 2014). These arthropod species are included in different ecosystem functional groups, from herbivores to decomposers, pollinators and species with carnivorous habits.

Alfalfa production presents some critical aspects derived from the interaction with certain phytophagous insects that can reduce production and decrease quality. Preventing foliage loss or damaged is very important for high quality livestock feed. The Spanish literature reports a long list of phytophagous arthropods that feed on alfalfa, going from Collembola to Diptera, and including Hemiptera, Coleoptera and Lepidoptera (Table 1). However, there is only a little number of species that can cause economic damages. In Spain they are some Coleoptera: *Hypera postica* Gyllenhal (alfalfa weevil), *Colaspidema barbarum* Fabricius (black beetle), and *Holotrichapion pisi* Fabricius (apion weevil); aphids: *Acyrthosiphon pisum* Harris (pea aphid), *Aphis craccivora* Koch (cowpea aphid), and *Therioaphis trifolii* Monell (spotted alfalfa aphid);

and lepidopteran leaf feeders, such as *Helicoverpa armigera* Hübner (cotton bollworm), *Spodoptera exigua* Hübner (beet armyworm), and *Loxostege sticticalis* Linnaeus (beet webworm) (Gurrea 1981; Gimeno and Perdiguer 1993; Martín et al. 2020; Pons and Nuñez 2020). In Aragon also the collembolan *Sminthurus viridis* Linnaeus (lucerne flea) may damage alfalfa stands sporadically (Gimeno and Perdiguer 1993; Baquero and Jordana 1999; Martín et al. 2020; Pons and Nuñez 2020). The alfalfa weevil and the apion weevil damage plants from the beginning of the plant development in spring to the first intercut. The alfalfa black beetle may damage plants during the second or third intercuts. Aphids are present on alfalfa during all the productive period but only produce damages in summer in case of very high populations and drought or plant hydric stress. Lepidopteran leaf feeders may attack the crop in the middle summer. Although these pests have a Palearctic origin, there is little information on their control in the European scientific literature and much of the information comes from professionals and technicians for dissemination to farmers. As an example, the technical reports and leaflets from Gimeno and Perdiguer (1993), Cambra et al. (2000), or Núñez et al. (2014). Among the scientific works developed, they mainly refer to insect species composition (Núñez 2002), to aphids (Pons 2002, 2019; Pons et al. 2005, 2011, 2013) or even to lepidopteran leaf feeders that can attack alfalfa (Cabello 1986; Pons and Eizaguirre 2008), but not related to other important pests, such as *C. barbarum* or *H. postica*.

On the other hand, it must be taken into account that alfalfa works also as a refuge to a very important number of species considered natural enemies, which provide ecosystem services such as biological pest control (predation and parasitism). Alfalfa is considered a reservoir and a source of natural enemies that exert natural biological pest control, not only on alfalfa, but also on other crops in the surrounding landscape (Summers 1998; Madeira et al. 2014, 2019; Meseguer et al. 2021). In the Ebro basin, during the past 10 years, more than 200 species of insects and arachnids with predatory habits and more than 50 species of insect parasitoids have been cited (Núñez 2002; Pons et al. 2011) . Therefore, control practices in alfalfa stands should always regard this important role of the crop and, for this reason, sustainable control strategies should be implemented. In this sense, recent works dealing with the role of alfalfa as a reservoir of natural enemies in a context of agroecosystem and landscape contribute for its better understanding (di Lascio et al. 2016; Clemente-Orta et al. 2020; Madeira et al. 2021)

Table 1: Phytophagous species considered as potential pests of alfalfa in Spain (Cabello 1986; Domínguez 1989; Cambra et al. 2000; Núñez 2002; Alfaro 2005; Pons et al. 2005).

Order	Family	Species
Collembola	Sminthuridae	*Sminthurus viridis*
Hemiptera	Miridae	*Lygus rugulipennis*
		Lygus pratensis
		Lygus punctatus
		Lygus wagneri
		Otros Lygus
		Adelphocoris lineolatus
		Polymerus cognatus
		Deraeocoris serenus

		Deraeocoris ribauti
		Creontiades pallidus
		Trigonotylus caelestialum
		Notostira erratica
	Cercopidae	*Philaenus spumarius*
	Cicadellidae	*Empoasca fabae*
	Aphididae	*Acyrthosiphon pisum*
		Aphis craccivora
		Therioaphis trifolii
Thysanoptera	Thripidae	*Frankliniella occidentalis*
		Other species
Coleoptera	Chrysomelidae	*Colaspidema barbarum*
	Curculionidae	*Holotrichapion pisi*
		Apion pisi
		Apion apricans
		Sitona lineatus
		Sitona discoideus
		Sitona humeralis
		Hypera postica
		Hypera zoilus
		Otiorrhynchus sp.
		Tychius aureolus
		Tychius medicaginis
	Coccinellidae	*Subcoccinella vingintiquatorpunctata*
Lepidoptera	Gelechiidae	*Dichomeris lotellus*
	Pyraliadae	*Loxostege sticticalis*
	Noctuidae	*Helicoverpa armigera*
		Spodoptera exigua
		Spodoptera litoralis
		Autographa gamma
		Trichoplusia sp.
	Pieridae	*Colias sp.*
Diptera	Cecidomyiidae	*Dasineura medicaginis*
	Agromyziidae	*Liriomyza sp.*

5. The alfalfa weevil pest status

One of the most voracious leaf eaters that affect alfalfa is the alfalfa weevil. The damage is concentrated during the first intercut of alfalfa, although there may be some affectation to the regrowth of the second one (Hoffmann 1963). In recent years this insect has become the most concerning pest of this crop in the Ebro Basin (Pons and Nuñez 2020), and one of the most important in the entire world (Hoff et al. 2002; Goosey 2012; Saeidi and Moharramipour 2017; Soroka et al. 2019). In Spain, damages caused by the larvae can account for 25–40% of the yield (Alfaro 2005). This pest is closely related with alfalfa and it is rarely found attacking other legume species or plants from other families (Hoffmann 1963). Although *H. postica* has origins in Eurasia (Hoffmann 1963), very little information about its ecology, pest status and control strategies has been reported in the scientific literature in Europe. Accordingly, it is unclear how agronomic practices, biotic stress and abiotic factors such as temperature affect its activity in European crops.

5.1. Biological cycle

The literature where the life cycle of *H. postica* in Europe has been described follows Hoffmann (1963). According to this, after a summer aestivation, females lay eggs in clusters inside alfalfa stems. The resulting larvae, hatched at the end of winter and the beginning of spring, feed on leaves and new plant buds, suffer three moults and at the end of the fourth larval development instar pupate between leaflets or under the windrow in white cocoons. Its feeding activity reduces forage yield and the quality of alfalfa. The adults emerge after a few days and continue in the field, but without causing any serious damage. Recent adults mate and females can lay egg in spring for a time entering later in a summer aestivation period to be newly active in autumn. However, field observations and studies conducted in the Ebro Basin (Pons and Nuñez 2020) suggest that differences in the described life cycle may exist under Spanish conditions. Therefore, it is needed to clearly establish the *H. postica* life cycle in order to properly manage this pest and to evaluate the consequences of environmental or crop management changes on its populations.

Since insects are ectothermic animals, temperature influences their survival, development, reproduction, and biological activities and, hence, their population dynamics (Price 1997; Gillott 2005; Régnière et al. 2012). Therefore, submitting populations of H. postica to different temperature regimes will allow determining the duration of the development stages, the reproduction capacity and growing population parameters and to develop prospective phenological models. The sampling of *H. postica* in alfalfa fields to determine its occurrence and phenology in real time will allow the validation of these models. Studies on the effect of temperature on the fitness of the alfalfa weevil have been developed with North American and Middle East Asian populations through the analysis of the temperature-dependent development rate and life tables (Sweetman and Wedemeyer 1933; Zahiri et al. 2010a, b; Ullah et al. 2020). However, no data have been reported by European populations, except those of Levi-Mourao et al. (2021a) for the alfalfa weevil embryonic development.

5.2. Control of *H. postica*

Several control methods against the alfalfa weevil have been commonly used in Spain (Martín et al. 2020; Pons and Nuñez 2020) and other world countries (Summers 1998), from crop management to insecticide spraying.

5.2.1 Chemical control

The principles of integrated pest management are mandatory in Spain since 2014 (Real Decreto 1311/2012, Ministerio de la Presidencia, 2012) for fields o farms with more than 5 ha surface (Plan de Acción Nacional). These principles consider chemical control as the last control strategy option, which should only be resorted to when other possible strategies have failed to keep the population levels of the pest, or its damage, below the tolerance thresholds limits. Alfalfa is mainly used to feed livestock, especially dairy farmers, and it is a natural insectary and a source of biodiversity in the agricultural ecosystem. Therefore, chemical control against the alfalfa weevil should be avoided to prevent the presence of insecticide residuals in livestock food and to preserve the ecological service provided by predators and parasitoids (Pons 2019). In Spain, despite these facts, farmers use one to three insecticide spraying applications. These insecticides contain active ingredients belonging to the pyrethroid family whose mode of action on the insect is the same. Beyond their detrimental effects on natural enemies, the efficacy of these treatments is questionable and may enhance the development of pest resistance (Rethwisch et al. 2019). In order to follow the principles of IPM and avoid the use of pesticides (reported as the last strategy option), it is necessary that alternative control methods such as crop management and biological control are effective and economically viable. However, not enough information on how to use these methods and their effectiveness is reported in the Spanish literature.

5.2.2. Crop management

Cultural control, i.e., early harvesting, grazing or intercropping, was proposed to combat the alfalfa weevil many years ago, and its efficacy has been reported, particularly in North American countries (Casagrande and Stehr 1973; Pellissier et al. 2017). Onstad and Shoemaker (1984) have claimed that a robust strategy is to always harvest early. Studies conducted in northern Catalonia close to the Pyrenees, in Spain, have concluded that, given the production alfalfa system there, this strategy is highly favourable (Pons and Nuñez 2020). Another form of control is to carry out a cut during the winter dormancy period of alfalfa, which should really be considered as a prevention method. Cutting the alfalfa shortly before it comes out of winter dormancy can eliminate a large number of eggs, significantly reducing the damage and population levels of this pest in spring (Núñez et al. 2015; Pons and Nuñez 2020). However, the studies supporting this methodology were developed under particular conditions and in a reduced number of fields. Therefore, the winter cutting control strategy would need to be contrasted when more diverse crop conditions occur in order to validate its effectiveness. Grazing alfalfa during winter dormancy can have a similar effect, sanitizing the crop by eliminating eggs and larvae. This practice has been common until a few years ago in many producing areas of Spain, but it is being abandoned in much of the Ebro basin, due to a shortage of herds and shepherds (Delgado 2020b).

5.2.3. Natural enemies and biological control

Alfalfa weevil is attacked by a reduced complex of natural enemies. Adults are parasitized by individuals of the genus *Microctonus* spp. (Drea, 1968; Van Driesche and Gyrisco, 1979; Mertins, 1984; Vink et al., 2012). In Europe, the information available on this native parasitoid as a control agent is very scarce, although it is known that it can attack adults of different Curculionidae species, i.e, *Sitona* spp. (Manglitz 2001; Barratt et al. 2012).

H. postica larvae are parasitized by solitary endoparasitoid wasps of the genus *Bathyplectes* Fröster (Hymenoptera: Ichneumonidae). These parasitoids, native from Eurasia, were imported into the United States (being the alfalfa weevil an exotic species there) in a classic biological control program which achieved notable success in the north-eastern states (Radcliffe and Flanders 1998; Rand 2013). In spite of this, the knowledge of the biology, phenology, population dynamics and control effectiveness in European and Mediterranean conditions is fairly unknown. *Bathyplectes anura* Thomson and *Bathyplectes curculionis* Thomson are usually found in alfalfa fields in the Ebro Basin, but their abundance and parasitism rates are very variable between years (Pons and Nuñez 2020; Levi-Mourao et al. 2021b). The identification of adult wasps is challenging because there are only slight morphological differences between species, especially in males (Soroka et al. 2020). The species can be more easily distinguished by the characteristics of the puparia (Day 1970; Dysart and Day 1976) but for definitive parasitoid identification and parasitism rate evaluation, each *H. postica* larva must therefore be reared until pupation. From this reason, developing DNA analysis techniques that allow predation and parasitism to be followed in a quicker way and do not require maintaining host insect in controlled-environment chambers would facilitate this task (Wolf et al. 2018; Agustí et al. 2020; Batuecas et al. 2021; Molina et al. 2021). Other studies otherwise inaccessible, such as trophic interactions, can be facilitated using the high sensitivity and fidelity of molecular methods (Traugott et al. 2013). Such methods require the development of specific molecular probes to detect target organisms. In arthropods, mitochondrial gene evolution has already been well studied, and divergent sequences in related populations provide a source of species-specific polymerase chain reaction (PCR) primers (Black et al. 1989; Simon et al. 1994).

Larvae of *H. postica* can also be infected by the entomopathogenic fungus *Zoophthora phytonomi* (Arthur) (Zygomycetes: Entomophthorales). Its role in the regulation of larval populations has not been sufficiently studied neither the interaction that can occur between the attack of this fungus and the parasitized *H. postica* larvae by *Bathyplectes* spp. Epizootics of *Z. phytonomy* have been reported in Europe (Papierok et al. 1986), including Spain (Pons and Nuñez 2020). It is known that this fungus remains in the soil and infects larvae of *H. postica* when environmental humidity conditions are adequate and when the density of alfalfa weevil larvae is higher (Los and Allen 1982, 1983). The infection is exacerbated in years with high rainfall during the winter and the beginning of spring (Radcliffe and Flanders 1998).

In addition, several species of lady beetles (Coleoptera: Coccinellidae) (Evans and Youssef 1992; Richards and Evans 1998; Davidson and Evans 2010; Meseguer et al. 2021), damsel bugs (Hemiptera: Nabidae) (Hussain 1975; Ouayogode and Davis 1981; Rand 2017) and lacewings (Neuroptera: Chrysopidae) (Lavallee and Shaw 1969; Pellissier et al. 2017) are also known consume *H. postica* as an alternative prey, when their natural prey is scarce.

6. Influence of landscape on the occurrence of the alfalfa weevil

Agriculture uses to occur in landscapes with a mosaic of habitats with different crop and non-crop plants, different phenology and different cultural practices. Agricultural pests are the result of the interactions between crop plants, herbivorous species natural enemies and the surrounding environment (Rand 2013; Rand and Lundgren 2019). Plant-insect and pest-natural enemy interactions can be mediated in a negative or positive way by the crop management (sowing date, mowing, harvesting, fertilization, pesticide use) and the physical and biological characteristics of the environment and landscape where the crops are located. In agroecosystems, the relationships between crops, herbivores and their natural enemies can be focused at field, farm or landscape levels (scales) but, in most cases, pest management has been targeted only at field level (scale) (Fahrig and Jonsen 1998; Fahrig et al. 2011). However, crops are usually a part of the farm crop rotation and there is a current tendency to expand the focus of these relationships to a wider scale where the landscape plays a determinant role (Fahrig and Jonsen 1998; Bianchi et al. 2006; Tscharntke et al. 2007; Rusch et al. 2016). Population dynamics and trophic interactions depend on processes at spatial scale larger than a single agricultural field and different species in a given area may experience that area quite differently. Landscape dimension of IPM is a consequence of two features of the biology of agricultural pests and their antagonists. The first is that most of them are capable to move between different habitats along their life; the second is that they need resources that often are not provided by a unique habitat. Therefore, movement between habitats will assure the access to the totality of resources needed for individual survival, development and reproduction. Crop pest and natural enemy populations may be affected by the landscape surrounding fields in positive, negative or indifferent effects (Tscharntke et al. 2007; Karp et al. 2018). The structure of the landscape is dynamic and influences the persistence, dispersal capacity and distribution of the organisms that inhabit these habitats, including pests and their natural enemies. In these circumstances, landscape composition (crops, natural areas, roads, rivers, etc.) and configuration (patch size, patch distribution and connectivity) will be major forces driving establishment, abundance and population dynamics of pests and their antagonists in crops.

So far, alfalfa has been described mainly as a unit independent of its environment analysing the pests and the control methodologies that can be applied at field level (scale). Only some recently studies have considered relationships of alfalfa and other crops at farm or landscape scale, mainly about predator species (Madeira et al. 2014, 2019, 2021; di Lascio et al. 2016; Clemente-Orta et al. 2020). However, none of these studies focused on the alfalfa weevil and its natural enemies. Little is known about the movement capacity of alfalfa weevil adults, and how the abundance of this pest may be affected by other alfalfa fields or by other landscape components. It has been reported that field margins provide overwintering and summer aestivation sites (Manglitz 1958; Dennis and Fry 1992; Holland and Fahrig 2000) for pest like the alfalfa weevil. No data is available on how landscape composition and configuration may affect the abundance of the alfalfa weevil and its main natural enemies.

REFERENCES

Agustí, N., Castañé, C., Fraile, I., Alomar, O., 2020. Development of a PCR-based method to monitor arthropod dispersal in agroecosystems: *Macrolophus pygmaeus* (Hemiptera: Miridae) from banker plants to tomato crops. Insect Sci. 27, 1125–1134.

Alfaro, A., 2005. Entomología Agraria. Los parásitos animales de las plantas cultivadas. Publicaciones de la Excma. Diputación de Soria. Soria, España.

Baquero, E., Jordana, R., 1999. Notas sobre la presencia de *Sminthurus viridis* (Linnaeus, 1758) como plaga de la alfalfa en la ribera del río Arga (Navarra). Boletín Sanid Veg Plagas 107–110.

Barratt, B.I.P., Oberprieler, R.G., Barton, D.M., Mouna, M., Stevens, M., Alonso-Zaragoza, M.A., Vink, C.J.,Ferguson, C.M., 2012. Could research in the native range, and non-target host range in Australia, have helped predict host range of the parasitoid *Microctonus aethiopoides* Loan (Hymenoptera: Braconidae), a biological control agent introduced for *Sitona discoideus* Gyllenhal (Coleoptera: Curculionidae) in New Zealand? BioControl 57, 735–750.

Basigalup, D., Irwin, J., Fugui, M.I., Abdelguerfi-Laour, M., 2014. Perspectives of alfalfa in Australia, China, Africa and Latin America. Legum Perspect 9–10.

Batuecas, I., Agustí, N., Castañé, C., Alomar, O., 2021. Molecular tracking of insect dispersal to verify arthropod predator movement from an alfalfa field to a peach orchard. Biol. Control 158, 104506.

Bianchi, F.J.J.A., Booij, C.J.H., Tscharntke, T., 2006. Proceeding of The Royal Society B. In: Sustainable pest regulation in agricultural landscapes: a review on ladscape cmposition, biodiversity and natural pest control. pp 273, 1715-1727.

Bortolazzo, E., Aldo, Dal A., Davolio, R., Ruozzi, F., 2016. Produttività e composizione dell'erba medica. L'informatore Agrario. pp 1, 40-43.

Black, W., McLain, D., Rai, K., 1989. Patterns of variation in the rDNA cistron within and among world populations of a mosquito, *Aedes albopictus* (Skuse). Genetics 121, 539–550.

Cabello, T., 1986. Natural enemies of noctuid pests (Lep.: Noctuidae) on alfalfa, corn, cotton and soybean in Southern Spain. J. Appl. Entomol. 108, 80–88.

Cambra, M., Gimeno, F., Martín, J., Mendoza, E., Perdiguer, A., 2000. Insectos que afectan a la producción de forraje de alfalfa. Informaciones técnicas 1/2000. Dirección General de Tecnología Agraria. Gobierno de Aragón.

Capistrós, J., 2020. Comercialización. In: Lloveras J, Delgado I, Chocarro C (eds) Alfalfa - Agronomía y utilización. Edicions de la Universitat de Lleida. Centro de Investigación y Tecnología Agroalimentaria de Aragón, Lleida- Zaragoza, España, pp. 343–364.

Casagrande, R.A., Stehr, F.W., 1973. Evaluating the effects of harvesting alfalfa on alfalfa weevil (Coleoptera: Curculionidae) and parasite populations in michigan. Can. Entomol. 105, 1119–1128.

Columela L circa 70 AC. Los doce libros de agricultura. En: CJ Castro (Trad.).1959. Obras Maestras. Ed. Iberia, Barcelona, España.

Clemente-Orta, G., Madeira, F., Batuecas, I., Sossai, S., Juárez-Escario, A., Albajes, R. 2020. Changes in landscape composition influence the abundance of insects on maize: The role of fruit orchards and alfalfa crops. Agric. Ecosyst. Environ. 291, 106805.

Cubero, J.I., 1999. El libro de agricultura de Al Awan. Empresa pública para el desarrollo Agrario y pesquero de Andalucía, Málaga.

Davidson, L.N., Evans, E.W., 2010. Frass analysis of diets of aphidophagous lady beetles (Coleoptera: Coccinellidae) in Utah alfalfa fields. Environ. Entomol. 39, 576–582.

Day, W.H., 1970. The survival value of its jumping cocoons to *Bathyplectes anurus*, a parasite of the Alfalfa Weevil. J. Econ. Entomol. 63, 586–589.

Delgado, I., 2020a. Botánica. In: Lloveras J, Delgado I, Chocarro C (eds) La alfalfa - Agronomia y Utilización. Edicions de la Universitat de Lleida. Centro de Investigación y Tecnología Agroalimentaria de Aragón, Lleida- Zaragoza, España, pp. 33–42.

Delgado, I., 2020b. La alfalfa en pastoreo. In: Lloveras J, Delgado I, Chocarro C (eds) La alfalfa - Agronomia y Utilización. Edicions de la Universitat de Lleida. Centro de Investigación y Tecnología Agroalimentaria de Aragón, Lleida- Zaragoza, España, pp. 315–323.

Delgado, I., Lloveras, J., 2020. Historia y distribución de la alfalfa. In: Lloveras J, Delgado I, Chocarro C (eds) La alfalfa, agronomía y utilización. Edicions de la Universitat de Lleida, Centro de Investigación y Tecnología Agroalimentaria de Aragón, Lleida- Zaragoza, España, pp. 17–32.

Dennis P., Fry, G.L.A., 1992. Field margins: can they enhance natural enemy population densities and general arthropod diversity on farmland? Agric. Ecosyst. Environ. 40, 95–115.

di Lascio, A., Madeira, F., Costantini, M.L., Rossi, L., Pons, X., 2016. Movement of three aphidophagous ladybird species between alfalfa and maize revealed by carbon and nitrogen stable isotope analysis. BioControl 61, 35–46.

Domínguez, F., 1989. Plagas y enfermedades de las plantas cultivadas, 8th edn. Mundi Prensa, Madrid, Spain.

Drea, J.J., 1968. Castration of male alfalfa weevils by *Microctonus spp.* J. Econ. Entomol. 61, 1291–1295.

Van Driesche, R.G., Gyrisco, G.G., 1979. Field studies of *Microctonus aethiopoides*, a parasite of the adult Alfalfa weevil, *Hypera postica*, in New York. Environ. Entomol. 8, 238–244.

Dysart, R.J., Day, W.H., 1976. Release and recovery of introduced parasites of the alfalfa weevil in Eastern North America, 1st edn. Agricultural Research Service - United States Department of Agriculture, Washington D.C.

Evans, E.W., Youssef, N.N., 1992. Numerical responses of aphid predators to varying prey density among Utah alfalfa fields. J. Kansas Entomol. Soc. 65, 30–39.

Fahrig, L., Baudry, J., Brotons, L., Burel, F.G., Crist, T.O., Fuller, R.J., Sirami, C., Siriwardena, G.M., Martin, J.L., 2011. Functional landscape heterogeneity and animal biodiversity in agricultural landscapes. Ecol. Lett. 14, 101–112.

Fahrig, L., Jonsen, I., 1998. Effect of habitat patch characteristics on abundance and diversity of insects in an agricultural landscape. Ecosyst. 12, 197–205.

Frame, J., Charlton, J., Laidlaw, A., 1998. Temperate forrage legumes. CAB international. London, UK.

Gardner, D., Putman, D.H., 2018. Proceedings. Second world alfalfa congress, Córdoba, Argentina. 11-14 noviembre. In: Alfalfa situation in the USA and Canada. Instituto Nacional de Tecnología Agropecuaria (INTA), pp. 30–33.

Gillott, C., 2005. Entomology, 3rd edn. Springer. Dordrecht, The Netherlands.

Gimeno, F., Perdiguer, A., 1993. Estudio sobre la evolución en Aragón de los insectos fitófagos en alfalfa. Boletín Sanid. Veg. Plagas, pp. 379–387.

Goosey, H.B., 2012. A degree-day model of sheep grazing influence on alfalfa weevil and crop characteristics. J. Econ. Entomol. 105, 102–112.

Gurrea, P., 1981. Ciclo biológico de *Hypera variabilis* Herst (Col.:Curculionidae) en la España central. Boletín Sanid. Veg. Plagas, pp. 147–156.

Hoff, K.M., Brewer, M.J., Blodgett, S.L., 2002. Alfalfa weevil (Coleoptera: Curculionidae) larval sampling: comparison of shake-bucket and sweep-net methods and effect of training. J. Econ. Entomol. 95, 748–753.

Hoffmann, A., 1963. Sous-famille des Curculionidae, Tribu des Hyperini, Les *Hypera* (syn: *Phytonomus*). In: Balachowsky, A.S. (ed). Entomologie appliquée a l'agriculture. Tome I. Coléoptères. Second volume., 2nd edn. Masson et Cíe., Paris, Francia, pp. 984–989.

Holland, J., Fahrig, L., 2000. Effect of woody borders on insect density and diversity in crop fields: a landscape-scale analysis. Agric. Ecosyst. Environ. 78, 115–122.

Humphries, A., Stewart, A.G., Newman, A.J., Ballard, R.A., 2018. Proceedings. Second World Alfalfa Congress, Córdoba, Argentina. 11-14 noviembre. In: Overview of the economic utilization and traits of interest of lucerne in Australia. Instituto Nacional de Tecnología Agropecuaria (INTA), pp. 20–24.

Hussain, M., 1975. Predators of the alfalfa weevil, *Hypera postica* in Western Nevada: a greenhouse study (Coleoptera: Curculionidae). J. New York Entomol. Soc. 83, 226–228.

Karp, D.S., Chaplin-Kramer, R., Meehan, T.D., Martin, E.A., DeClerck, F., Grab, H., Gratton, C., Hunt, L., Larsen, A.E., Martínez-Salinas, A., O'Rourke, M.E., Rusch, A., Poveda, K., Jonsson, M., Rosenheim, J.A., Schellhorn, N.A., Tscharntke, T., Wratten, S.D., Zhang, W., Iverson, A.L., Adler, L.S., Albrecht, M., Alignier, A., Angelella, G.M., Anjum, M.Z., Avelino, J., Batáry, P., Baveco, J.M., Bianchi, F.J.J.A., Birkhofer, K., Bohnenblust, E.W., Bommarco, R., Brewer, M.J., Caballero-López, B., Carrière, Y., Carvalheiro, L.G., Cayuela, L., Centrella, M., Ćetković, A., Henri, D.C., Chabert, A., Costamagna, A.C., De la Mora, A., de Kraker, J., Desneux, N., Diehl, E., Diekötter, T., Dormann, C.F., Eckberg, J.O., Entling, M.H., Fiedler, D., Franck, P., van Veen, F.J.F., Frank, T., Gagic, V., Garratt, M.P.D., Getachew, A., Gonthier, D.J., Goodell, P.B., Graziosi, I., Groves, R.L., Gurr, G.M., Hajian-Forooshani, Z., Heimpel, G.E., Herrmann, J.D., Huseth, A.S., Inclán, D.J., Ingrao, A.J., Iv, P., Jacot, K., Johnson, G.A., Jones, L., Kaiser, M., Kaser, J.M., Keasar, T., Kim, T.N., Kishinevsky, M., Landis, D.A., Lavandero, B., Lavigne, C., Le Ralec, A., Lemessa, D., Letourneau, D.K., Liere, H., Lu, Y., Lubin, Y., Luttermoser, T., Maas, B., Mace, K., Madeira, F., Mader, V., Cortesero, A.M., Marini, L., Martinez, E., Martinson, H.M., Menozzi, P., Mitchell, M.G.E., Miyashita, T., Molina, G.A.R., Molina-Montenegro, M.A., O'Neal, M.E., Opatovsky, I., Ortiz-Martinez, S., Nash, M., Östman, Ö., Ouin, A., Pak, D., Paredes, D., Parsa, S., Parry, H., Perez-Alvarez, R., Perović, D.J., Peterson, J.A., Petit, S., Philpott, S.M., Plantegenest, M., Plećas, M., Pluess, T., Pons, X., Potts, S.G., Pywell, R.F., Ragsdale, D.W., Rand, T.A., Raymond, L., Ricci, B., Sargent, C., Sarthou, J.P., Saulais, J., Schäckermann, J., Schmidt, N.P., Schneider, G., Schüepp, C., Sivakoff, F.S., Smith, H.G., Whitney, K.S., Stutz, S., Szendrei, Z., Takada, M.B., Taki, H., Tamburini, G., Thomson, L.J., Tricault, Y., Tsafack, N., Tschumi, M., Valantin-Morison, M., van Trinh, M., van der Werf, W., Vierling, K.T., Werling, B.P., Wickens, J.B., Wickens, V.J., Woodcock, B.A., Wyckhuys, K., Xiao, H., Yasuda, M., Yoshioka, A., Zou, Y., 2018. Crop pests and predators exhibit inconsistent responses to surrounding landscape composition. Proc. Natl. Acad. Sci. 115, E7863–E7870.

Lavallee, A.G., Shaw, F.R., 1969. Preferences of golden-eye lacewing larvae for pea aphids, leafhopper and plant bug nymphs, and alfalfa weevil larvae. J. Econ. Entomol. 62, 1228–1229.

Levi-Mourao, A., Madeira, F., Meseguer, R., García, A., Pons, X., 2021a. Effects of temperature and relative humidity on the embryonic development of *Hypera postica* Gyllenhal (Col.: Curculionidae). Insects 12, 3, 250.

Levi-Mourao, A., Meseguer, R., Pons, X., 2021b. Parasitism of the alfalfa weevil *Hypera postica* Gyllenhal by *Bathyplectes* spp. in Spain. In: Proceedings of the 1st International electronic conference on entomology (https://doi.org/10.3390/IECE-10505).

Lloveras, J., Delgado, I., Chocarro, C., Cavero, J., 2020. Riegos. In: Lloveras J, Delgado I, Chocarro C (eds) La alfalfa - Agronomia y Utilización. Edicions de la Universitat de Lleida. Centro de Investigación y Tecnología Agroalimentaria de Aragón, Lleida- Zaragoza, España, pp. 93–104.

Los, L.M., Allen, W.A., 1983. Incidence of *Zoophthora phytonom*i (Zygomycetes: Entomophthorales) in *Hypera postica* (Coleoptera: Curculionidae) larvae in Virginia. Environm. Entomol. 12, 1318–1321.

Los, L.M., Allen, W.A., 1982. Visual technique for determining presence and stage of *Zoophthora phytonomi* in dead alfalfa weevil larvae. Entomol. Soc. Am. 75, 375–377.

Madeira, F., Clemente-Orta, G., Alomar, O., Batuecas, I., Sossai, S., Albajes, R., 2021. Land use alters the abundance of herbivore and predatory insects on crops: the case of alfalfa. J. Pest Sci. 1, 1–19.

Madeira, F., di Lascio, A., Carlino, P., Constantini, M.L., Rossi, L., Pons, X., 2014. Stable carbon and nitrogen isotope signatures to determine predator dispersal between alfalfa and maize. Biol. Control 77, 66–75.

Madeira, F., di Lascio, A., Costantini, M.L., Rossi, L., Rosch, V., Pons, X., 2019. Intercrop movement of heteropteran predators between alfalfa and maize examined by stable isotope analysis. J. Pest Sci. 92, 757–767.

Manglitz, G.R., 2001. A spatial model for the successful biological control of *Sitona discoideus* by *Microctonus aethiopoides*. J. Appl. Ecol. 38, 162–169.

Manglitz, G.R., 1958. Aestivation of the alfalfa weevil. J. Econ. Entomol. 51, 506–508.

MAPA (Ministerio de Agricultura, Pesca y Alimentación), 2019. Anuario de estadística. https://www.mapa.gob.es/es/estadistica/temas/publicaciones/anuario-de-estadistica/2020/default.aspx?parte=3&capitulo=07. Accessed in March 23, 2022.

Martín, Á., Núñez, E., Rodríguez, E., 2020. Guía de gestión integrada de plagas (GIP) - Alfalfa. Ministerio de Agricultura, Pesca y Alimentación, Madrid, España.

Mertins, J.W., 1984. Notes on *Microctonus* spp. (Hymenoptera: Braconidae) introduced to Iowa against the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae). Gt. Lakes Entomol.

17, 10.

Meseguer, R., Levi-Mourao, A., Pons, X., 2021. Species complex and temporal associations between coccinellids and aphids in alfalfa stands in Spain. Insects 12, 971.

Michaud, R., Lehman, W.F., Rumbaugh, M.D., 1988. World distribution and historical development. In: Hanson, A.A., Barnes, D.K., Hill, R.R. (eds) Alfalfa and alfalfa improvement. Agronomy 29. American Society of America, Agronomy, Crop Science Society of America, Soil Science Society of America, Madison,WI., USA, pp. 25–91.

Ministerio de la Presidencia. Gobierno de España, 2012. Real Decreto 1311/2012, de 14 de septiembre, por el que se establece el marco de actuación para conseguir un uso sostenible de los productos fitosanitarios. https://www.boe.es/eli/es/rd/2012/09/14/1311. Accessed in January 12, 2021.

Molina, P., Martínez-Ferrer, M.T., Campos-Rivela, J.M., Riudavets, J., Agustí, N., 2021. Development of a PCR-based method for the screening of potential predators of the African citrus psyllid *Trioza erytreae* (Del Guercio). Biol. Control 160, 104661.

Nastis, S.A., Papanagiotou, E., Zmanidis, S., 2012. Productive efficiency of subsidized organic alfalfa farms. J. Agric. Resour. Econ. 37, 280–288.

Núñez, E., 2002. La alfalfa como reservorio de enemigos naturales. PhD thesis. Universitat de Lleida.

Núñez, E., Rodríguez, E., Perdiguer, A., 2014. Insectos y arácnidos beneficiosos para la agricultura. Informaciones técnicas. Dirección General de Alimentación Agraria y Centro de Investigación y Tecnología Agroalimentaria de Aragón.

Núñez, E., Taberner, A., Muñoz, F., Delgado, I., 2015. Efecto del corte de la alfalfa en parada invernal en el control de *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae). Oral comunication, SEEA Valencia.

Onstad, D.W., Shoemaker, C.A., 1984. Management of alfalfa and the alfalfa Weevil (*Hypera postica*): An example of systems analysis in forage production. Agric. Syst. 14, 1–30.

Orloff, S.B., 1997. Introduction. In: Orloff, S.B., Karlson, H.L., Tauber, L.R. (eds) Intermountain alfalfa management. Publication 3366. University of California. Division of Agriculture and Natural Resources, Oakland, pp. 2–3.

Ouayogode, B.V., Davis, D.W., 1981. Feeding by selected predators on alfalfa weevil larvae. Environ. Entomol. 10, 62–64.

Papierok, B., Aeschlimann, J.P., Loan, C., 1986. Two entomophthoralean fungi occurring on *Hypera postica* in southern France. J. Invertebr. Pathol. 48, 377–380.

Pellissier, M.E., Nelson, Z., Jabbour, R., 2017. Ecology and management of the alfalfa weevil (Coleoptera: Curculionidae) in Western United States alfalfa. J. Integr. Pest Manag. 8, 5.

Pons, X., 2002. Niveles poblacionales de pulgones en alfalfa libre de tratamientos aficidas. In: Lloveras J (ed) Producción de pastos, forrajes y céspedes. XLII Reunión Científica de la Sociedad Española para el Estudio de los Pastos, Lleida, Spain, pp. 349–353.

Pons, X., 2019. Control químico de plagas de alfalfa, situación actual y perspectivas. Vida Rural 462 48–52.

Pons, X., Eizaguirre, M., 2008. Cultivos extensivos en regadío: cereales, maíz y alfalfa. In: Jacas, J.A., Urbaneja, A. (eds). Control biológico de plagas agrícolas. Phytoma España, Valencia, pp. 384–398.

Pons, X., Lumbierres, B., Albajes, R., 2009. Heteropterans as aphid predators in inter-mountain alfalfa. Eur. J. Entomol. 106, 369–378.

Pons, X., Lumbierres, B., Antoni, R., Starý, P., 2011. Parasitoid complex of alfalfa aphids in an IPM intensive crop system in northern Catalonia. J. Pest Sci. 84, 437–445.

Pons, X., Lumbierres, B., Comas, J., Madeira, F., Starý, P., 2013. Effects of surrounding landscape on parasitism of alfalfa aphids in an IPM crop system in northern Catalonia. BioControl 58, 733–744.

Pons, X., Nuñez, E., 2020. Plagas da la alfalfa: Importancia, daños y estrategias de control. In: Lloveras, J., Delgado, I., Chocarro, C. (eds). La alfalfa, agronomía y utilización. Edicions de la Universitat de Lleida, Centro de Investigación y Tecnología Agroalimentaria de Aragón, Lleida-Zaragoza, España, pp. 167–202.

Pons, X., Núñez, E., Lumbierres, B., Albajes, R., 2005. Epigeal aphidophagous predators and the role of alfalfa as a reservoir of aphid predators for arable crops. Eur. J. Entomol. 519–525.

Price, P.W., 1997. Insect Ecology, 3rd edn. John Wiley and Sons. New York.

Radcliffe, E.B., Flanders, K.L., 1998. Biological control of alfalfa weevil in North America. Integr. Pest Manag. Rev. 3, 225–242.

Rand, T.A., 2013. Host density drives spatial variation in parasitism of the alfalfa weevil, *Hypera postica*, across dryland and irrigated alfalfa cropping systems. Environ. Entomol. 42, 116–122.

Rand, T.A., 2017. Assessing the role of generalist predators in the biological control of alfalfa weevil (Coleoptera: Curculionidae). Can. Entomol. 149, 525–533.

Rand, T.A., Lundgren, J.G., 2019. Quantifying temporal variation in the benefits of aphid honeydew for biological control of alfalfa weevil (Coleoptera: Curculionidae). Environ. Entomol. 48, 141–146.

Régnière, J., Powell, J., Bentz, B., Nealis, V., 2012. Effects of temperature on development, survival and reproduction of insects: experimental design, data analysis and modeling. J. Insect Physiol. 58, 634–647.

Rethwisch, M.D., Peairs, F., Pierce, J., Mostafa, A., Price, S., Ramirez, R., Rondon, S.,Schell, S., Vardiman, J., Walsh, D.B., Wanner, K., Wenninger, E., 2019. Insecticide resistance in alfalfa weevil and related implications in other alfalfa insect pests. In: Proceedings. Western Alfalfa and Forage Symposium, Reno, NV, Nov.19-21.

Richards, D.R., Evans, E.W., 1998. Reproductive responses of aphidophagous lady beetles (Coleoptera: Coccinellidae) to nonaphid diets: an example from alfalfa. Ann. Entomol. Soc. Am. 91, 632–640.

Rusch, A., Chaplin-Kramer, R., Gardiner, M.M., Hawro, V., Holland, J,, Landis, D., Thies, C., Tscharntke, T., Weisser, W.W., Winqvist, C., Woltz, M., Bommarco, R., 2016. Agricultural landscape simplification reduces natural pest control: a quantitative synthesis. Agric. Ecosyst. Environ. 221, 198–204.

Saeidi, M., Moharramipour, S., 2017. Physiology of cold hardiness, seasonal fluctuations, and cryoprotectant contents in overwintering adults of *Hypera postica* (Coleoptera: Curculionidae). Environ. Entomol. 46, 960–966.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., Flook, P., 1994. Evolution weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am. 87, 651–701.

Soroka, J., Bennett, A.M.R., Kora, C., Schwarzfeld, M.D., 2020. Distribution of alfalfa weevil (Coleoptera: Curculionidae) and its parasitoids on the Canadian Prairies, with a key to described species of Nearctic *Bathyplectes* (Hymenoptera: Ichneumonidae). Can. Entomol. 152, 663–701.

Soroka, J., Grenkow, L., Cárcamo, H., Meers, S., Barkley, S., Gavoski, J., 2019. An assessment of degree-day models to predict the phenology of alfalfa weevil (Coleoptera: Curculionidae) on the Canadian Prairies. Can. Entomol. 152, 110–129.

Summers, C.G., 1998. Integrated pest management in forage alfalfa. Integr. Pest Manag. 3, 127–154.

Sweetman, H.L., Wedemeyer, J., 1933. Further studies of the physical ecology of the alfafa weevil, *Hypera postica* (Gyllenhal). Ecology 14, 46–60.

Thiébeau, P., Parnaudeau, V., Guy, P., 2003. Quel avenir pour la luzerna en France et en Europe? Courrier de l'environnement de l'INRA, 49.

Traugott, M., Kamenova, S., Ruess, L., Seeber, J., Plantegenest, M., 2013. Empirically characterising trophic networks: whats emerging DNA-based methods, stable isotope and fatty acid analyses can offer. Adv. Ecol. Res. 49, 177–224.

Tscharntke, T., Bommarco, R., Clough, Y., Crist, T.O., Kleijn, D., Rand, T.A., Tylianakis, J.M., Van Nouhuys, S., Vidal, S., 2007. Conservation biological control and enemy diversity on a landscape scale. Biol. Control 43, 294–309.

Ullah, M.S., Kamimura, T., Gotoh, T., 2020. Effects of temperature on demographic parameters of *Bryobia praetiosa* (Acari: Tetranychidae). J. Econ. Entomol. 113, 211–221.

Vink, C.J., Barratt, B.I.P., Phillips, C.B., Barton, D.M., 2012. Moroccan specimens of *Microctonus aethiopoides* spice our understanding of genetic variation in this internationally important braconid parasitoid of adult weevils. BioControl 57, 751–758.

Wolf, S., Zeisler, C., Sint, D., Romeis, J., Traugott, M., Collatz, J., 2018. A simple and cost-effective molecular method to track predation on *Drosophila suzukii* in the field. J. Pest Sci. 91, 927–935.

Zahiri, B., Fathipour, Y., Khanjani, M., Moharramipour, S., Zalucki, M.P., 2010a. Modeling demographic response to constant temperature in *Hypera postica* (Coleoptera: Curculionidae). J. Econ. Entomol. 103, 292–301.

Zahiri, B., Fathipour, Y., Khanjani, M., Moharramipour, S., Zalucki, M.P., 2010b. Preimaginal development response to constant temperatures in *Hypera postica* (Coleoptera: Curculionidae): picking the best model. Environ. Entomol. 39, 177–189.

RESEARCH OBJECTIVES

The general aim of this thesis focuses on to achieve more accurate information about the biology and the ecology of one of the most important alfalfa pest over the world, *H. postica*, in order to forecast its seasonal occurrence in the western Mediterranean region and to develop sustainable control methods to prevent damages. For this reason we have studied the effect of temperature on the fitness of this pest; the efficacy of crop management and its effect on the alfalfa weevil natural enemies; the host-prey relationships between this pest and its natural parasitoid using molecular methods; and the influence of the surrounding landscape on the abundance of this pest, a specific pest of alfalfa with low mobility and therefore more influenced by crop characteristics and management.

<u>The specific objectives of this thesis are:</u>

1) To determine the effect of temperature on the fitness of *H. postica* to forecast its phenology in the Ebro basin

Although the effect of temperature on *H. postica* has been studied with North American and Middle East Asian populations, no reports with European populations exist in spite of being an Eurasian species. In this chapter the fitness of Spanish alfalfa weevil populations raised at constant different temperatures is studied, using life tables and models describing the temperature-dependent development rate. The results obtained will be used to predict the life cycle and population dynamics in the Mediterranean crop conditions of the Ebro Basin. This objective is addressed to Chapter I of this thesis.

2) To evaluate the effectiveness of an extra winter cutting as an alternative control strategy against the alfalfa weevil and its effect on its larval parasitoids: *Bathyplectes* spp.

This objective aims to corroborate the previous results obtained by the CITA Aragón and by the UdL's Entomology group, which showed that a winter alfalfa cutting may reduce spring populations of the alfalfa weevil. In this chapter we evaluate the effectiveness of this method in reducing *H. postica* populations and how it affects the rates of parasitism due to *Bathyplectes* spp. in an extended number of alfalfa growing areas of the Ebro Basin, that includes much more commercial fields with a wider range of surfaces and two mowing machinery. This objective is addressed to Chapter II of this thesis.

3) To determine host-prey relationships between *H. postica* and *Bathyplectes* spp. using molecular methods

This objective deals with the set-up of a molecular tool for distinguishing the two main *H. postica* parasitoid species, *Bathyplectes anura* and *Bathyplectes curculionis*, by developing specific primers to detect early parasitism and evaluate the parasitism rates at field conditions. This objective is addressed to Chapter III of this thesis.

4) To determine the effect of the landscape composition on the field abundance of *H. postica* and its natural enemies

This objective will analyse the effect of landscape composition, determined by the percentage of different cover types at different scales (250, 500, and 1000m from the sampled field), and field variables on the field abundance of eggs, larvae, and adults of *H. postica*, and the abundance and effectiveness of its natural enemies. This objective is addressed to Chapter IV of this thesis.

OUTLINE OF CHAPTERS

Chapter I:

Levi-Mourao, Alexandre; Madeira, Filipe; Meseguer, Roberto; Pons, Xavier (2022). Effects of temperature on the fitness of the alfalfa weevil (*Hypera postica* Gyllenhal). This chapter has been published in *Pest Management Science*. **DOI:** 10.1002/ps.7040. Impact factor: 4.85.

Levi-Mourao, Alexandre; Madeira, Filipe; Meseguer, Roberto; García, Addy; Pons, Xavier (2021). Effects of temperature and relative humidity on the embryonic development of *Hypera postica* Gyllenhal (Col.: Curculionidae). This chapter has been published in *Insects*. **DOI:** 10.3390/insects12030250. Impact factor: 2.82.

Chapter II:

Levi-Mourao, Alexandre; Núñez, Eva; García, Addy; Meseguer, Roberto; Pons, Xavier (2022). Alfalfa winter cutting: Effectiveness against the alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) and effect on its rate of parasitism due to *Bathyplectes* spp. (Hymenoptera: Ichneumonidae). This chapter has been published in *Crop Protection*. **DOI:** 10.1016/j.cropro.2021.105858. Impact factor: 2.68.

Chapter III:

Levi-Mourao, Alexandre; Muñoz, Pilar; Cerda-Bennasser, Pedro; Meseguer, Roberto; Pons, Xavier (2022). Molecular and morphological identification of *Bathyplectes anura* and *Bathyplectes curculionis* in alfalfa weevil larvae to estimate the rate of parasitism. This chapter has been published in *Biocontrol*. **DOI:** 10.1007/s10526-022-10141. Impact factor: 3.68.

Chapter IV:

Levi-Mourao, Alexandre; Madeira, Filipe; Meseguer, Roberto; Pons, Xavier. Influence of landscape composition on the abundance of the alfalfa weevil (*Hypera postica*) and its natural enemies in alfalfa crops of Northeast Iberian Peninsula. This chapter is on preparation and will be submitted to *Agriculture, Ecosystems & Environment.* Impact factor: 5.56

CHAPTER I

Effects of temperature on the fitness of the alfalfa weevil (*Hypera postica*)

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Effects of temperature on the fitness of the alfalfa weevil (*Hypera postica*)

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Abstract

Background: Temperature directly influences the survival, development and reproduction of insects and limits their biological activity. The alfalfa weevil (*Hypera postica* Gyllenhal) is a destructive pest of alfalfa crops in Spain and in most of the alfalfa production countries. The knowledge of how temperature affects the fitness of this pest can be used to predict its activity in current or forecasted environmental conditions and to develop more accurate control strategies.

Results: We evaluated the fitness of alfalfa weevil at eight constant temperatures (8–36 °C) at 4 °C intervals by using life tables and models for the description of temperature-dependent development rates. We found that most of the parameters we studied were temperature dependent. Development was observed at all temperatures. However, postembryonic survival was optimal at 16 °C but very low at 36 °C. Adults did not reproduce at 8, 32 or 36 °C. The highest fecundity and net reproductive rate were observed at 20 °C, but the highest intrinsic rate of increase occurred at 24 °C. We predicted the phenology of the pest based on the heat needed for development, the cumulative degree days from the beginning of October to the end of May, and the day length in the study region.

Conclusion: Our prediction was validated using field data reporting the first occurrence of larval instars and adults, revealing that no more than two generations are possible within an annual cycle. In a hypothetical case where average monthly temperatures increase by 1–2 °C, the number of generations would not change.
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Supporting information may be found in the online version of this article.

Keywords: two-sex life table; pest control; alfalfa; development; survival; reproduction

1 INTRODUCTION

Hypera postica Gyllenhal (Coleoptera: Curculionidae), commonly known as the alfalfa weevil, is one of the most destructive pests of alfalfa and has spread globally.[1–4] In Spain, the weevil larvae cause serious damage to the first crop cutting (from March to the end of April) by defoliation, reducing forage quality and yield.[5] Although the weevil originates from Eurasia,[6] little is known about its biology, life cycle or ecology in Europe. The European literature where the *H. postica* life cycle has been described follows Hoffmann.[6] According to this, after a summer aestivation, females lay eggs in clusters inside alfalfa stems. The resulting larvae, hatched at the end of winter and the beginning of spring, feed on leaves and new plant buds, suffer three moults and at the end of the fourth larval development instar pupate between leaflets or under the windrow in white cocoons. Its feeding activity reduces forage yield and the quality of alfalfa. The adults emerge after a few days and continue in the field, but without causing any serious damage. Recent adults mate and females can lay eggs in spring for a time, entering later in a summer aestivation period to be newly active in autumn. The number of generations is variable depending on the climate, and from one to four generations can occur. This life cycle has been also reported in the scarce Spanish literature, where a single generation seems to occur.[7] However, field observations conducted in the Ebro Basin[5] suggest that differences in the described life cycle may exist. Therefore, it is necessary to clearly establish the *H. postica* life cycle to properly manage this pest and evaluate the consequences of environmental or crop management changes on its populations.

Insects are ectothermic animals so the temperature is an important abiotic factor that directly influences their survival, development and reproduction, and limits their biological activity.[8–10] The study of the relationship between temperature and development allows to know how long the different developmental stages and foreseen life cycle in a region will be. Beside this, temperature also affects the reproductive capacity, adult life span and estimate how a population will increase. Therefore, the

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knowledge of how temperature affects the fitness of *H. postica* can be used to predict its activity in current or forecasted environmental conditions and to develop more accurate control strategies.

The relationship between temperature and development time is widely studied and has been described using various models.[11–17] Such models differ in terms of parameter numbers and basic assumptions about temperature effects near the upper and lower limits. The linear model is the easiest to build, but nonlinear models provide a more realistic description of development rate as a function of temperature by estimating the low, high and optimal temperature thresholds for development. Temperature-driven rate models are often used to predict the activity and seasonal population dynamics of pests or their natural enemies, but these models do not consider other factors that also influence insect fitness, such as larval mortality, female fecundity, or adult reproductive time and longevity.[18]

A life table[19–22] is an alternative approach to determine the impact of environmental factors on pest survival, development and fecundity, as well as population dynamics.[23–26] More precisely, life tables contain information on survival rate, developmental rate and fecundity, but also on stage differentiation. Therefore, insect populations should not be structured only by age but by age-stage.[18]

The effect of temperature on the fitness of the alfalfa weevil has been studied using North American and Middle East Asian populations by analysing the temperature-dependent development rate and life tables, and differences between populations of these geographic areas have been reported.[16,27,28] However, to the best of our knowledge, only the embryonic development of European populations has been studied in detail.[29] To address this knowledge gap, we determined the fitness of Spanish alfalfa weevil populations raised at eight constant temperatures, using life tables and models describing the temperature-dependent development rate to obtain essential information that can be used to predict field activity and population dynamics in the current climate and in a perspective of warming due to the climatic change. The data can be used to increase the effectiveness of pest control methods under Mediterranean and European crop conditions.

2 MATERIALS AND METHODS

2.1 Insect rearing

Adult specimens of *H. postica* were collected from a commercial field of alfalfa in Lleida, north-east Spain, in spring 2018. They were reared in 2000-mL glass jars covered with mousseline for proper ventilation, and were maintained at 20 °C and 60–70% relative humidity with an 8-h photoperiod. Fresh alfalfa stems were provided daily for egg laying and feeding. The stems were placed in a glass vial filled with water and sealed with Parafilm to prevent dehydration of the plants and drowning of adult insects. Rearing was refreshed yearly with different location field captured adult individuals to reduce the effects of consanguinity. All *H. postica* individuals used in the experiments were derived from this laboratory population. Experiments were performed during 2019 and 2020.

2.2 Experimental set-up

Separate climate chambers were set to eight different temperatures ranging from 8 to 36 °C at 4 °C intervals, while maintaining the standard parameters of 60–70% relative humidity and an 8-h photoperiod.

The effect of temperature on larval development and survival was studied using eggs laid in the previous 24 h, which were placed in transparent PVC rearing cages (diameter = 53 mm, height = 32 mm) in each of the climate chambers. Hatched larvae were individualized in the same rearing cages. Hatching was confirmed when the larva was able to break through the chorion and leave the eggshell. Accordingly, only eggs producing living larvae were considered (no mortality during the egg stage). The larvae were fed regularly on fresh alfalfa. Instar changes were confirmed by the presence of the exuvia. Instar survival and development were recorded daily, along with the pupal stage and adult emergence. The experiment was complete when all individuals had either died or passed through postembryonic development and emerged as adults. For all calculations, events were assumed to occur at the midpoint between two observations. The experiment was carried out in triplicate.

For the analysis of adult reproduction and longevity, individuals were reared as above until postembryonic development was complete. Healthy male and female pairs were then maintained in the same environment under which they were reared. Only adults that emerged on the same day in each group were paired, to rule out the effect of age on survival. Alfalfa was provided on a regular basis for feeding and oviposition. Female fecundity and adult male and female longevity were recorded every 2 days. The experiment was complete when all paired adults had died. The experiment was carried out in triplicate.

2.3 Data analysis

Data were analysed using an age-stage, two-sex life table[30,31] in TWOSEX-MSChart.[32] This takes into account the stage differentiation, the variable developmental rate among individuals, and both sexes. Accordingly, it can properly describe the development, survival and reproduction of all individuals.[18] The egg/larval/pupal data were collected from an individual-reared cohort of 90–160 individuals, whereas the adult data were collected from a group-reared cohort of 105 individuals per temperature. Adult life table data were converted into individual-reared life tables.[33] The bootstrap-random match technique was then used to construct the life table.[34] The bootstrap technique with 100 000 resamples was used to estimate the standard errors of the parameters.[35] The 0.5th percentile life table was used to calculate the population parameters, whereas the 0.025th and 0.975th percentiles were used to calculate the confidence intervals. The individual parameters were then compared between temperatures using a paired bootstrap test.[36,37]

The following life table parameters were calculated: (a) the age-stage specific survival rate (s_{xj}), the probability that a newly laid egg survives to age x and stage j; (b) the age-specific survival rate (l_x), the probability of survival to age x; (c) the age-stage-specific fecundity (f_{xj}), the fecundity of an individual at age x and stage j; (d) the age-specific fecundity (m_x), the fecundity of the surviving population (number of eggs/individual) at age x; (e) the age-specific net maternity ($l_x.m_x$) the number of offspring produced by an individual at age x when the survival rate is taken into consideration; (f) the female adult pre-oviposition period (APOP), the time between female emergence and the onset of reproduction; (g) the ovipositional period, the period during which female oviposition occurs; (h) the total fecundity, the total number of eggs produced per female during her lifetime.

The following population parameters were calculated[25,38]: (i) the finite rate of increase (λ), the population will increase λ-fold per day when it settles down to the stable age-stage distribution as time

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approaches infinity; (j) the intrinsic rate of increase (r), the population instantaneous growth rate as time approaches infinity and the population reaches the stable age-stage distribution ($e^r = \lambda$); (k) the gross reproduction rate (GRR), the summation of m_x from age 0 to the last age, ignoring the survival rate; (l) the net reproduction rate (R_0), the total number of offspring that an individual can produce during its lifetime; (m) the mean generation time (T), the length of time taken by a population to increase by R_0-fold when the population reaches a stable age-stage distribution.

The Kaplan–Meier method was used to estimate postembryonic and adult survival curves at each temperature. Log-rank tests were used to compare overall survival curves between the eight temperatures. We used the R packages 'survival'[39] and 'survminer'.[40] The adult sex ratio was analysed using a chi-square test. The effect of temperature, sex and their interaction on adult longevity was assessed by two-way analysis of variance (ANOVA). Data were square root transformed [sqrt(x + 0.5)] to achieve, as far as possible, a normal distribution. When significant differences were detected, Tukey's honest significant difference (HSD) test was used for multiple comparisons in the 'multcomp' R package.[41]

We also determined the duration of all developmental stages: egg, larval (different instars and total larval development), pupal, adult (both sexes), postembryonic and whole immature stages, only including individuals that completed development of those stages. We then calculated the corresponding rates of development. To describe the relationship between temperature and developmental rate, we used four mathematical models: a linear model[11] and the nonlinear models Brière-1, Brière-2 and Lactin-2.[13,15] Lower, optimal and upper temperatures were obtained for all immature stages. The three nonlinear models were chosen because they are often used to model coleopteran developmental rates, particularly in species of Curculionidae[42,43] and Coccinellidae.[17,44–46] The equations for each of the adjusted models are shown in Table S1. Only nonlinear models allowed the calculation of the optimum temperature (t_{opt}) and the maximum (t_{max}) developmental threshold, whereas all models allowed us to estimate the lower developmental threshold (t_{min}) as the value intercepting the temperature axis. The goodness of fit in the linear and nonlinear models was evaluated by calculating the coefficient of determination (R^2, a higher value indicating a better fit), the residual sum of squares (RSS, a lower value indicating a better fit), the Akaike information criteria (AIC, a lower value indicating a better fit) and biological criteria. We used the 'devRate' R package for data fitting.[47] Initial parameter estimation for the Lactin-2 model was based on Logan.[14] All statistical analyses referring to model adjustment and comparisons were carried out using R v3.5.2 (R Core Team, 2018). In addition, we calculated the thermal constant (K) of each stage, which can only be estimated using the linear model as the reciprocal of the slope b ($K = 1/b$). This allowed us to predict the occurrence of different development stages in the field. To adjust the linear model, the data value representing 36 °C was omitted because it deviated from the straight line. This was necessary to calculate the parameters K and t_{min} correctly.[48]

We recently determined the *H. postica* oviposition window under Ebro basin conditions.[29] This period starts in the second week of October and ends at the beginning of February. To predict the occurrence of different development stages in the field, we extended this period from the first week of October to the third week of February. Then, we calculated thermal accumulation (degree days or DD) from the start of oviposition in October to the end of May at weekly intervals, according to the mean temperature method, considering the average weekly temperature of the last 10 years and the lower temperature threshold (t_{min}). No upper development threshold was taken into account. Data for weekly temperatures were obtained from the Spanish Agency of Meteorology (AEMET) based on reports from four different meteorological stations along the Ebro basin region (Urgell, Segrià, Baja Cinca and Monegros), covering a total distance of 150 km in a straight line. To validate the predicted occurrence of instar larvae, pupae and adults, we contrasted the data with records of insect phenology from >50 alfalfa fields in the Ebro basin during the period 2018–2020. To obtain these records, we sampled commercial alfalfa fields by stem cutting (200 stems) and net-sweeping (five sweeps in 12 sites of the field) from October to May. Samples were brought to the laboratory where they were maintained in a fridge at 5 °C, in the case of the stems, or frozen before sorting, in case of the sweep-net samples. Stems were dissected and the number of eggs and larvae was recorded. Sweep-net samples were observed under stereoscopic microscope and the larval number and development stage, pupae and adults were recorded.

3 RESULTS

3.1 Pre-imaginal survival and development

The alfalfa weevil was able to complete all developmental stages at all temperatures. The age-stage-specific survival rate (S_{xj}) for the different larval instars and pupa are shown in Fig. 1. Overall, the survival rates mainly decreased at the larval stage. The age-specific survival rate (l_x) is shown in Fig. 2. The overall postembryonic survival curves differed significantly between temperatures ($X^2 = 690$, df = 7, $P < 0.001$). The highest survival rate was recorded at 16 °C and the lowest at 36 °C. The temperature had no significant effect on the sex ratio of the emerging adults (ratio 1:1; $X^2 = 8.59$, df = 7, $P = 0.25$). The developmental time for eggs, each larval instar, total larval development and pupae at each temperature is shown in Table 1. The duration of development was significantly affected by temperature ($P < 0.05$) at all stages and decreased as the temperature rose from 8 to 32 °C.

The linear model and all three nonlinear models closely fitted the relationships between temperature and the rate of development of the egg, larval, pupal, postembryonic and entire immature stages (Table S2). The minimum thresholds for development (t_{min}) in the linear and Lactin-2 models were very close for postembryonic development (6.68 and 6.50, respectively) and total development (6.64 and 6.60, respectively), whereas the Brière-1 model yielded lower values and the Brière-2 yielded some negative values (Table S2). The optimal temperature (t_{opt}) for rapid development was 32–35 °C according to the nonlinear models (Table S2).

The relationship between temperature and total or postembryonic development rates fitted with the Linear and Lactin-2 models is shown in Fig. 3. The thermal constant K was 122 DD for eggs, 284 DD for larvae and 74 DD for pupae. The heat needed to complete postembryonic development was 358 DD, and the heat needed to develop from egg to adult was 484 DD.

According to the extended oviposition period (and taking into account the heat needed for the completion of different developmental stages), the occurrence of larvae in the field would last from the end of October to the third week of April. Eggs laid in first week of October should become adults by the second week of March, whereas eggs laid in the last week of February should become adults by the end of April (Fig. 4). Field data records revealed that first-instar larvae were first recorded in the second

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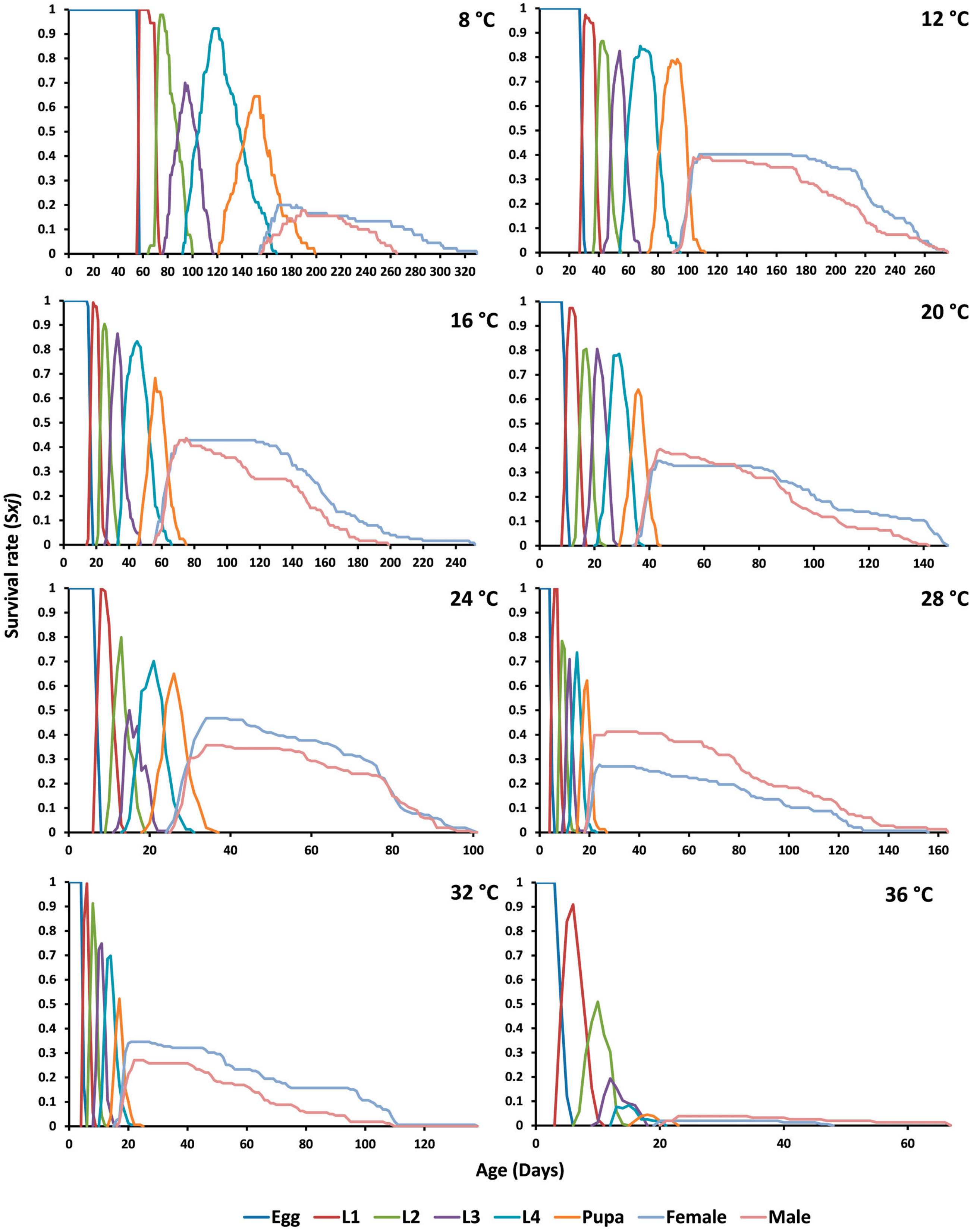

Figure 1. Age-stage-specific survival rates (S_{xj}) of *H. postica* eggs, first to fourth instar larvae (L1–L4), pupae and adults (male and female) at different rearing temperatures.

week of November and the second, third and fourth larval instars in the middle of November, at the end of January and in the middle of March, respectively (Fig. 4). The first adults were recorded in the field during the fourth week of March (Fig. 4). These records confirm that the oviposition period started in the third week of October and not before. Furthermore, given that the highest

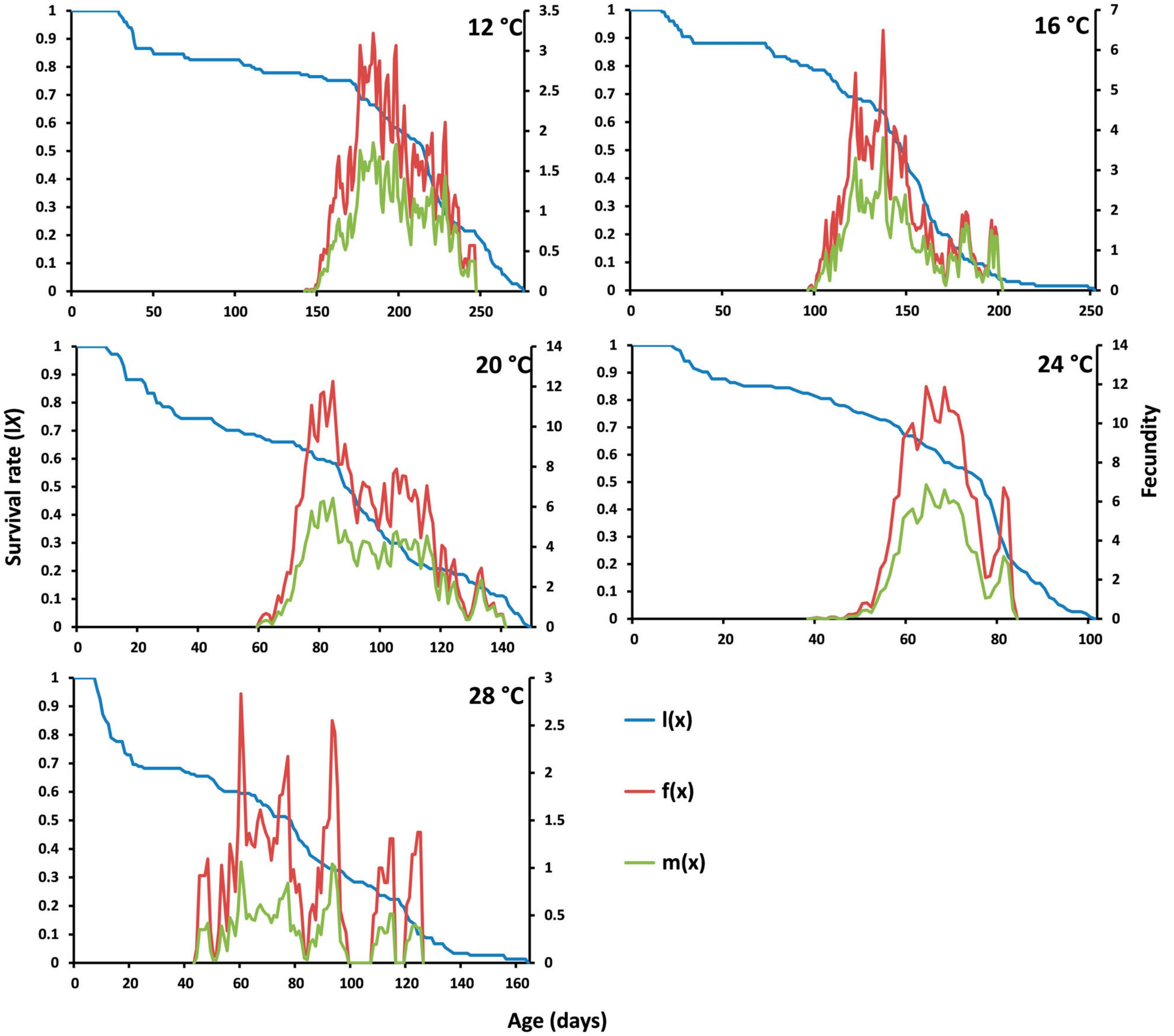

Figure 2. Age-specific survival rate (l_x), age-stage-specific fecundity (f_x) and age-specific fecundity (m_x) of *H. postica* at the five temperatures compatible with reproduction.

proportion of larvae was recorded in the field between the fourth week of March and the third week of April, and that the highest proportion of adults was recorded between the last week of April and the second week of May, our results suggest that the main oviposition period was between the end of January and the second week of February (Fig. S1).

3.2 Adult survival and longevity

The temperature significantly affected both female and male survival ($P < 0.0001$). The higher the temperature, the lower the survival rate ($X^2 = 522$, df = 15, $P < 0.0001$). The female survival rate was higher than that of males at 8 °C ($X^2 = 7.8$, df = 1, $P = 0.05$), 12 °C ($X^2 = 6.3$, df = 1, $P = 0.01$), 16 °C ($X^2 = 14.5$, df = 1, $P < 0.0001$) and 20 °C ($X^2 = 15.7$, df = 1; $P < 0.0001$). Adult survival decreased with advancing age as expected. Survival rates were higher at the beginning of adulthood but decreased more or less abruptly from the middle or last third of the adult lifespan (Fig. 1).

Temperature significantly affected longevity (F = 48.94, df = 7, $P < 0.0001$). The longevity of both male and female adults decreased strikingly when the temperature increased from 20 to 36 °C (Table 2). We found a significant interaction between sex and temperature, but only observed significant differences between the longevity of males and females at 12 °C ($F = 5.16$; $P < 0.0001$) and 16 °C ($F = 4.91$; $P < 0.0001$).

3.3 Reproduction

Reproduction was significantly affected by the temperature ($P < 0.005$). No reproduction was observed at 8, 32 or 36 °C (Table 3). The APOP (time required for the onset of egg laying) was also temperature dependent. It significantly decreased as the temperature rose from 12 °C to 24 °C and then significantly increased at 28 °C ($P < 0.005$). Age-stage-specific fecundity (f_x) and age-specific fecundity (m_x) are shown in Fig. 2, whereas age-specific maternity ($l_x m_x$) is shown in Fig. 5. All values differed significantly between temperatures ($P < 0.05$), increasing as the temperature rose from 12 to 20 °C and then decreasing from 24 °C ($P < 0.05$). Females laid the most eggs (277.02 eggs per individual) at 20 °C ($P < 0.005$) (Table 3).

Table 1. Mean (± SE) developmental time for different developmental stages (egg, larval and pupal) of *H. postica* reared at eight temperatures

Developmental stage	Developmental period															
	8 °C		12 °C		16 °C		20 °C		24 °C		28 °C		32 °C		36 °C	
	n	Days	*n*	Days	*n*	Days	*n*	Days	*n*	Days	*n*	Days	*n*	Days	*n*	Days
Egg	90	56.69 ± 0.05a	149	29.10 ± 0.07b	126	16.76 ± 0.07c	144	9.94 ± 0.05d	154	7.47 ± 0.04e	148	5.22 ± 0.03f	159	5.16 ± 0.03f	155	4.66 ± 0.06ef
L1	88	14.45 ± 0.16a	129	10.02 ± 0.10b	118	6.19 ± 0.10c	127	5.08 ± 0.09d	142	4.18 ± 0.07e	131	3.37 ± 0.07f	155	2.39 ± 0.04g	94	4.07 ± 0.08cdef
L2	86	17.12 ± 0.70a	126	9.76 ± 0.22b	113	6.38 ± 0.15c	127	4.61 ± 0.09d	135	3.60 ± 0.09e	122	2.75 ± 0.07f	148	2.47 ± 0.05g	47	3.51 ± 0.16cdef
L3	83	15.78 ± 0.51a	126	10.84 ± 0.30b	111	8.56 ± 0.23c	115	5.34 ± 0.11d	133	2.98 ± 0.08e	115	2.44 ± 0.06f	129	2.51 ± 0.07fg	23	2.74 ± 0.22efg
L4	64	34.78 ± 0.74a	123	22.07 ± 0.24b	111	15.56 ± 0.25c	107	8.49 ± 0.12d	131	5.95 ± 0.12e	107	3.79 ± 0.09f	110	3.81 ± 0.10f	13	3.23 ± 0.12ef
Total larval stages	64	81.11 ± 1.38a	123	52.76 ± 0.39b	111	36.77 ± 0.44c	107	23.59 ± 0.19d	131	16.64 ± 0.19e	107	12.24 ± 0.11f	110	11.23 ± 0.14g	13	13.77 ± 0.53g
Pupa	43	30.84 ± 1.05a	123	18.41 ± 0.29b	110	9.76 ± 0.23c	107	5.78 ± 0.11d	131	5.28 ± 0.07e	102	3.62 ± 0.10f	98	2.66 ± 0.08g	9	3.00 ± 0.29fg
Postembryonic	43	111.95 ± 1.34a	123	71.17 ± 0.24b	110	46.53 ± 0.54c	107	29.37 ± 0.23d	131	21.92 ± 0.29e	102	15.86 ± 0.22f	98	13.89 ± 0.19g	9	16.77 ± 0.39f
Pre-imaginal	43	166.2 ± 1.40a	123	100.26 ± 0.32b	110	63.19 ± 0.39c	107	39.32 ± 0.20d	131	29.43 ± 0.22e	102	21.11 ± 0.14f	98	18.86 ± 0.13g	9	21.33 ± 0.44f

Different letters in the same row indicate significant differences between temperatures at $P < 0.05$. Standard errors were estimated by 100 000 bootstrap resampling. n = number of individuals.

3.4 Population parameters

Population parameters such as the intrinsic rate of increase (r), GRR and R_0 were also affected by temperature ($P < 0.005$) (Table 4). The r was zero at 8, 32 and 36 °C due to the absence of reproduction at these temperatures. The highest r was observed at 24 °C but the highest GRR and R_0 were observed at 20 °C. The shortest generation time was observed at 24 °C ($P < 0.05$). These values contrasted with the optimal temperature for development predicted by the nonlinear models.

4 DISCUSSION

4.1 Overview

Quantifiable thermal responses in a demographic context are required to understand insect population dynamics, adaptations to climatic conditions and phenological responses in the field. As anticipated, temperature affected the fitness of Spanish *H. postica* populations, influencing individual parameters such as survival, developmental time and reproduction. In turn, population parameters such as the intrinsic rate of increase and the generation time were also affected. This basic knowledge allows us to predict field activity periods and potential adaptations to the environmental warming that is foreseen in the near future.

4.2 Postembryonic survival and development

The effect of temperature on the postembryonic survival of Spanish *H. postica* populations is similar to the impact reported on populations from the Asian Middle East and North America.[27,28] However, we observed the highest larval survival rate at 16 °C, compared to 24 and 22 °C, respectively, in these earlier studies. We also found that eggs were able to hatch at 8 °C and the survival rate at this temperature was high, whereas no hatching was observed at 9 °C[28] and the larval survival rate at 11.5 °C was low. However, eggs have been hatched successfully at 8 °C.[49]

The increasing developmental rate from 8 to 32 °C in our experiments was consistent with North America and Asian Middle East populations.[27,28,49,50] All four models fitted well with the temperature-dependent developmental rate of the *H. postica* population from the Ebro basin. The three nonlinear models predicted an optimal temperature (t_{opt}) of between 32 °C (Brière-1) and 35 °C (Lactin-2) for postembryonic and total development. Such high temperatures only occur in the warmest summer period. The Brière-1 prediction was very similar to that reported for an Iranian population.[28] On the other hand, the minimum developmental thresholds ($t_{\min}$) predicted by the linear and Lactin-2 models were very similar for postembryonic and total development (linear = 6.7 and 6.6, respectively; Lactin-2 = 6.5 and 6.6, respectively), whereas the values predicted by the Brière-1 model were lower (5.0 and 4.3, respectively), suggesting that some development occurs at temperatures slightly below 5 °C. The Briere-2 model estimated a negative minimum temperature threshold, which is not realistic. The $t_{\min}$ reported for all immature developmental stages in Iranian populations was 8.5 °C,[28] higher than in our experiments. This supports the presumption that higher latitudes correspond to lower $t_{\min}$ values.[51]

4.3 Adult fitness

The survival and longevity of *H. postica* adults declined at higher temperatures, as previously reported,[16] suggesting that constant high temperatures are detrimental. We observed higher female longevity at 12 and 16 °C. Similarly, cold weather increased the longevity of females, reflecting their reduced activity and/or lower

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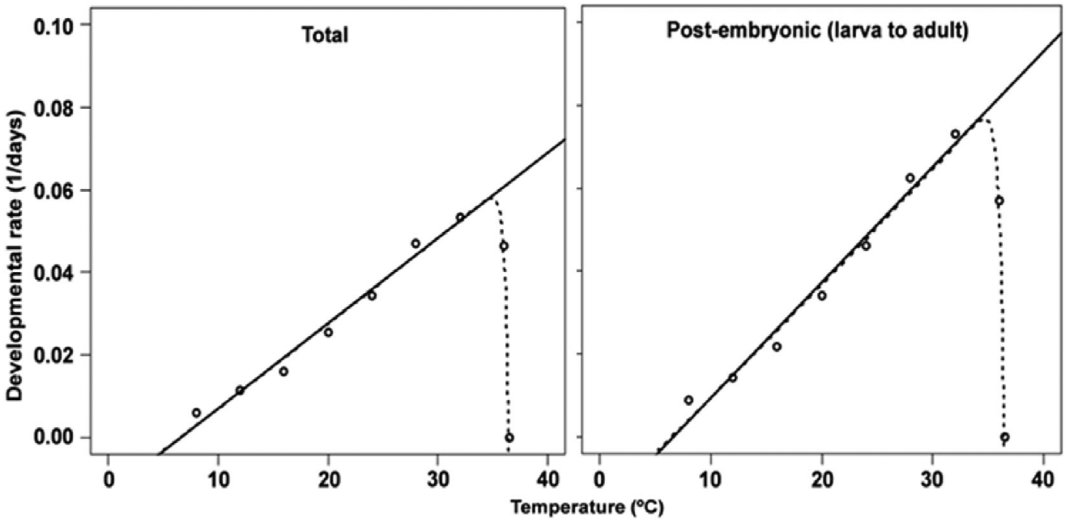

Figure 3. The relationship between temperature and the developmental rate (total and postembryonic) of *H. postica* described by the linear model (solid line) and the Lactin-2 nonlinear model (dashed line).

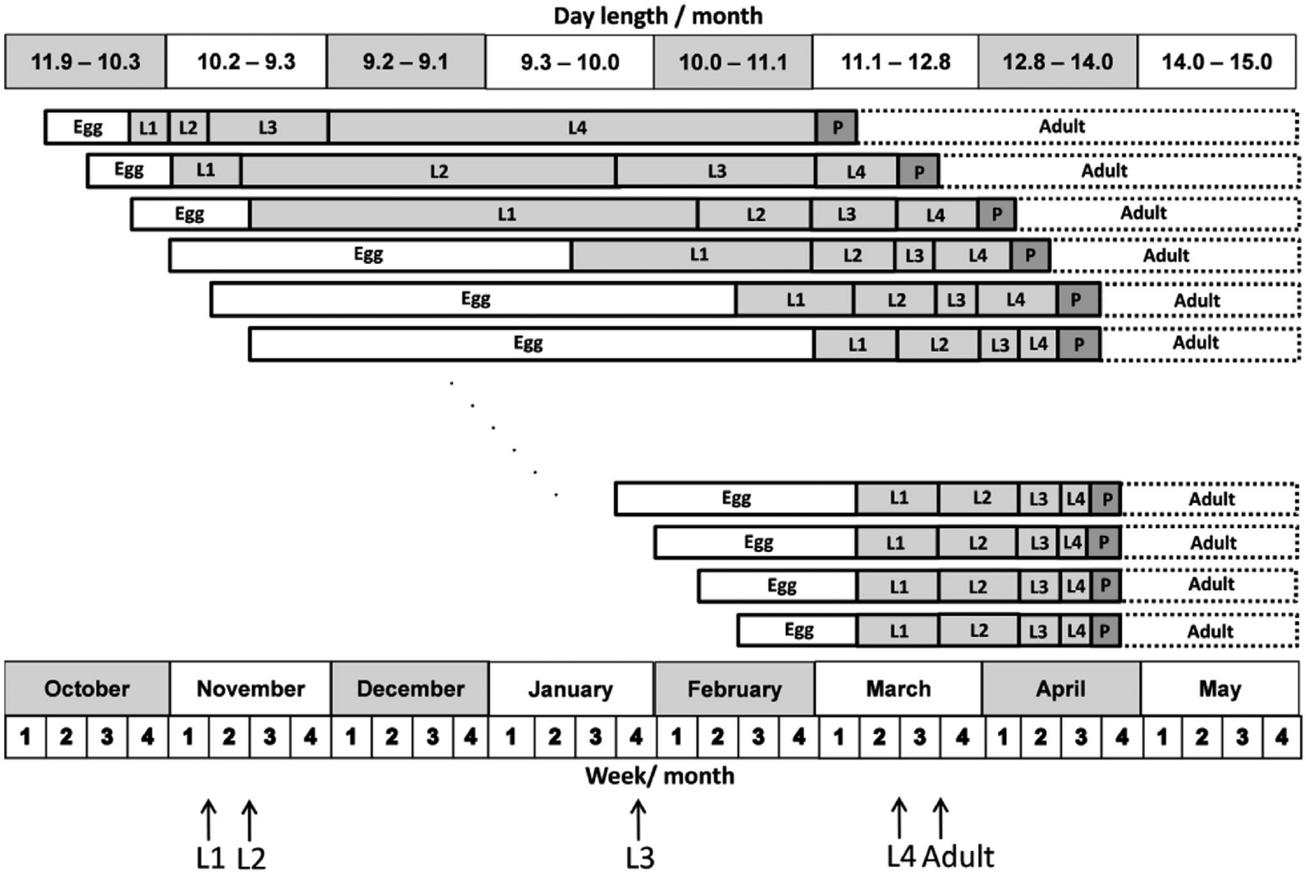

Figure 4. Predicted occurrence of larval instars, pupae and adults according to the degree-days (DD) needed to complete development with an oviposition window from October to February, at weekly intervals (Levi-Mourao *et al*., 2021). Vertical arrows indicate the first record in the field for each of the larval instars and adults. Dots indicate the period when no reproduction occurred.

energy expenditure during reproduction.[52] We also found that female APOP and ovipositional periods decreased significantly as the temperature increased from 12 to 24 °C, as previously reported.[16] Fecundity was also temperature dependent, and females reared at 8, 32 and 36 °C did not lay eggs. These results largely agree with studies of American populations[27,53–55] but partially contrast with adults from Iran, which were able to lay eggs at 31.5 °C.[16] These variations indicate the differences between geographically separated *H. postica* populations. When the age-specific fecundity (m_x) was tempered with the age-specific rate of survival (l_x), the highest reproductive rate was observed at 20 °C, decreasing at 24 and 28 °C. These results

Table 2. Mean (± SE) adult longevity (in days) of *H. postica* males and females reared at eight different temperatures

	Longevity (days)			
Temperature (°C)	*n*	Female	*n*	Male
8 °C	22	80.45 ± 11.23a	21	51.05 ± 7.27a
12 °C	60	130.03 ± 3.21a	63	98.98 ± 5.82b
16 °C	54	101.63 ± 3.92a	56	71.36 ± 4.42b
20 °C	50	71.52 ± 4.10a	57	54.25 ± 3.28a
24 °C	73	44.00 ± 1.88a	58	44.62 ± 2.15a
28 °C	41	69.76 ± 4.70a	61	76.72 ± 3.84a
32 °C	55	59.96 ± 3.68a	43	46.42 ± 3.25a
36 °C	3	24.67 ± 2.40a	6	30.00 ± 5.06a

Standard errors were estimated by 100 000 bootstrap resampling. Different letters in the same row indicate significant differences between sexes at $P < 0.05$. n = number of individuals.

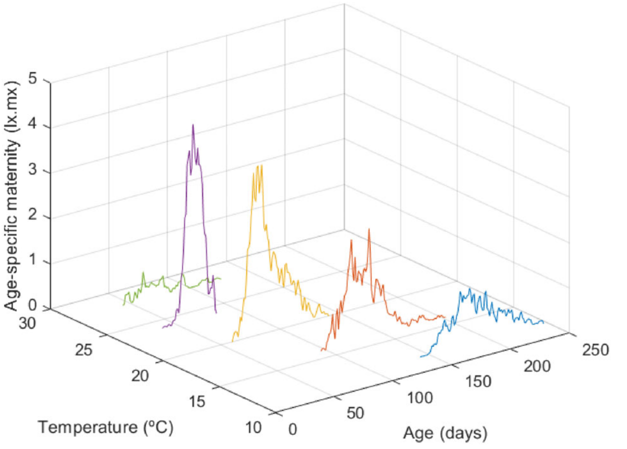

Figure 5. Age-specific temperature dependent maternity ($l_x.m_x$) of *H. postica* at the five temperatures compatible with reproduction.

suggest that Spanish populations of *H. postica* do not perform optimally at high temperatures. Indeed, most of the activity of this insect in the study area is observed during early spring.[29]

4.4 Population fitness

Two-sex life table analysis allows the estimation of important population fitness parameters and integrates data concerning pre-imaginal and adult fitness.[56,57] The intrinsic rate of increase (*r*) integrates the effects of survival and fecundity in a single value determined by the pre-imaginal survival, developmental rate, sex ratio and female fecundity and longevity, all of which are affected by temperature.[18] In our study, *r* increased in the range 12–28 °C and decreased at higher temperatures. A similar profile was described previously,[28] but there were also noticeable differences between the studies. Our maximum *r* (0.0065) occurred at 24 °C whereas the maximum value in the earlier study was 0.1138 at 29 °C.[16] Furthermore, our *r* values were higher than those previously reported in the low temperature range of 12–14 °C.[16] These results suggest that Spanish *H. postica* populations are less well-adapted to higher temperatures than Iranian populations, again highlighting the differences between geographically separated populations.

The highest *r* at 24 °C was offset by the net reproductive rate (R_0) and gross reproduction rate (GRR), which reached maximum values at 20 °C. These results suggest that the temperature range associated with the maximum fitness of Spanish *H. postica* populations is 20–24 °C. This is lower than the temperature predicted for the faster developmental rate because the latter parameter refers to development and not the overall effect on the population fitness, which also takes survival and fecundity into account.[18]

4.5 Phenology and the damage risk period of *H. postica* in the Ebro basin

We predicted the occurrence of *H. postica* at different developmental stages, taking the estimated values of t_{min} and K from the linear model into account, as previously reported.[42,43,58,59] The occurrence of late-stage larvae at the beginning of the productive cycle (late March) increases the risk of damage caused by this pest.[5,29,60,61] Previous studies have suggested that *H. postica* produces more than one generation under the conditions in the Ebro basin,[5,29] thus affecting both the first and second alfalfa intercuts.[7] This is possible given the fast development and high reproductive capacity of this insect at 20–24 °C.

Another factor that should be taken into account is the effect of photoperiod on the induction of adult diapause and aestivation. Larval stages reared under long-day conditions with a day length >12 h induce imaginal diapause.[54,62–68] In the Ebro basin, the day length is <12 h from November to the middle of March, but it increases to 12.8 h by the end of March and to 14 h in April (Fig. 4). Larvae developing early in the season (reaching the final instar before April) therefore do not produce adults with reproductive diapause, whereas this is the more likely fate of larvae

Table 3. Mean (± SE) pre-oviposition (APOP) and ovipositional periods in days, and fecundity as the total number of eggs produced per *H. postica* female reared at the five temperatures compatible with reproduction

	Temperature (°C)									
	12		16		20		24		28	
Female reproduction parameters	*n*	Days	*n*	Days	*n*	Days	*n*	Days	*n*	Days
APOP	55	69.24 ± 1.98a	50	50.88 ± 1.17b	46	32.48 ± 1.21c	56	27.67 ± 0.87d	30	51.60 ± 3.95b
Ovipositional period	55	25.05 ± 1.82a	50	20.04 ± 1.97a	46	22.13 ± 1.73a	56	13.73 ± 0.86b	30	7.47 ± 1.12c
Fecundity (eggs/♀)	60	124.55 ± 14.86b	54	152.35 ± 17.05b	50	277.02 ± 32.25a	73	148.21 ± 15.82b	41	45.22 ± 10.61c

Different letters in the same row indicate significant differences between temperatures at $P < 0.05$. Standard errors were estimated by 100 000 bootstrap resampling. n = number of individuals.

Table 4. *H. postica* population parameters (mean ± SE)

Temperature (°C)	*n*	Finite rate of increase λ (days^{-1})	Intrinsic rate of increase *r* (days^{-1})	Gross reproduction rate (GRR)	Net reproduction rate R_0	Mean generation time *T* (days)
12	149	1.02 ± 0.00084d	0.020 ± 0.00083d	89.07 ± 12.77b	50.14 ± 7.78b	187.59 ± 1.96a
16	126	1.03 ± 0.00121c	0.032 ± 0.00118c	130.67 ± 22.08b	65.29 ± 9.88ab	130.15 ± 1.67b
20	144	1.05 ± 0.00199b	0.053 ± 0.00191b	234.02 ± 38.13a	96.18 ± 15.64a	85.80 ± 1.41c
24	154	1.06 ± 0.00246a	0.065 ± 0.00231a	120.21 ± 14.70b	70.25 ± 9.55ab	65.02 ± 0.61d
28	148	1.04 ± 0.00518c	0.036 ± 0.005c	28.04 ± 6.33c	12.52 ± 3.35c	68.94 ± 3.55d

Finite rate of increase, intrinsic rate of increase, gross reproduction, net reproduction rate and mean generation time were calculated for the five temperatures that were compatible with reproduction. Different letters in the same column indicate significant differences at $P < 0.05$. Standard errors were estimated by 100 000 bootstrap resampling. *n* = number of individuals.

developing from eggs laid at the end of the autumn–winter period, which reach the final larval instar during April.

The first occurrence of spring adults was predicted by the end of March, as confirmed by field sampling (Levi-Mourao *et al*., unpublished). At this time, the mean air temperature is ~12 °C and mated females are not able to lay eggs for nearly 70 days. Although the temperature increases from the end of April to June, it is unlikely that this pre-oviposition period is <1 month (Table 3). In such cases, a theoretical new generation could arise in 2–3 months (Table 4), but the larvae would develop under conditions that induce reproductive diapause and no further generations would arise in the same year. In the context of a warming climate, a temperature increase of 1–2 °C would radically change the first spring adult occurrence. If the temperature were to increase by 1 °C, the first spring adults would appear in the middle of February, but if it increased by 2 °C, they would appear in the third week of December. Even in these hypothetical situations, the temperature would never reach the minimum threshold that allows reproduction (8 °C) until the middle of February, and females would need nearly 2 months to become reproductive (Table 3). Accordingly, the larvae would always develop under the day-length conditions (end of March and April) that induce diapause in the adults.

The success of these two potential generations would be influenced by crop management practices in the Ebro basin. The first alfalfa cutting is usually performed by the end of April or the beginning of May, removing most of the larvae and pupae present on the foliage. However, some larvae may remain in the alfalfa furrow and can infest new alfalfa buds if the forage is not withdrawn quickly.[5,69] Advancing this cutting as far as possible without compromising crop development may therefore help to reduce damage and economic loss. Further cuttings would also help to limit the growth of weevil populations. An additional winter cutting is a good control method to reduce the population and the damage caused in the study region during the first intercut.[69] Based on the annual cycle of the weevil in the region, the best time for cutting is late February.

5 CONCLUSION

We found that the two-sex life table and developmental rate models can help to predict the effect of temperature on the fitness of *H. postica*. Two-sex life tables provide more realistic data on whole-life performance. For example, under Ebro basin conditions, the optimal temperature range for *H. postica* is 20–24 °C. The models (especially the linear model) can determine the minimum development threshold temperature and the heat needed to accomplish each developmental stage. The information derived from both types of approaches allowed us to predict the annual cycle of the alfalfa weevil in the Ebro basin, which has been validated by field records. This knowledge will facilitate the introduction of more successful control strategies, such as the management of cutting to prevent weevil population growth.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION STATEMENT

Conceptualization: Xavier Pons and Alexandre Levi-Mourao. Methodology and validation: Alexandre Levi-Mourao and Xavier Pons. Formal analysis: Alexandre Levi-Mourao, Filipe Madeira and Xavier Pons. Investigation: Alexandre Levi-Mourao and Roberto Meseguer. Resources: Xavier Pons. Writing—original draft preparation: Alexandre Levi-Mourao and Xavier Pons. Writing—review and editing: Alexandre Levi-Mourao, Filipe Madeira, Roberto Meseguer and Xavier Pons. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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REFERENCES

1 Goosey HB, A degree-day model of sheep grazing influence on alfalfa weevil and crop characteristics. *J Econ Entomol* **105**:102–112 (2012).

2 Hoff KM, Brewer MJ and Blodgett SL, Alfalfa weevil (Coleoptera: Curculionidae) larval sampling: comparison of shake-bucket and sweep-net methods and effect of training. *J Econ Entomol* **95**:748–753 (2002).

3 Saeidi M and Moharramipour S, Physiology of cold hardiness, seasonal fluctuations, and cryoprotectant contents in overwintering adults of *Hypera postica* (Coleoptera: Curculionidae). *Environ Entomol* **46**:960–966 (2017).

4 Soroka J, Grenkow L, Cárcamo H, Meers S, Barkley S and Gavloski J, An assessment of degree-day models to predict the phenology of alfalfa weevil (Coleoptera: Curculionidae) on the Canadian Prairies. *Can Entomol* **152**:110–129 (2019).

5 Pons X and Nuñez E, Plagas da la alfalfa: Importancia, daños y estrategias de control, in *La alfalfa, agronomía y utilización*, ed. by Lloveras J, Delgado I and Chocarro C. Edicions de la Universitat de Lleida, Lleida, Spain, pp. 167–202 (2020).

6 Hoffmann A, Sous-famille des Curculionidae, Tribu des Hyperini, Les Hypera (syn: Phytonomus), in *Entomologie appliquée a l'agriculture. Tome I. Coléoptères*, Vol. **2**, ed. by Balachowsky AS. Masson et Cíe, Paris, Francia, pp. 984–989 (1963).

7 Alfaro A, *Entomología Agraria*. Los parásitos animales de las plantas cultivadas. Publicaciones de la Excma, Diputación de Soria (2005).

8 Price PW, *Insect Ecology*, 3rd edn. John Wiley and Sons, New York (1997).

9 Régnière J, Powell J, Bentz B and Nealis V, Effects of temperature on development, survival and reproduction of insects: experimental design, data analysis and modeling. *J Insect Physiol* **58**:634–647 (2012).

10 Gillott C, *Entomology*, 3rd edn. Springer, Dordrecht, The Netherlands (2005).

11 Campbell A, Frazer BD, Gilbert N, Gutierrez AP and Mackauer M, Temperature requirements of some aphids and their parasites. *J Appl Ecol* **11**:431 (1974).

12 Wagner TL, Wu H-I, Sharpe PJH and Coulson RN, Modeling distributions of insect development time: a literature review and application of the Weibull function. *Ann Entomol Soc Am* **77**:475–483 (1984).

13 Lactin DJ, Holliday NJ, Johnson DL and Craigen R, Improved rate model of temperature-dependent development by arthropods. *Environ Entomol* **24**:68–75 (1995).

14 Logan JA, Toward an expert system for development of pest simulation models. *Environ Entomol* **17**:359–376 (1988).

15 Briere JF, Pracros P, Le Roux AY and Pierre JS, A novel rate model of temperature-dependent development for arthropods. *Environ Entomol* **28**:22–29 (1999).

16 Zahiri B, Fathipour Y, Khanjani M, Moharramipour S and Zalucki MP, Modeling demographic response to constant temperature in *Hypera postica* (Coleoptera: Curculionidae). *J Econ Entomol* **103**:292–301 (2010).

17 Kontodimas DC, Eliopoulos PA, Stathas GJ and Economou LP, Comparative temperature-dependent development of *Nephus includens* (Kirsch) and *Nephus bisignatus* (Boheman) (Coleoptera: Coccinellidae) preying on *Planococcus citri* (Risso) (Homoptera: Pseudococcidae): evaluation of a linear and variou. *Environ Entomol* **33**:1–11 (2004).

18 Chi H, You M, Atlıhan R, Smith CL, Kavousi A, Özgökçe MS *et al.*, Age-stage, two-sex life table: an introduction to theory, data analysis, and application. *Entomol Gen* **40**:103–124 (2020).

19 Birch LC, The intrinsic rate of natural increase of an insect population. *J Anim Ecol* **17**:15–26 (1948).

20 Andrewartha HG and Birch C, *The Distribution and Abundance of Animals*, 6th edn. University of Chicago Press, California (1954).

21 Southwood TR, *Ecological Methods: With Particular Reference to the Study of Insect Populations*, 2nd edn. Chapman and Hall. London, U.K., London (1978).

22 Price PW, Denno Robert F, Eubanks MD, Finke DL and Kaplan I, *Insect Ecology: Behavior, Populations and Communities*, 1st edn. Cambridge University Press, Cambridge, UK (2011).

23 Chi H and Getz WM, Mass rearing and harvesting based on an age-stage, two-sex life table: a potato tuberworm (Lepidoptera: Gelechiidae) case study. *Environ Entomol* **17**:18–25 (1988).

24 Bellows TS, Van Driesche RG and Elkinton JS, Life-table construction and analysis in the evaluation of natural enemies. *Annu Rev Entomol* **37**:587–614 (1992).

25 Chi H and Su HY, Age-stage, two-sex life tables of *Aphidius gifuensis* (Ashmead) (Hymenoptera: Braconidae) and its host *Myzus persicae* (Sulzer) (Homoptera: Aphididae) with mathematical proof of the relationship between female fecundity and the net reproductiv. *Environ Entomol* **35**:10–21 (2006).

26 Ren JL, Tu XB, Ge J, Zhao L and Zhang ZH, Influence of temperature on the development, reproduction, and life table of *Calliptamus italicus* (L.) (Orthoptera: Acridoidea). *J Asia-Pac Entomol* **19**:203–207 (2016).

27 Sweetman HL and Wedemeyer J, Further studies of the physical ecology of the alfafa weevil, *Hypera postica* (Gyllenhal). *Ecology* **14**:46–60 (1933).

28 Zahiri B, Fathipour Y, Khanjani M, Moharramipour S and Zalucki MP, Preimaginal development response to constant temperatures in *Hypera postica* (Coleoptera: Curculionidae): picking the best model. *Environ Entomol* **39**:177–189 (2010).

29 Levi-Mourao A, Madeira F, Meseguer R, García A and Pons X, Effects of temperature and relative humidity on the embryonic development of Hypera postica Gyllenhal (Col.: Curculionidae). *Insects* **12**:250 (2021).

30 Chi H and Liu H, Two new methods for the study of insect population ecology. *Bull Inst Zool* **24**:225–240 (1985).

31 Chi H, Life-table analysis incorporating both sexes and variable development rates among individuals. *Environ Entomol* **17**:26–34 (1988).

32 Chi H, Two-sex.-MS Chart: a computer program for the age-stage, two-sex life table analysis, 2021. http://140.120.197.173/ecology/prod02.htm [20 August 2021].

33 Chang C, Huang CY, Dai SM, Atlihan R and Chi H, Genetically engineered ricin suppresses *Bactrocera dorsalis* (Diptera: Tephritidae) based on demographic analysis of group-reared life table. *J Econ Entomol* **109**:987–992 (2016).

34 Amir-Maafi M, Chi H, Chen Z-Z and Xu Y-Y, Innovative bootstrap-match technique for life table set up. *Entomol Gen* (2022). https://doi.org/10.1127/entomologia/2022/1334

35 Akca I, Ayvaz T, Yazici E, Smith CL and Chi H, Demography and population projection of *Aphis fabae* (Hemiptera: Aphididae): with additional comments on life table research criteria. *J Econ Entomol* **108**:1466–1478 (2015).

36 Efron B and Tibshirani RJ, *An Introduction to the Bootstrap*. Chapman and Hall, New York (1993).

37 Wei M, Chi H, Guo Y, Li X, Zhao L and Ma R, Demography of *Cacopsylla chinensis* (Hemiptera: Psyllidae) reared on four fultivars of *Pyrus bretschneideri* (Rosales: Rosaceae) and *P. communis* pears with estimations of confidence intervals of specific life table statistics. *J Econ Entomol* **113**:2343–2353 (2020).

38 Tuan SJ, Lin YH, Yang CM, Atlihan R, Saska P and Chi H, Survival and reproductive strategies in two-spotted spider mites: demographic analysis of *Arrhenotokous parthenogenesis* of *Tetranychus urticae* (Acari: Tetranychidae). *J Econ Entomol* **109**:502–509 (2016).

39 Therneau TM, A package for survival analysis in R, R Packag Version 32-3, 2020. https://cran.r-project.org/web/packages/survival/index.html [14 August 2020].

40 Kassambara A, Kosinski M, and Biecek P, Survminer: Drawing survival curves using "ggplot2," 2020. https://cran.rproject.org/web/packages/survminer/survminer.pdf [14 August 2020].

41 Horton T, Bretz F, Westfall P, Heiberger RM, Schuetzenmeister A, and Scheibe S, Package "multcomp," (2020). https://cran.r-project.org/web/package/multcomp/multcomp.pdf [14 August 2020].

42 Azrag AGA, Yusuf AA, Pirk CWW, Niassy S, Mbugua KK and Babin R, Temperature-dependent development and survival of immature stages of the coffee berry borer *Hypothenemus hampei* (Coleoptera: Curculionidae). *Bull Entomol Res* **110**:207–218 (2020).

43 Davídková M and Doležal P, Temperature-dependent development of the double-spined spruce bark beetle *Ips duplicatus* (Sahlberg, 1836) (Coleoptera; Curculionidae). *Agric For Entomol* **21**:388–395 (2019).

44 Jalali MA, Tirry L and de Clercq P, Effect of temperature on the functional response of *Adalia bipunctata* to *Myzus persicae*. *BioControl* **55**:261–269 (2010).

45 Papanikolaou NE, Milonas PG, Kontodimas DC, Demiris N and Matsinos YG, Temperature-dependent development, survival, longevity, and fecundity of *Propylea quatuordecimpunctata* (Coleoptera: Coccinellidae). *Ann Entomol Soc Am* **106**:228–234 (2013).

46 Lumbierres B, Madeira F, Roca M and Pons X, Effects of temperature and diet on the development and reproduction of the ladybird *Oenopia conglobata*. *Entomol Gen* **41**:197–208 (2020).
47 Rebaudo F, Struelens Q and Dangles O, Modelling temperature-dependent development rate and phenology in arthropods: the DEVRATE package for R. *Methods Ecol Evol* **9**:1144–1150 (2018).
48 De Clercq P and Degheele D, Development and survival of *Podisus maculiventris* (say) and *Podisus sagitta* (fab.) (Heteroptera: Pentatomidae) at various constant temperatures. *Can Entomol* **124**:125–133 (1992).
49 Guppy JC and Mukerji MK, Effects of temperature on developmental rate of the immature stages of the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae). *Can Entomol* **106**:93–100 (1974).
50 Koehler CS and Gyrisco GG, Responses of the alfalfa weevil, *Hypera postica*, to controlled environments. *J Econ Entomol* **54**:625–627 (1961).
51 Hodek I and Honěk A, *Ecology of Coccinellidae*. Kluwer academics, Dordrecht (1996).
52 Armbrust EJ, White CE and Dewitt JR, Lethal limits of low temperature for the Alfalfa Weevil in Illinois. *J Econ Entomol* **62**:464–467 (1969).
53 Parks TH, Effect of temperature upon the oviposition of the alfalfa weevil (*Phytonomus posticus* Gyllenhal). *J Econ Entomol* **7**:417–421 (1914).
54 Litsinger JA and Apple JW, Thermal requirements for embryonic and larval development of the alfalfa weevil in Winsconsin. *J Econ Entomol* **66**:309–311 (1973).
55 Hsieh F and Armbrust EJ, Temperature limits of alfalfa weevil oviposition and egg density in Illinois. *J Econ Entomol* **67**:203–206 (1974).
56 Huang YB and Chi H, Age-stage, two-sex life tables of *Bactrocera cucurbitae* (Coquillett) (Diptera: Tephritidae) with a discussion on the problem of applying female age-specific life tables to insect populations. *Insect Sci* **19**:263–273 (2012).
57 Li WB, Gao Y, Cui J and Sen SS, Effects of temperature on the development and fecundity of *Atractomorpha sinensis* (Orthoptera: Pyrgomorphidae). *J Econ Entomol* **113**:2530–2539 (2020).
58 Jalali M, Mehrnejad MR and Kontodimas DC, Temperature-dependent development of the five psyllophagous ladybird predators of *Agonoscena pistaciae* (Hemiptera: Psyllidae). *Ann Entomol Soc Am* **107**: 445–452 (2014).
59 Jalali M, Sakaki S, Ziaaddini M and Daane KM, Temperature-dependent development of *Oenopia conglobata* (Col.: Coccinellidae) fed on *Aphis gossypii* (Hem.: Aphididae). *Int J Trop Insect Sci* **38**:410–417 (2018).
60 Casagrande RA and Stehr FW, Evaluating the effects of harvesting alfalfa on alfalfa weevil (Coleoptera: Curculionidae) and parasite populations in Michigan. *Can Entomol* **105**:1119–1128 (1973).
61 Dowdy AK, Berberet RC, Stritzke JF, Caddel JL and McNew RW, Late fall harvest, winter grazing, and weed control for reduction of alfalfa weevil (Coleoptera: Curculionidae) populations. *J Econ Entomol* **85**: 1946–1953 (1992).
62 Huggans JL and Blickenstaff CC, Effects of photoperiod on sexual development in alfalfa weevil. *J Econ Entomol* **57**:167–169 (1964).
63 Rosenthal S and Koehler CS, Photoperiod in relation to diapause in *Hypera postica* from California. *Ann Entomol Soc Am* **61**:531–536 (1968).
64 Roberts SJ, DeWitt JR and Armbrust EJ, Predicting spring hatch of the alfalfa weevil. *J Econ Entomol* **63**:921–923 (1970).
65 Dewitt JR and Armbrust EJ, Photoperiodic sensitivity of the alfalfa weevil during larval development. *J Econ Entomol* **65**:1289–1292 (1972).
66 Latheef MA, Parr JC and Pass BC, Factors affecting survival of Kentucky populations of the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae). *Environ Entomol* **8**:1032–1036 (1979).
67 Ohto K, Effects of photoperiod on the adult diapause in the alfalfa weevil, *Hypera postica* (Gyllenhal). *Res Bull Plant Prot Jpn* **32**:1–6 (1996).
68 Schroder RFW and Steinhauer AL, Effects of photoperiod and temperature regimens on the biology of European and United States alfalfa weevil populations. *Ann Entomol Soc Am* **69**:701–706 (1976).
69 Levi-Mourao A, Núñez E, García A, Meseguer R and Pons X, Alfalfa winter cutting: effectiveness against the alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) and effect on its rate of parasitism due to *Bathyplectes* spp. (Hymenoptera: Ichneumonidae). *Crop Prot* **152**:105858 (2022).

SUPPLEMENTARY MATERIAL

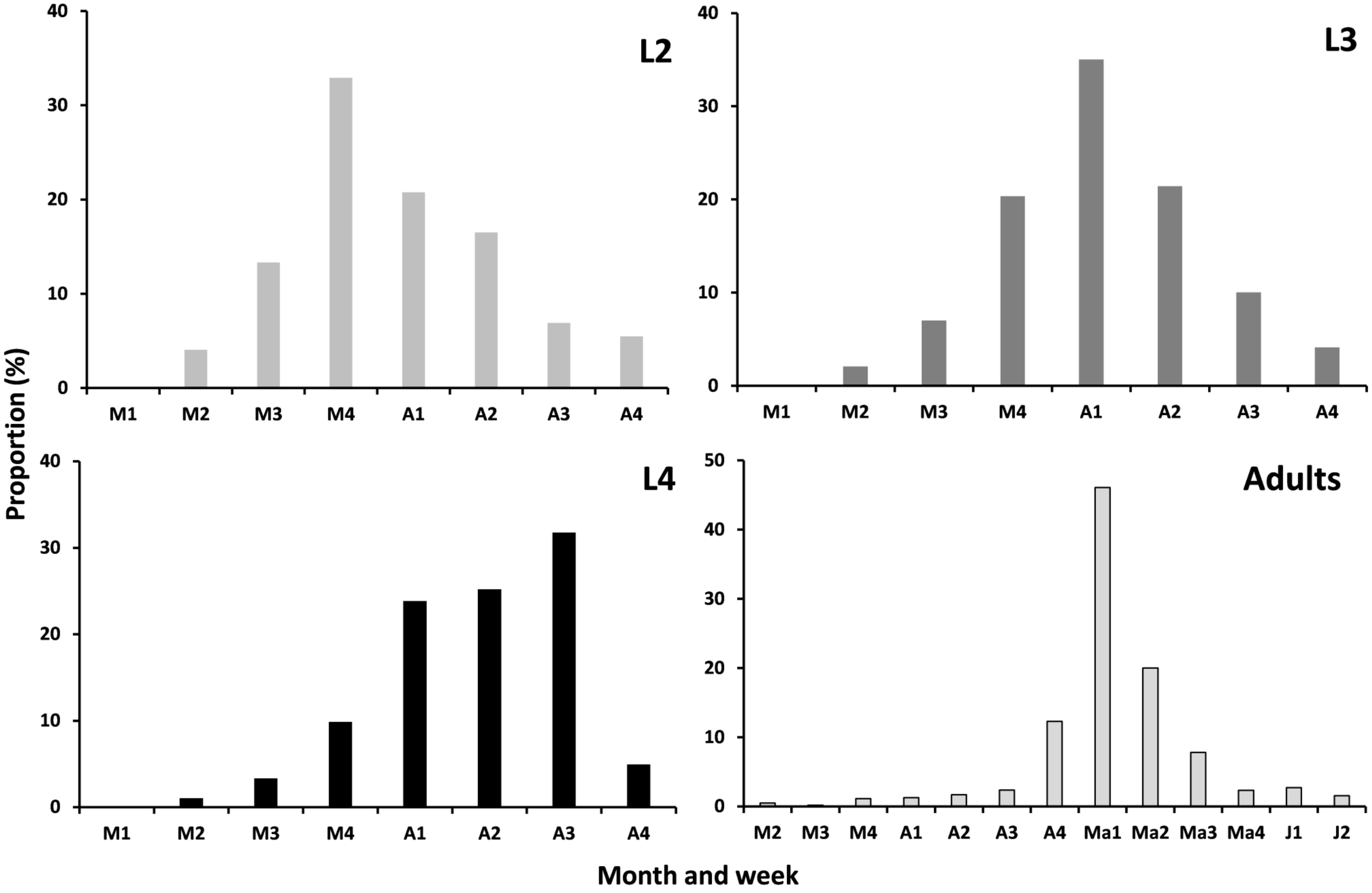
Table S1. Models and corresponding mathematical equations used to fit the relationship between temperature (*T*) and developmental rate (1/*D*).

Model	Equation	Calculated parameters
Linear	$1/D = a + bT$	a, b = constants
Brière-1	$1/D = aT\,(T-T_0)\,(T_L-T)^{1/2}$	a = constant T_0 = low developmental threshold T_L = lethal threshold
Brière-2	$1/D = aT\,(T-T_0)\,(T_L-T)^{1/d}$	d = constant; ≠ in Brière-1
Lactin-2	$1/D = e^{(pT)} - e^{(pTL - (TL - T)/\Delta T)} + \lambda$	*p* = constant TL = lethal maximum temperature ΔT = temp. range over which physiological breakdown becomes the overriding influence Δ = constant

Table S2. Model parameter values (mean ± SE) for the four adjusted models describing the *H. postica* developmental rate.

Model	Parameters	Egg	± SE	Larvae	± SE	Pupa	± SE	Post-embryonic	± SE	Total	±se
Linear	aa	-0.059521	0.012136	-0.022701	0.005336	-0.098725	0.023295	-0.018670	0.003953	-0.013705	0.002765
	bb	0.008204	0.000563	0.003522	0.000248	0.013443	0.001081	0.002794	0.000184	0.002065	0.000128
	K	121.899189		283.921524		74.388157		357.961054		484.378784	
	T_{min} (T_0)	7.255513		6.445303		7.343971		6.683061		6.638460	
	R^2	0.977000		0.975900		0.968700		0.978900		0.981000	
	RSS	0.000711		0.000138		0.002620		0.000075		0.000037	
	AIC	-38.498052		-50.001057		-29.368858		-54.201194		-59.207865	
Lactin-2	ρ	0.007116	0.000410	0.003305	0.000205	0.010871	0.000596	0.002615	0.000145	0.001987	0.000114
	T_L (T_{max})	36.772996	0.113078	37.632734	0.171705	36.762329	0.075405	37.564355	0.145105	37.487186	0.146338
	ΔT	0.164180	0.069454	0.480569	0.072266	0.203013	0.059705	0.411577	0.056356	0.346010	0.052036
	λ	-1.050268	0.010597	-1.020956	0.004809	-1.077447	0.017014	-1.017076	0.003340	-1.013079	0.002587
	t_{opt}	35.700000		34.500000		35.500000		34.700000		35.000000	
	t_{min}	6.900000		6.300000		6.900000		6.500000		6.600000	
	R^2	0.988583		0.985215		0.987753		0.988827		0.988806	
	RSS	0.000656		0.000122		0.001850		0.000058		0.000034	
	AIC	-50.195246		-65.309703		-40.865989		-72.036236		-76.789277	
Brière-1	a	0.000047	0.000015	0.000037	0.000006	0.000082	0.000026	0.000028	0.000005	0.000019	0.000003
	t_{min} (T_0)	2.182000	3.539000	5.150000	2.469000	2.400000	3.738000	5.048000	2.440000	4.285000	2.210000
	t_{max} (T_L)	54.080000	8.944000	38.890000	0.911500	50.360000	6.922000	39.630000	1.107000	40.840000	1.260000
	t_{opt}	43.400000		31.600000		40.600000		32.200000		33.200000	
	R^2	0.989369		0.966597		0.985098		0.971897		0.983037	
	RSS	0.000525		0.000202		0.001749		0.000110		0.000038	
	AIC	-46.351473		-54.005312		-36.721710		-58.852041		-67.297788	
Brière-2	a	0.000000	0.000030	0.000071	0.000006	0.000268	0.000037	0.000057	0.000004	0.000041	0.000003
	t_{min} (T_0)	3.511000	6.776000	-3.411000	4.686000	-4.733000	8.454000	-3.906000	3.689000	-2.468000	3.892000
	t_{max} (T_L)	94.640000	522.400000	36.020000	0.075440	36.240000	1.813000	36.010000	0.037930	36.170000	0.327200
	d	0.665500	5.620000	9.549000	6.063000	20.000000	47.700000	12.480000	7.688000	8.518000	4.708000
	t_{opt}	54.800000		34.100000		35.300000		34.600000		34.100000	
	R^2	0.989615		0.994782		0.992165		0.997293		0.996279	
	RSS	0.000513		0.000032		0.000876		0.000011		0.000008	
	AIC	-44.538853		-66.856954		-40.253702		-75.573651		-77.433844	

Figure S1. Field proportion (%) captures of the three different larval instars and adults of *H. postica* during the sampling periods. Codes on the x-axes refer to the four weeks of March (M), April (A), May (Ma) and June (J).



CHAPTER II

Alfalfa winter cutting: Effectiveness against the alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) and effect on its rate of parasitism due to *Bathyplectes* spp. (Hymenoptera: Ichneumonidae)

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Alfalfa winter cutting: Effectiveness against the alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) and effect on its rate of parasitism due to *Bathyplectes* spp. (Hymenoptera: Ichneumonidae)

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ABSTRACT

The alfalfa weevil *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) is a major pest of alfalfa worldwide. In Spain, insecticide sprayings are mainly used for control with low efficacy. As a potential more sustainable control method, we determined the effectiveness of an alfalfa winter cutting for reducing pest populations in the spring and its interaction with parasitism rate due to *Bathyplectes* spp. (Hymenoptera: Ichneumonidae). Forty-two commercial fields were split in two parts and one was mowed during winter. Larval abundance in each part of the field was sampled by sweep netting in 2019 and 2020 before the first alfalfa spring cutting, when damage is caused. The rates of parasitism due to *Bathyplectes anura* (Thomson) and *Bathyplectes curculionis* (Thomson) were estimated by rearing larvae in the laboratory. Winter cutting significantly reduced the spring larval weevil populations and favored the rate of larval parasitism. Our results suggest that winter cutting can be a useful cultural method for alfalfa weevil control that has potential to be a component of an integrated pest management program.

1. Introduction

Alfalfa, *Medicago sativa* L., is the world's most valuable cultivated forage crop (Orloff, 1997). In Spain, alfalfa is a traditional component of crop rotations. Plant stands remain in the field from 3 to 6 years. Alfalfa covers more than 250000 ha, accounting for approximately 20% of the alfalfa cultivation area in Europe (Delgado and Lloveras, 2020). Spain is the main European country exporting alfalfa (dehydrated or pellets), particularly to the Middle East and China (Capistrós, 2020). Alfalfa management in Spain consists of periodic cutting during the growing season (usually five cuttings from the end of April to the end of September in 30–40 days intervals).

The alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae), is a highly destructive pest in most world regions where alfalfa is cultivated (Goosey, 2012; Saeidi and Moharramipour, 2017), including Spain (Pons and Núñez, 2020). After a summer aestivation, females lay eggs in clusters inside alfalfa stems (Hoffmann, 1963). Studies in Spain have shown that the main egg laying period occurs during the autumn and winter (Pons and Núñez, 2020; Levi-Mourao et al., 2021). The resulting larvae, hatched from the end of winter to the beginning of spring, feed on leaves and new plant buds, thus reducing forage yield and the quality of the first alfalfa intercut, and causing economic losses (Pons et al., 2011; Pons and Núñez, 2020). Damages caused by the larvae can account for 25–40% of the yield (Alfaro, 2005). At the end of the fourth instar larval development, the insects pupate between leaflets in white cocoons. Emerging adults only cause negligible damage. An additional incomplete generation can sometimes occur (Pons and Núñez, 2020).

Alfalfa is a reservoir of natural enemies that contribute to minimizing primary and secondary pest outbreaks not only in alfalfa but also in surrounding crops (Summers, 1998; Madeira et al., 2019). These natural enemies can play an important role in reducing populations of the alfalfa weevil (Summers, 1998; Soroka et al., 2020). *Hypera postica* larvae can be parasitized by *Bathyplectes anura* (Thomson) and *Bathyplectes curculionis* (Thomson) (Hymenoptera, Ichneumonidae). These species are native to Europe and other regions of the Old World (Kingsley et al., 1993; Kuhar et al., 1999; Radcliffe and Flanders, 1998). They were successfully introduced in the USA to control alfalfa weevil (Radcliffe and Flanders, 1998) but have been more effective in eastern than in western USA (Rand, 2013). In Spain, the incidence of these parasitoids

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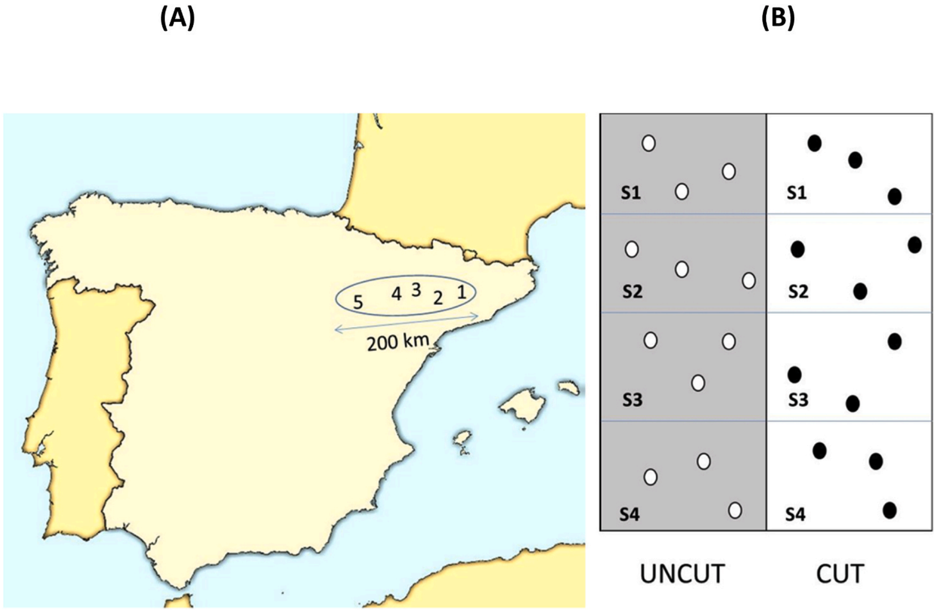

Fig. 1. (A) Map of areas where sampled fields were located in 2019 and 2020. Counties within the Ebro Valley: 1. Urgell; 2. Segrià; 3; Baja Cinca; 4. Monegros; 5. Zaragoza Central. (B) Schematic of one sampled field, each divided into two sections (UNCUT and CUT) of similar area, with each divided into four sectors (S1, S2, S3 and S4). In each sector, three points consisting of five net sweeps were sampled (total sampling points per section = 12).

has not been studied in depth and the few data available show highly variable parasitism rates (Pons and Núñez, 2020).

Chemical control against the alfalfa weevil should be avoided to preserve the ecological services of predators and parasitoids. However, farmers in Spain use one to three applications of pyrethroids (the only authorized class of insecticide) against the alfalfa weevil during the period when most damage occurs (Pons and Levi-Mourao, 2020; Pons and Núñez, 2020). Beyond their detrimental effects on natural enemies, the efficacy of these treatments is questionable and may lead to the development of resistance (Rethwisch et al., 2019). Therefore, alternative control methods should be used to implement integrated pest management (IPM), which has been mandatory in Spain since 2014.

Cultural control such as early harvesting of first crop, autumn cutting, intercropping or grazing have been proposed to combat the alfalfa weevil many years ago, particularly in North American (Pellissier et al., 2017). Onstad and Shoemaker (1984) claimed that a robust strategy is to always harvest early. Studies conducted in northern Catalonia in Spain concluded that this strategy is effective in reducing the impact of *H. postica* (Pons and Núñez, 2020). However, under the common production system in most of the Ebro Valley in Spain, this strategy is not easily applied, because most farmers/producers sell the alfalfa to dehydrating forage companies who decide and manage when to cut according to their needs. Dowdy et al. (1992) reported that late autumn cutting in the USA reduced the number of alfalfa weevil eggs by half. However, no data of the effectiveness of cutting during the overwintering period of *H. postica* in Spain or Europe exist, apart from those reported in Núñez et al. (2015). The Núñez et al. (2015) study was conducted in a single county in small fields (<2 ha) using a laser mower, which only rarely used today. Our study was conducted over a broader geographical range in larger fields using a disk mower in most of the fields. Intercropping is not used in commercial alfalfa production in Spain. Grazing was another effective practice reported in some countries as useful to reduce weevils in alfalfa (Gossey, 2012; Sanaei and Seiedy, 2016; Wynn-Williams et al., 1991). This practice is currently in disuse in Spain, mainly because of the decrease in sheep flocks and intestinal bloating risk by foraging (Delgado, 2020). Therefore, cutting the alfalfa during winter to eliminate overwintering population of the pest needs to be further investigated as a control method under Spanish crop conditions.

The aim of this work was to determine the efficacy of one winter alfalfa cutting as a cultural strategy to reduce the population of the alfalfa weevil under Spanish crop conditions and to evaluate the interaction of this management method with parasitism rates due to the parasitoid *Bathyplectes* spp. We expected that winter cutting would remove a substantial number of eggs and larvae of *H. postica*, thus resulting in significantly lower spring populations (Hypothesis 1). Because host density can have cascading effects on parasitoids, with positive (Eveleigh et al., 2007) and negative (Costamagna et al., 2004) effects reported, and because higher host densities may enhance the parasitism of *Bathyplectes* spp. (Rand, 2013), we predicted a higher level of parasitism in fields without winter cutting management (Hypothesis 2).

2. Materials and methods

2.1. Field site description

The study was performed in the Ebro Valley region, where 60% of Spanish alfalfa is cultivated (Delgado and Lloveras, 2020), mostly under irrigation. The Ebro Valley is a geographic region of Northeast of the Iberian Peninsula. Mean temperatures range from 1 °C in winter to 30 °C in summer. Annual rainfall is variable and ranges from 200 to 800 mm, and is mainly concentrated in spring and autumn. Mean altitude is 200 m (asl).

A total of 42 commercial fields in five counties in the region (Urgell, Segrià, Baja Cinca, Monegros and Zaragoza Central) were selected during 2019 and 2020 crop seasons (Fig. 1A). When possible, sampling was repeated in the same fields in the two study years. The fields in each county were separated by at least 2 km. Most of the selected fields were 2 or 3 years old, and were sown with the Aragon variety. This commercial variety, obtained from the ecotype Aragon, has been cultivated in the Ebro Valley for decades. In addition to tolerating temperatures down to −15 °C, it has a short dormancy period, fast development in spring and after cutting regrowth, and it may be cut 5–6 times under irrigation (Lloveras et al., 2020; Delgado 2020). No insect resistance traits are known for this variety. The field size ranged from 1 to 7 ha, and fields were sprinkler or blanket irrigated. During the study period no pesticides were used. Field characteristics are shown in Table S1.

2.2. Sampling plan

Each field was divided into two sections with approximately equivalent area, and each section was randomly assigned to one of the cutting management treatments (Fig. 1B). One section was not subject to any management practice during the winter (UNCUT, hereafter), whereas in the other section, alfalfa was cut in winter once, and the forage was removed after cutting (CUT hereafter). Alfalfa was cut as short as the mowing machinery allowed (always below 4 cm in height). In most fields in Zaragoza Central, alfalfa cutting was performed with a laser mower (a mower guided by a laser land level equipment), whereas fields in the other counties were cut with a disc mower (Table S1). Cutting dates depended on the weather conditions and farmer availability, and varied among fields from the beginning of January to the middle of February (Table S1). Each section of the field (UNCUT and CUT) was divided into four sectors of approximately equivalent size (Fig. 1B).

All fields were sampled before winter cutting management (to determine whether differences existed between the experimental sections) and during the first alfalfa intercut (period between the beginning of the vegetative growing season and the first spring alfalfa cutting; see Pons et al., 2011 for details), when *H. postica* larvae damage the crop. Sampling before winter cutting management was performed in 2019 (during January) with a sweep-net (procedure described below), but very few records of the occurrence of larvae and eggs were obtained (stem pieces occasionally collected with net sweeping were dissected for that purpose). Therefore, in 2020, the sampling method (from middle December to the second fortnight of January) consisted of collecting 25 stems in each of the four sectors (100 stems in each UNCUT and CUT management section) and gently excising them from the plant crown with scissors. Stems were brought to the laboratory of entomology of the University of Lleida, kept in a refrigerator at 5 °C and dissected during the next 2 weeks.

In the following spring, samplings were performed by conducting 180° sweeping with a 38 cm diameter net. In each UNCUT and CUT section, three samples, consisting of five sweeps for each of the four sectors, were collected. Therefore, for each field, 12 samples were obtained for each section (Fig. 1B). Because of the small size of the fields in Zaragoza Central County, only six samples per field section were collected. Spring sampling was performed twice in 2019, with the first at the beginning of alfalfa vegetative growing (mid-March) and the second when alfalfa was well developed (mid- or second fortnight of April). Because of the COVID-19 pandemic, only one sampling was performed in 2020. Collected field samples were transported to the laboratory and frozen at −20 °C until processing.

In addition to the sampling pattern described, on the same sampling date, we collected an additional sample consisting of 20 sweeps in each management section. Within the next 24 h, 25–150 L3 or L4 instar larvae, depending on the larval abundance of *H. postica* per sample, were selected (usually 100–150). Larvae were kept in 500 ml rearing polyethylene cages (maximum 50 larvae/cage), covered by a mesh to facilitate aeration. Fresh alfalfa was provided every 2 days. Larvae were maintained until pupation in a climatic chamber at 22 °C, with an 8:16 (light: dark) photoperiod and 50% relative humidity.

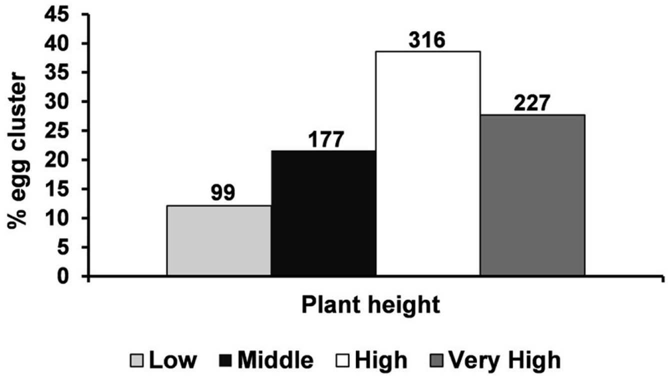

Fig. 2. Percentage of *H. postica* egg clusters found at different height intervals in alfalfa stems: low (below ¼ stem height), middle (between ¼ and ½ stem height), high (between ½ and ¾ stem height) and very high (above ¾ stem height). Data from all fields were combined. Total numbers of clusters are shown above the bars.

Table 1
ANOVA statistics for the fixed effects in the mixed model for the abundance of *H. postica* larvae.

Source	df	Approximate df denominator	F	*p*
County	4	26	4.07	0.0107
Year	1	23	6.66	0.0167
County*Year	4	22	2.90	0.0501
Sampling date [Year]	1	15	17.87	0.0007
Sampling date * County [Year]	4	15	6.53	0.0032
Winter Cutting	1	23	34.80	<0.0001
County * Winter Cutting	4	23	2.79	0.0502
Year * Winter Cutting	1	20	2.42	0.1349
County * Year * Cutting	4	19	3.53	0.0254
Sampling date * Winter Cutting [Year]	1	13	0.01	0.9327
Sampling date * County * Winter Cutting [Year]	4	13	4.19	0.0209

2.3. Data collection

Before winter cutting, the numbers of egg clusters and larvae per sampling point (in 2019) and per stem (in 2020) in each field were recorded. We also recorded the height of the stem and the height from the stem base at which the egg clusters were found. Based on the relative height location of the egg cluster on the stem height, we classified the location of the egg cluster as low (below ¼ stem height), middle (between ¼ and ½ stem height), high (between ½ and ¾ stem height) and very high (above ¾ stem height). These samplings were made between middle December until middle January.

From the sweep net samples taken after winter cutting we recorded 1) the number of *H. postica* larvae; 2) the number of larvae that were dead or with disease symptoms; 3) the number of adults of the parasitoid *Bathyplectes* spp. (species were not identified, because slight morphological differences make identification difficult).

From the rearing cages, we recorded the number of *H. postica* pupae and those of *B. anura* and *B. curculionis* puparia; dead larvae were removed from the cage, and their number was recorded daily. Both species of *Bathyplectes* spp. can be easily distinguished on the basis of the characteristics of the puparium. This additional sampling could not be performed in the Zaragoza Central fields.

2.4. Data analysis

For ANOVA analysis data from the samples taken in each section of the field (UNCUT and CUT) were averaged and the field was considered as a replication. Box-Cox's lambda was used to verify normality and homoscedasticity of variance and data of abundances. Data of abundance were square root transformed before analysis. Percentage data were transformed to arcsine $(\times /100)^{1/2}$. Comparisons within statistically significant factors ($p < 0.05$) were performed by Tukey HSD test. All analyses were performed with JMP PRO 15 software (JMP,).

1) Hypera postica
    1.1) Before winter cutting management, abundance of *H. postica* eggs and larvae was analyzed by a multifactorial mixed model ANOVA where county and winter cutting were considered fixed factors. Fields were nested to counties and all field interactions

Table 2
H. postica larval abundance (mean ± se) after winter cutting in UNCUT and CUT sections of the 2019 and 2020 fields (n = 12). Efficacy of the cutting management was calculated as ((1-C/NC)*100), where C and NC are the abundance of larvae in CUT and UNCUT sections.

Year												
2019								2020				
County	Field	Sampling 1 (March)			Sampling 2 (April)			County	Field	Sampling 1 (March/April)		
		UNCUT	CUT	Efficacy	UNCUT	CUT	Efficacy			UNCUT	CUT	Efficacy
Urgell	1	60.9 ± 3.8	45.6 ± 4.5	25	654.6 ± 50.3	337.6 ± 26.7	48	**Urgell**	1	777.8 ± 49.1	220.3 ± 11.9	72
	2	13.5 ± 2.9	8.9 ± 1.4	34	246.1 ± 18.9	143.4 ± 11.1	42		2	296.3 ± 32.8	148.6 ± 8.1	50
	3	307.2 ± 43.1	106.7 ± 9.6	65	372.9 ± 31.2	149.9 ± 12.3	60		3	391.9 ± 32.8	210.5 ± 17.7	46
	4	90.5 ± 11.5	29.5 ± 6.4	67	523.0 ± 34.6	118.2 ± 8.9	77		4	121.0 ± 19.1	159.9 ± 35.1	−32
	5	9.8 ± 1.2	4.9 ± 1.2	50	196.2 ± 17.8	83.3 ± 15.8	58		5	187.5 ± 44.8	178.4 ± 21.6	5
	6	80.2 ± 5.6	45.2 ± 3.3	44	263.7 ± 15.2	162.7 ± 6.1	38		6	176.2 ± 24.0	181.7 ± 19.3	−3
	7	4.1 ± 1.0	2.4 ± 0.8	41	149.9 ± 13.3	84.28 ± 6.7	44					
	8	33.4 ± 3.5	24.3 ± 3.9	27	79.7 ± 7.9	48.9 ± 6.1	39	**Segrià**	7	819.4 ± 56.1	462.9 ± 65.2	44
	9	71.4 ± 8.1	23.9 ± 3.5	67	222.8 ± 19.4	152.7 ± 11.9	31		8	133.2 ± 20.8	238.7 ± 33.8	−79
Segrià	10	12.3 ± 3.3	12.2 ± 3.9	1	322.2 ± 29.5	202.7 ± 22.7	37		9	152.7 ± 34.1	142.7 ± 23.4	7
	11	11.7 ± 1.7	6.6 ± 1.2	44	102.5 ± 11.3	55.8 ± 6.9	46	**Baja Cinca**	10	348.7 ± 37.1	222.3 ± 24.9	36
	12	23.9 ± 3.3	15.5 ± 2.4	35	259.9 ± 26.4	176.2 ± 13.7	32		11	175.6 ± 7.8	261.2 ± 15.3	−48
	13	5.3 ± 1.3	1.7 ± 0.5	67	93.9 ± 10.6	48.4 ± 6.5	48		12	318.1 ± 43.6	135.3 ± 8.3	57
	14	20.0 ± 4.6	7.2 ± 1.6	64	229.7 ± 17.9	100.7 ± 8.4	56	**Monegros**	13	71.8 ± 7.4	43.5 ± 5.0	39
	15	66.8 ± 3.3	49.5 ± 5.2	26	221.0 ± 20.0	184.8 ± 17.3	16		14	78.0 ± 8.2	7.2 ± 1.3	91
Baja Cinca	16	151.1 ± 16.6	132.6 ± 11.9	12	318.9 ± 24.3	289.0 ± 32.2	9		15	110.8 ± 12.3	39.0 ± 3.7	65
	17	557.0 ± 33.2	173.5 ± 14.6	69					16	136.7 ± 21.2	71.8 ± 9.9	47
Monegros	18	109.8 ± 7.4	53.1 ± 5.2	52	291.6 ± 14.5	202.8 ± 16.8	30		17	124.8 ± 16.1	56.3 ± 7.4	55
	19	67.8 ± 8.6	31.2 ± 3.2	54	91.9 ± 9.3	82.9 ± 7.0	10					
Zaragoza Central (n = 6)	20	25.0 ± 3.5	4.2 ± 0.9	83	2.5 ± 0.4	1.2 ± 0.3	52	**Zaragoza Central** (n = 6)	18	31.3 ± 4.1	9.7 ± 2.4	69
	21	98.8 ± 9.8	20.3 ± 4.2	79	1.5 ± 0.6	0.2 ± 0.1	87		19	342.7 ± 19.7	4.33 ± 0.4	99
	22	90.0 ± 15.2	29.2 ± 10.7	68	57.8 ± 12.6	25.8 ± 4.1	55		20	321.3 ± 23.1	21.8 ± 5.5	93

were considered as random factors. Analysis was performed for 2019 and 2020 separately, since sampling methods were different between both years.

1.2) After winter cutting management, *H. postica* abundance in spring was analyzed by a multifactorial mixed model ANOVA where county, year, and winter cutting were considered as fixed factors. As samplings were performed at different times in 2019 and 2020, the sampling date was nested to year and considered as a fixed factor. Fields were nested to counties and all interactions involving the random term field [counties] were also considered random.

1.3) Winter cutting efficacy, for each field, was calculated according to Abbot (1925) as [(1-C/UC)*100], where C and UC were the abundance of alfalfa weevil larvae in the CUT and UNCUT sectors, respectively. The efficacy was analyzed through a multifactorial mixed model ANOVA where county and year were considered as fixed factors. Sampling date was nested to year and field was nested to county. All field interactions were considered as random factors. The 4 fields where the efficacy was negative were not included in the analysis. The relationship between efficacy and area of a field was analyzed by Pearson correlation. The relationship between efficacy and mowing method and irrigation type, since they were binary variables, were analyzed by a Point-biserial correlation (see Table S1).

2) *Bathyplectes* spp.

The abundance of collected adults in field sampling and the rate of parasitism were analyzed with the same ANOVA model described in 1.2.

3) Fungal disease

Because of an unexpected epizootic of *Zoophthora phytonomi* (Arthur) (Zygomycetes: Entomophthorales) in 2020, the influence of this fungal disease on the efficacy of the cutting management and on the rate of parasitism of *Bathyplectes* spp. was evaluated for this year.

3.1) The proportion of larvae showing symptoms of infection by *Z. phytonomi* in the UNCUT and CUT sections of each field sampled in 2020 was calculated as (Li/Lt*100), where Li is the number of larvae with symptoms of infection, and Lt is the total number of larvae in the sample. This rate was analyzed by

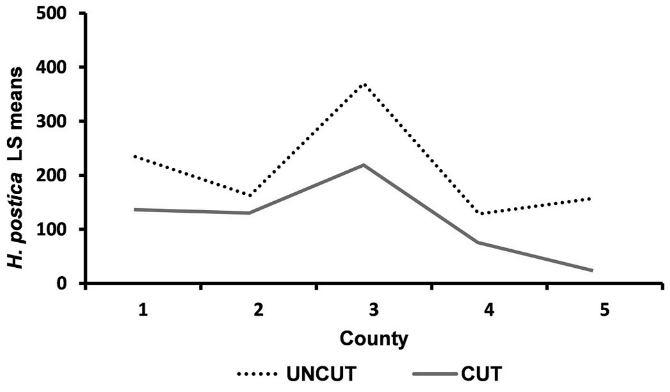

Fig. 3. Abundance of *H. postica* larvae expressed as LS means in UNCUT and CUT sections in each of the five sampled counties: 1. Urgell; 2. Segrià; 3; Baja Cinca; 4. Monegros; 5. Zaragoza Central.

ANOVA with county and cutting as fixed factors. Field factor was nested to county and considered as random.

3.2) The relationship between the rate of parasitism by *Bathyplectes* spp. and the proportion of larvae showing symptoms of infection by *Z. phytonomi* was determined by Spearman correlation.

3. Results

3.1. Effect of winter cutting management on H. postica

H. postica data before winter cutting are presented on Table S2. In both the 2019 (sweep net) and the 2020 (stem) samplings, there were no significant differences between CUT and UNCUT sections in the abundance of *H. postica* eggs (2019: $F_{1,\ 15} = 0.032$, $p = 0.86$; 2020: $F_{1,\ 13} = 0.39$, $p = 0.54$) and larvae (2019: $F_{1,\ 17} = 0.0035$, $p = 0.95$; 2020: $F_{1,\ 15} = 0.033$, $p = 0.63$). Most of the egg clusters (around 65%) were found on the upper part of the stem, mainly between the middle and the three quarter parts of their height (Fig. 2). Only around 10% of egg clusters were located in the first quarter of the plant.

After winter cutting, the most significant factor determining abundance across years, county and field was the winter cutting management ($p < 0.0001$, Table 1). The abundance of *H. postica* larvae in UNCUT sections was very high in April 2019 and 2020 (Table 2), exceeding the economic threshold of 20 larvae/sweep (Martin et al., 2020). In CUT sections the abundance was lower and in some cases below the economic threshold (Table 2). Although the effects of sampling date, year and county were also significant, the cutting management factor interacted with these other factors in a quantitative manner, as can be seen for the interaction County * Cutting (Fig. 3).

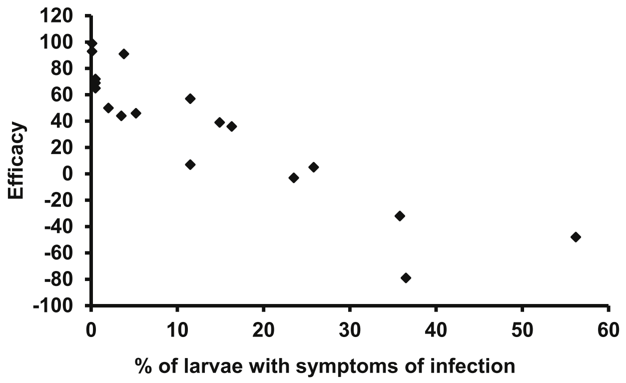

Fig. 4. Relationship between the percentage of *H. postica* larvae with symptoms of infection by *Z. phytonomi* in the UNCUT sections and the efficacy of the winter cutting management, calculated as [1-(C/UC)]*100, where C and UC are the abundance in CUT and UNCUT field sections (rho = −0.93).

Table 3
ANOVA statistics for the fixed effects in the mixed model for *Bathyplectes* spp. adult abundance.

Source	Df	Approximate Df denominator	F	*p*
County	4	10	62.02	<0.0001
Year	1	30	0.006	0.9375
County*Year	4	30	7.71	0.0004
Sampling date [Year]	1	27	22.02	<0.0001
Sampling date * County [Year]	4	26	12.02	<0.0001
Winter Cutting	1	31	12.44	0.0013
County * Winter Cutting	4	31	3.00	0.0333
Year * Winter Cutting	1	21	0.76	0.3919
County * Year * Cutting	4	20	2.63	0.0649
Sampling date * Winter Cutting [Year]	1	24	3.91	0.0598
Sampling date * County * Winter Cutting [Year]	4	22	0.81	0.5290

When the efficacy of the method was analyzed, the only significant factor was county ($F_{4,\ 21} = 5.67$, $p = 0.0029$; ANOVA table not shown) with higher efficacy in Zaragoza Central than in the other counties. The efficacy was associated with mowing method ($r = 0.54$; $p = 0.0004$) and to the field surface ($r = -0.32$; $p = 0.0351$) but not to the irrigation system ($r = -0.16$; $p = 0.2956$).

In some fields, the proportion of larvae with symptoms of *Z. phytonomi* infection exceeded 35% in 2020. The efficacy of the winter cutting was negatively correlated with the proportion of larvae showing symptoms of fungal infection ($rho = -0.93$; $t = -10.09$; $p < 0.0001$) (Fig. 4).

3.2. Effect of winter cutting management on Bathyplectes spp.

The total number of *Bathyplectes* spp. adults collected in the sweep net sampling was low but varied between the cutting management (Table 3). Although there was an interaction between county and cutting, this was once again quantitative and the abundance of *Bathyplectes* spp. adults was significantly higher in CUT than in UNCUT sections. No other interactions with cutting occurred.

H. postica collected in the field and reared in the laboratory resulted in variable rates of parasitism, ranging from 5 to more than 50%, and the prevalent parasitoid species was *B. anura*, with more than 90% of the puparia belonging to this species (Table 4). ANOVA results showed a significant effect of winter cutting, with higher rates of parasitism in CUT than in UNCUT sections (Table 5).

The rate of *H. postica* larvae with symptoms of fungal infection was evaluated in 2020, when the epizootic occurred. ANOVA results showed a significant effect of the winter cutting ($p < 0.0001$). Higher rates of infection were found in UNCUT than in CUT sections, resulting in a significant negative correlation ($rho = -0.82$; $t = -8.14$; $p < 0.0001$) between the rate of infection by *Z. phytonomi* and the rate of parasitism by *Bathyplectes* spp. (Fig. 5).

4. Discussion

In the study area, female alfalfa weevils start egg laying in the second fortnight of October. Consequently, some larvae are already present at the end of November and eggs can easily be found during January and February when our fields were sampled (Levi-Mourao et al., 2021). Although the natural abundance of *H. postica* markedly varied across fields and counties, the results of the comparison of UNCUT and CUT sections at the field and regional levels revealed the value of winter cutting in significantly reducing the *H. postica* larval population in the spring. The efficacy of the winter cutting was previously reported for some areas of the region (Núñez et al., 2015; Pons and Núñez, 2020), but the results presented in this study include a greater number of fields, located along the main Spanish alfalfa growing area. Therefore, we

Table 4
Rates of parasitism (%) by *B. anura* (Ba), *B. curculionis* (Bc) and total (Ba + Bc) in larval laboratory rearing in the UNCUT and CUT sections of the study fields in samplings of A) 2019 (March and April) and B) 2020. The rate of larval killing by *Z. phytonomi* is also shown (Zooph). Ni = initial number of larvae.

A) 2019											
March											
County	UNCUT						CUT				
	Field	Ni	Ba	Bc	Total	Zooph	Ni	Ba	Bc	Total	Zooph
Urgell	1	122	9	0	9	6.6	51	16	0	15.7	0
	2	100	13	2	15	0	100	19	0	19	0
	3	125	11	0	11.2	0	125	20	0	20	0
	4	142	11	2.1	12.7	0	42	33	17	50	0
	5	100	19	0	19	0	100	28	0	28	0
	6	39	44	0	43.6	0	54	19	0	18.5	0
	7	45	2.2	0	2.2	0	37	5.4	5.4	10.8	0
	8	84	4.8	0	4.8	0	53	5.7	3.8	9.5	0
	9	34	12	0	11.8	0	55	9.1	0	9.1	0
Segrià	10	50	6	2	8	0	38	18	0	18.4	0
	11	36	83	0	83.3	0	25	100	0	100	0
	12	25	60	0	60	0	25	60	15	75	0
	13	100	7	0	7	0	75	16	0	16	0
	14	100	3	0	3	0	100	9	0	9	0
	15	40	2.5	0	2.5	0	40	10	0	10	0
Baja Cinca	16	100	37	2	39	0	100	36	1	37	0
	17	150	19	0	18.7	0	150	22	0	22	0
Monegros	18	110	11	0	10.9	0	120	2.5	0.8	3.3	0
	19	115	12	0	12.2	0	105	43	0	42.9	0
April											
	UNCUT						**CUT**				
Urgell	1	150	1.3	0	1.3	0	150	11	0	10.7	0
	2	150	8.7	0	8.7	0	120	18	0	18.3	0
	3	150	4	0	4	0	100	23	0	23	0
	4	150	7.3	0	7.3	0	100	27	0	27	0
	5	150	21	0	21.3	0	109	30	0	30.3	0
	6	150	14	0	14	0	150	11	0	11.3	0
	7	100	17	0	17	0	40	18	0	17.5	0
	8	60	6.7	0	6.7	0	50	24	0	24	0
	9	110	2.7	0	2.7	0	100	12	0	12	0
Segrià	10	150	6.7	0	6.7	40	150	21	0	20.7	8.7
	11	100	24	0	24	0	75	36	0	36	0
	12	150	17	0	17.3	6.7	130	46	0	46.2	0
	13	100	23	0	23	0	75	33	0	33.3	0
	14	100	18	0	18	0	67	42	0	41.8	0
	15	100	5	0	5	64	100	13	0	13	0
Baja Cinca	16	140	23	0	22.9	0	140	38	0	37.9	0
	17	150	8.9	0	8.9	0	150	13	0	12.7	0
Monegros	18	60	8.3	13	21.7	0	150	2	19	20.7	0
	19	90	16	0	15.6	51	70	1.4	34	35.7	50
B) 2020											
	UNCUT						**CUT**				
County	**Field**	**Ni**	**Ba**	**Bc**	**Total**	**Zooph**	**Ni**	**Ba**	**Bc**	**Total**	**Zooph**
Urgell	1	150	8.7	2	10.7	43.3	150	17	1	18.7	18
	2	100	0	0	0	79	100	1	1	2	71.4
	3	25	4	0	4	75	28	7.1	0	7.1	57.4
	4	100	0	0	0	100	100	15	2	17	65
	5	100	0	0	0	100	60	1.7	0	1,7	80
	6	100	0	0	0	96	100	3	1	4,0	75
Segrià	7	150	13	3	16	58	150	17	3	20	22
	8	97	1	0	1	96.9	100	2	1	3	71
	9	100	0	0	0	100	100	11	2	13	75
Baja Cinca	10	26	3.9	0	3.9	96.1	58	1.7	2	3.4	81
	11	100	0	0	0	100	100	1	0	1	92
	12	150	4.7	1	6	84.7	40	2.5	0	2.5	97.5
Monegros	13	50	0	0	0	100	40	15	5	20	80
	14	25	0	0	0	100	25	0	0	0	83.3
	15	25	0	0	0	100	25	0	0	0	83.3
	16	115	7.8	0	7.8	14.8	95	7.4	2	9.5	0
	17	137	18	0	17.5	0	70	19	6	24.3	0

assume that the applicability of our results could be extended to other Spanish alfalfa crop conditions and potentially to other European regions.

The average efficacy of winter cutting in 2019 was approximately 50%, a similar value to the late autumn cutting efficacy reported by Dowdy et al. (1992) in USA. However, in our study, the efficacy varied across counties. The highest efficacies were recorded in Zaragoza Central, where a laser mower was used, which allows for cutting the alfalfa at the plant crown level and automatically collecting the cut plant material. In the fields where a disk mower was used, the plants were cut at 2–4 cm from the soil and left in the field for 1–2 days before collection. There was a significant correlation between efficacy and mowing

Table 5
ANOVA statistics for the fixed effects in the mixed model for the rate of parasitism by *Bathyplectes* spp.

Source	df	Approximate df denominator	F	*p*
County	4	14	0.58	0.6338
Year	1	54	15.60	0.0002
County*Year	4	52	0.3449	0.7930
Sampling date [Year]	1	22	0.01	0.9139
Sampling date * County [Year]	4	20	0.31	0.8181
Winter Cutting	1	12	20.74	0.0006
County * Winter Cutting	4	12	1.96	0.1747
Year * Winter Cutting	1	20	0.01	0.9196
County * Year * Cutting	4	20	0.27	0.8440
Sampling date * Winter Cutting [Year]	1	17	1.70	0.2091
Sampling date * County * Winter Cutting [Year]	4	16	0.57	0.6449

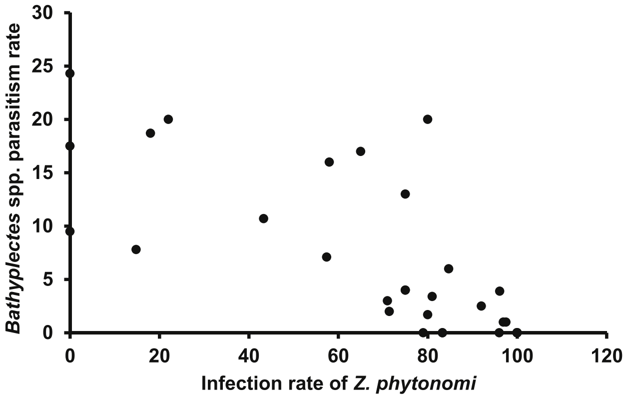

Fig. 5. Relationship between the parasitism rate of *Bathyplectes* spp. and the rate of larvae of *H. postica* showing symptoms of infection by *Z. phytonomi* (rho = −0.82).

method. Although both mowing methods cut stems less than 4 cm from the soil level and potentially remove most of the egg clusters (90% are located above the bottom quarter of the stem height; Fig. 2), laser mower cuts and collects nearly all stems, whereas the cutting efficiency of disc mower is lower. Furthermore, cut stems remain in the field for a longer time before collection with disc mower. Plant pieces remaining in the field or under the windrow may allow for the survival of eggs and small larvae (Summers, 1998; Blodgett et al., 2000; Blodgett and Lensen, 2004), even during the winter. Larvae already developed or emerging from the remaining eggs can infest new alfalfa stems. Furthermore, a significant correlation was found between the field size and the efficacy of the winter cutting, with lower efficacies in larger fields. In these larger fields, with their more variable topography and higher numbers of sprinkler supply pipes, whose bases hinder the cutting of alfalfa with the disc mower, the collection of alfalfa was less precise. In addition to local field management, the potential effect of nearby unmanaged alfalfa fields that can act as a source for adult alfalfa weevil immigration during the first crop intercut (Prokopy et al., 1967), should be further studied.

Epizootics of *Z. phytonomy* have been reported in Europe (Papierok et al., 1986), including in Spain (Pons and Núñez, 2020). The efficacy of the winter cutting method recorded in 2020 was lower than that in 2019, and a negative relationship was observed between the efficacy of the method and the occurrence of *Z. phytonomy*. This fungus remains in the soil and infects larvae of *H. postica* when environmental humidity conditions are adequate (Radcliffe and Flanders, 1998). The infection is exacerbated in years with high rainfall during the winter and the beginning of spring, as occurred in 2020 (Table S3). These results indicate that in epizootic years, the effects of winter cutting management may be less evident. Additionally, the rate of infected larvae was higher in UNCUT than in CUT sections in several fields, suggesting that the epizootic was more severe where the density of alfalfa weevil larvae was higher (Los and Allen, 1983).

A clear predominance of *B. anura* over *B. curculionis* was observed in both study years; the former represented 90% of the total parasitoid individuals. This predominance has been observed previously in the study area (Pons and Núñez, 2020). In many regions where the two species live together, *B. anura* has been found to be predominant or even to displace *B. curculionis*, because the former has greater reproductive capacity, more rapid search and handling, and more aggressive behaviour (Harcourt, 1990).

Contrary to our hypothesis, winter cutting management did not negatively affect the rate of parasitism by *B. anura*. The rates of parasitism obtained were higher in the CUT than in UNCUT sections. These results further underline the value of winter cutting and suggest that this method may enhance conservation biological control of *B. anura*. Rand (2013) has reported that *B. curculionis* parasitizes a lower proportion of hosts at high alfalfa weevil densities, thus potentially explaining the results obtained with *B. anura* in our study. The mechanism through which high host density negatively affects *Bathyplectes* spp. is unknown but may occur through hindering the selection of hosts or increasing the host handling time. Specific studies should be performed to elucidate this relationship.

Parr et al. (1993) reported that *B. anura* and *Z. phytonomi* were able to coexist in the USA. Our results support these findings, because we have found both *H. postica* parasitoid species in our region for years. Although *Z. phytonomi* causes high mortality of *H. postica* larvae in wet seasons and is considered an important biological control agent (Harcourt and Guppy, 1991; Giles and Obrycki, 1997), our results show that fungal epizooties can negatively affect the role of *B. anura* by reducing the rate of parasitism. Because the disease also kills parasitized larvae of the alfalfa weevil (Giles et al., 1994; Kuhar et al., 1999), epizootics of *Z. phytonomy* disrupt the alfalfa weevil-parasitoid system.

5. Conclusion

The results show that winter cutting management can be a useful cultural tool that has potential as a component of an IPM program against the alfalfa weevil in Spain and potentially in other European regions. This cultural method not only reduces overwintering stages but also the larval density of this pest in the spring. Furthermore, the method increases the rate of parasitism of *Bathyplectes* spp., particularly *B. anura*, and can be considered a strategy for enhancing conservation biological control. However, despite this potential control capacity, the occurrence of *Z. phytonomy* epizootics, which strongly depends on weather conditions, may conceal the value of the winter cutting strategy, which should be applied in winter, before it is known whether a fungal epizootic will occur in the spring.

Declaration of competing 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.

Table S1
Sampled field coordinates and characteristics during a) 2019 and b) 2020.

a) 2019

County	Field	Latitude	Longitude	Altitude (m)	Age (years)	Irrigation type	Type of mowing	Mowing height	Winter cut date	Surface (ha)
Urgell	1	41°41′22.36″N	1°3′58.83″E	302	2	Blanket	Disc	2–4 cm	February 05, 2019	3.35
	2	41°38′58.94″N	0°45′6.98″E	187	3	Blanket	Disc	≤2 cm	January 29, 2019	2.36
	3	41°39′9.61″N	0°44′15.41″E	183	3	Blanket	Disc	≤2 cm	January 29, 2019	4.87
	4	41°39′42.59″N	0°48′9.19″E	200	3	Blanket	Disc	≤2 cm	February 04, 2019	4.79
	5	41°40′59.75″N	0°49′44.68″E	205	3	Blanket	Disc	2–4 cm	February 07, 2019	2.94
	6	41°38′13.42″N	0°57′3.90″E	262	3	Blanket	Disc	2–4 cm	February 06, 2019	3.95
	7	41°42′20.05″N	0°58′50.76″E	256	3	Blanket	Disc	2–4 cm	February 05, 2019	6.34
	8	41°35′5.45″N	0°50′12.09″E	222	3	Blanket	Disc	2–4 cm	February 06, 2019	6.96
	9	41°39′16.48″N	0°55′15.53″E	246	2	Blanket	Disc	2–4 cm	February 06, 2019	4.12
Segrià	10	41°46′49.35″N	0°31′12.15″E	287	2	Sprinkler	Disc	2–4 cm	January 11, 2019	2.76
	11	41°43′42.73″N	0°20′54.59″E	187	2	Sprinkler	Disc	≤2 cm	January 04, 2019	2.58
	12	41°42′46.44″N	0°20′53.02″E	178	2	Sprinkler	Disc	≤2 cm	January 04, 2019	4.75
	13	41°47′30.82″N	0°16′52.37″E	259	3	Sprinkler	Disc	≤2 cm	January 09, 2019	1.24
	14	41°47′54.43″N	0°17′25.43″E	259	6	Sprinkler	Disc	≤2 cm	January 09, 2019	1.58
	15	41°44′9.59″N	0°47′17.84″E	216	3	Blanket	Disc	2–4 cm	February 06, 2019	2.47
Baja Cinca	16	41°57′17.82″N	0°3′14.55″E	315	2	Sprinkler	Disc	2–4 cm	February 09, 2019	4.60
	17	41°54′33.19″N	0°3′11.14″E	304	2	Sprinkler	Disc	2–4 cm	February 09, 2019	2.91
Monegros	18	41°28′12.75″N	0°4′27.75″W	315	2	Sprinkler	Disc	2–4 cm	February 15, 2019	4.7
	19	41°31′0.66″N	0°10′33.68″E	363	3	Sprinkler	Disc	2–4 cm	February 15, 2019	2.76
Zaragoza Central	20	41°33′30.90″N	0°41′32.29″W	179	3	Blanket	Laser	≤2 cm	February 06, 2019	0.9
	21	41°34′43.53″N	0°45′48.37″E	188	2	Blanket	Laser	≤2 cm	February 06, 2019	1.3
	22	41°42′21.63″N	0°51′11.01″E	217	2	Blanket	Disc	≤2 cm	February 08, 2019	1.52

b) 2020

County	Field	Latitude	Longitude	Altitude (m)	Age (years)	Irrigation type	Type of mowing	Mowing height	Winter cut date	Surface (ha)
Urgell	1	41°39′9.61″N	0°44′15.41″E	183	4	Blanket	Disc	2–4 cm	February 14, 2020	4.87
	2	41°39′42.59″N	0°48′9.19″E	200	4	Blanket	Disc	2–4 cm	February 13, 2020	4.79
	3	41°40′59.75″N	0°49′44.68″E	205	4	Blanket	Disc	2–4 cm	February 13, 2020	2.94
	4	41°38′13.42″N	0°57′3.90″E	262	4	Blanket	Disc	2–4 cm	February 08, 2020	3.95
	5	41°35′5.45″N	0°50′12.09″E	222	4	Blanket	Disc	2–4 cm	February 11, 2019	6.96
	6	41°39′16.48″N	0°55′15.53″E	246	3	Blanket	Disc	2–4 cm	February 06, 2019	4.12
Segrià	7	41°46′49.35″N	0°31′12.15″E	287	3	Sprinkler	Disc	2–4 cm	January 17, 2020	2.76
	8	41°38′23.86″N	0°32′34.20″E	214	3	Sprinkler	Disc	2–4 cm	January 17, 2020	7.84
	9	41°47′30.82″N	0°16′52.37″E	259	4	Sprinkler	Disc	≤2 cm	February 11, 2020	1.24
Baja Cinca	10	41°57′17.82″N	0°3′14.55″E	315	3	Sprinkler	Disc	≤2 cm	February 27, 2020	4.60
	11	41°53′27.92″N	0°2′9.58″E	328	4	Sprinkler	Disc	≤2 cm	February 27, 2020	6.40
	12	41°54′33.19″N	0°3′11.14″E	304	3	Sprinkler	Disc	≤2 cm	February 27, 2020	2.91
Monegros	13	41°29′24.18″N	0°6′6.49″W	327	4	Sprinkler	Disc	2–4 cm		1.60

(*continued on next page*)

Table S1 (*continued*)

a) 2019										
County	Field	Latitude	Longitude	Altitude (m)	Age (years)	Irrigation type	Type of mowing	Mowing height	Winter cut date	Surface (ha)
									February 12, 2020	
	14	41°28′12.75″N	0°4′27.75″W	315	3	Sprinkler	Disc	2–4 cm	February 12, 2020	4.7
	15	41°29′2.29″N	0°5′46.44″E	282	3	Sprinkler	Disc	2–4 cm	February 12, 2020	5.0
	16	41°29′0.41″N	0°6′11.15″E	325	3	Sprinkler	Disc	2–4 cm	February 12, 2020	3.96
	17	41°32′25.74″N	0°9′42.93″E	372	4	Sprinkler	Disc	2–4 cm	February 12, 2020	2.07
Zaragoza Central	18	41°33′30.90″N	0°41′32.29″E	179	4	Blanket	Laser	≤2 cm	February 14, 2020	0.9
	19	41°34′43.53″N	0°45′48.37″E	188	3	Blanket	Laser	≤2 cm	February 14, 2020	1.3
	20	41°42′21.63″N	0°51′11.01″E	217	3	Blanket	Laser	≤2 cm	February 27, 2020	1.52

Table S2

Mean (±s.e.) abundance of larvae and eggs of *H. postica* in the UNCUT and CUT sectors of the alfalfa fields sampled before winter cutting management during 2019 and 2020. Samplings in 2019 were performed in five net-sweeps in 12 points (3 × 4), and alfalfa was collected and dissected to detect the occurrence of eggs. In 2020, 25 stems per sector of UNCUT and CUT sections (25 × 4 = 100 stems per section) were collected and dissected.

YEAR											
2019						2020					
County	Field	Larvae/5 net-sweeps		Eggs/5 net-sweeps		County	Field	Larvae/stem		Eggs/stem	
		UNCUT	**CUT**	**UNCUT**	**CUT**			**UNCUT**	**CUT**	**UNCUT**	**CUT**
Urgell	1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	Urgell	1	0.00 ± 0.00	0.01 ± 0.01	5.34 ± 1.13	3.72 ± 1.45
	2	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00		2	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
	3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00		3	0.03 ± 0.03	0.01 ± 0.01	0.39 ± 0.20	0.43 ± 0.19
	4	0.17 ± 0.17	0.17 ± 0.17	1.33 ± 0.90	0.00 ± 0.00		4	0.01 ± 0.01	0.02 ± 0.01	0.41 ± 0.20	0.50 ± 0.33
	5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00		5	0.05 ± 0.04	0.21 ± 0.16	2.52 ± 1.43	0.68 ± 0.31
	6	0.25 ± 0.18	0.33 ± 0.19	1.83 ± 1.33	4.00 ± 1.95		6	0.11 ± 0.08	0.02 ± 0.02	6.26 ± 0.75	7.69 ± 1.46
	7	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	Segrià	7	0.52 ± 0.22	0.07 ± 0.06	1.83 ± 0.83	1.61 ± 0.78
	8	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00		8	0.16 ± 0.10	0.02 ± 0.02	0.52 ± 0.30	2.09 ± 1.45
	9	0.01 ± 0.01	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00		9	0.19 ± 0.14	0.05 ± 0.03	0.37 ± 0.19	0.12 ± 0.12
Segrià	10	0.00 ± 0.00	0.00 ± 0.00	4.25 ± 3.66	0.92 ± 0.45	Baja Cinca	10	0.15 ± 0.08	0.06 ± 0.04	1.95 ± 1.04	2.03 ± 1.21
	11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00						
	12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00		11	0.02 ± 0.02	0.06 ± 0.06	0.48 ± 0.16	0.38 ± 0.21
	13	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00		12	0.12 ± 0.09	0.00 ± 0.00	1.68 ± 0.69	1.22 ± 0.77
	14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	Monegros	13	0.01 ± 0.01	0.00 ± 0.00	7.00 ± 1.20	5.85 ± 1.21
	15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00		14	0.56 ± 0.28	0.11 ± 0.06	8.68 ± 0.83	8.65 ± 1.06
Baja Cinca	16	0.50 ± 0.19	0.58 ± 0.26	0.42 ± 0.42	2.33 ± 1.74		15	0.04 ± 0.01	0.01 ± 0.01	0.42 ± 0.24	0.18 ± 0.18
	17	0.75 ± 0.28	1.25 ± 0.39	1.50 ± 1.18	0.50 ± 0.50		16	2.50 ± 1.11	1.33 ± 0.80	4.17 ± 1.87	5.83 ± 1.90
Monegros	18	0.08 ± 0.08	0.00 ± 0.00	2.50 ± 1.50	2.25 ± 2.25		17	0.33 ± 0.20	0.00 ± 0.00	3.00 ± 2.3	1.00 ± 1.00
	19	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	Zaragoza Central (n = 6)	18	0.00 ± 0.00	0.00 ± 0.00		
Zaragoza Central (n = 6)	20	0.00 ± 0.00	0.00 ± 0.00				29	0.01 ± 0.01	0.00 ± 0.00		

(*continued on next page*)

Table S2 (*continued*)

YEAR											
2019						2020					
County	Field	Larvae/5 net-sweeps		Eggs/5 net-sweeps		County	Field	Larvae/stem		Eggs/stem	
		UNCUT	**CUT**	**UNCUT**	**CUT**			**UNCUT**	**CUT**	**UNCUT**	**CUT**
	21	0.33 ± 0.19	0.25 ± 0.18								
	22	0.00 ± 0.00	0.00 ± 0.00				20	0.01 ± 0.01	0.00 ± 0.00		

Table S3

Cumulative monthly rainfall (l/m^2), from January to April, in 2019 and 2020 in the five counties in the study region (Urgell, Segrià, Baja Cinca, Monegros and Zaragoza Central). Source: AEMET.

County	2019					2020				
	Jan.	Feb.	Mar.	Apr.	Sum	Jan.	Feb.	Mar.	Apr.	Sum
Urgell	14.1	9.7	0.5	42.5	**66.8**	86.6	0.8	29.5	122.8	**239.7**
Segrià	12.1	3.2	0.6	28.7	**44.6**	85.4	0.6	35.8	75.2	**197.0**
Baja Cinca	12.2	7.8	3.4	55.9	**79.3**	101.1	2.2	74.7	66.8	**244.8**
Monegros	11.1	5.9	1.6	26.7	**45.3**	98.9	1.2	41.8	40.0	**181.9**
Zaragoza C.	20.2	2.1	13.6	33.6	**57.5**	62.8	0.6	71.2	35.4	**169.4**

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References

Alfaro, A., 2005. In: Santiago-Álvarez, C. (Ed.), Entomología Agraria: Los parásitos animales de las plantas cultivadas, Pblicaciones de la Excma. Diputación de Soria, Soria.

Abbot, W.S., 1925. A method of computing effectiveness of an insecticide. J. Econ. Entomol. 18, 265–267.

Blodgett, S.L., Lensen, A.W., 2004. Distribution of alfalfa weevil (Coleoptera: Curculionidae) larvae among post-cutting locations. J. Econ. Entomol. 94, 1319–1322.

Blodgett, S.L., Lenssen, A.W., Cash, S.D., 2000. Harvest with raking for control of alfalfa weevil (Coleoptera: Curculionidae). J. Entomol. Sci. 35, 129–135.

Capistrós, J., 2020. Comercialización. In: Lloveras, J., Delgado, I., Chocarro, C. (Eds.), La Alfalfa - Agronomía Y Utilización. Edicions de la Universitat de Lleida. Centro de Investigación y Tecnología Agroalimentaria de Aragón. Lleida-Zaragoza, pp. 343–364.

Costamagna, A.C., Menalled, F.D., Landis, D.A., 2004. Host density influences parasitism of the armyworm *Pseudaletia unipuncta* in agricultural landscapes. Basic Appl. Ecol. 5, 347–355.

Delgado, I., 2020. La alfalfa en pastoreo. In: Lloveras, J., Delgado, I., Chocarro, C. (Eds.), La Alfalfa - Agronomía Y Utilización. Edicions de la Universitat de Lleida. Centro de Investigación y Tecnología Agroalimentaria de Aragón. Lleida-Zaragoza, pp. 315–323.

Delgado, I., Lloveras, J., 2020. Historia y distribución de la alfalfa. In: Lloveras, J., Delgado, I., Chocarro, C. (Eds.), La Alfalfa - Agronomía Y Utilización. Edicions de la Universitat de Lleida. Centro de Investigación y Tecnología Agroalimentaria de Aragón. Lleida-Zaragoza, pp. 17–32.

Dowdy, A.K., Berberet, R.C., Stritzke, J.F., Caddel, J.L., McNew, R.W., 1992. Late fall harvest, Winter grazing and weed control for reduction of alfalfa weevil (Coleoptera: Curculionidae) populations. J. Econ. Entomol. 85, 1946–1953.

Eveleigh, E.S., McCann, K.S., McCarthy, P.C., Pollock, S.J., Lucarotti, C.J., Morin, B., McDougall, G.A., Strongman, D.B., Huber, J.T., Umbanhowar, J., 2007. Fluctuations in density of an outbreak species drive diversity cascades in food webs. Proc. Natl. Acad. Sci. USA 104, 16976–16981.

Giles, K.L., Obricky, J.J., Degooyer, T.A., Orr, C.J., 1994. Seasonal occurrence and impact of natural enemies of *Hypera postica* (Coleoptera: Curculionidae) larvae in Iowa. Environ. Entomol. 23, 167–176.

Giles, K.L., Obrycki, J.J., 1997. Reduced insecticide rates and strip-harvesting effects on alfalfa weevil (Coleoptera: Curculionidae) larval populations and prevalence of *Zoophthora phytonomi* (Entomophthorales: Entomophthoraceae). J. Econ. Entomol. 90, 933–944.

Goosey, H.B., 2012. A degree-day model of sheep grazing influence on alfalfa weevil and crop characteristics. J. Econ. Entomol. 105, 102–112. https://doi.org/10.1603/EC11171.

Harcourt, D.G., 1990. Displacement of *Bathyplectes curculionis* (Thomson) (Hymenoptera: Ichneumonidae) by *B. anurus* (Thomson) in eastern Ontario populations of the alfalfa weevil, *Hypera postica* (Gyllenhal.) (Coleoptera: Curculionidae). Can. Entomol. 122, 641–645.

Hardcourt, D.G., Guppy, J.C., 1991. Numerical analysis of an outbreak of the alfalfa weevil (Coleoptera: Curculionidae) in eastern Ontario. Environ. Entomol. 20, 217–223.

Hoffmann, A., 1963. Sous famille des Curcilionidae. Tribu des Hyperini. Les (hypera (Syn. Phytonomus). In: Balachowsky, A. (Ed.), Entomologe Appliquée a L'agriculture. Tome I : Coleoptères, Second Volume. Masson et Cie, París, pp. 984–989.

JMP®, Version Pro 15. SAS Institute Inc., Cary, NC, 1989-2019.

Kingsley, P.C., Bryan, M.D., Day, W.H., Burger, T.L., Dysart, R.J., Schwalbe, C.P., 1993. Alfalfa weevil (Coleoptera: Curculionidae) biological control: spreading the benefits. Environ. Entomol. 22 (6), 1234–1250.

Kuhar, T.P., Youngman, R.R., Laub, C.A., 1999. Alfalfa weevil (Coleopteran: Curculionidae) pest status and incidence of *Bathyplectes* spp. (Hymenoptera: Ichneumonidae) and *Zoophthora phytonomi* (Zygomycetes: Entomophthorales) in Virginia. J. Econ. Entomol. 92, 1184–1189.

Levi-Mourao, A., Madeira, F., Messeguer, R., García, A., Pons, X., 2021. Effects of temperature and relative humidity on the embryonic development *of Hypera postica,* Gyllenhal (Col. Curculionidae). Insects 12 (3), 250. https://doi.org/10.3390/insects12030250.

Los, L.M., Allen, W.A., 1983. Incidence of *Zoophthora phytonomi* (Zygomycetes: Entomophthorales) in *Hypera postica* (Coleoptera: Curculionidae) larvae in Virginia. Environ. Entomol. 12, 1318–1321.

Madeira, F., di Lascio, A., Costantini, L., Rossi, L., Rösch, V., Pons, X., 2019. Intercrop movement of heteropteran predators between alfalfa and maize examined by stable isotope analysis. J. Pest. Sci. 92, 757–767.

Núñez, E., Taberner, A., Muñoz, F., Delgado, I., 19-23 October 2015. Unpublished results. Efecto del corte de la alfalfa en parada invernal en el control de Hypera postica (Gyllenhal) (Coleoptera: Curculionidae). Communication presented at IX Congreso Nacional de Entomología Aplicada. Valencia.

Onstad, D.W., Shoemaker, C.A., 1984. Management of alfalfa and the alfalfa weevil (*Hypera postica*): an example of system analysis in forage production. Agric. Syst. 14, 1–30.

Orloff, S.B., 1997. Introduction. In: Orloff, S.B., Carlson, H.L., Tauber, L.R. (Eds.), Intermountain Alfalfa Management. Publication 3366. University of California. Division of Agriculture and National Resources, Oakland, pp. 2–3.

Parr, J.C., Pass, B.C., Nordin, G.L., 1993. Compatibility of *Zoophthora phytonomi* (Entomophthorales: Entomophthoraceae) and *Bathyplectes anurus* (Hymenoptera: Ichneumonidae) in Kentucky alfalfa fields. Environ. Entomol. 22, 674–678. https://doi.org/10.1093/ee/22.3.674.

Papierok, B., Aeschlimann, J.-P., Loan, C., 1986. Two entomophthoralean fungi occurring on *Hypera postica* in southern France. J. Invertebr. Pathol. 48, 377–380.

Pellissier, M.E., Nelson, Z., Jabbour, R., 2017. Ecology and management of the alfalfa weevil (Coleoptera: Curculionidae) in western United States Alfalfa. J. Integr. Pest Manag. 8, 1–7.

Pons, X., Levi-Mourao, A., 2020. El gusano verde, una plaga clave de la alfalfa. Vida Rural 479, 10–14.

Pons, X., Núñez, E., 2020. Plagas de la alfalfa: importancia, daños y estrategias de control. In: Delgado, L., Chocarro, C. (Eds.), La Alfalfa - Agronomía Y Utilización. Lloveras J. Edicions de la Universitat de Lleida. Centro de Investigación y Tecnología Agroalimentaria de Aragón. Lleida-Zaragoza, pp. 167–202.

Pons, X., Lumbierres, Ribes A., Starý, P., 2011. Parasitoid complex of alfalfa aphids in an IPM intensive crop system in northern Catalonia. J. Pest. Sci. 84, 437–445.

Prokopy, R.J., Armbrust, E.J., Cothran, W.R., Gyrisco, G.G., 1967. Migration of the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae) to and from aestivation. Ann. Entomol. Soc. Am. 60, 26–31. https://doi.org/10.1093/aesa/60.1.26.

Radcliffe, E., Flanders, K., 1998. Biological control of alfalfa weevil in North America. Integr. Pest Manag. Rev. 3, 225–242.

Rand, T.A., 2013. Host density drives spatial variation of the alfalfa weevil, *Hypera postica*, across dryland and irrigated alfalfa cropping systems. Environ. Entomol. 42, 116–122.

Rethwisch, M.D., Peairs, F., Pierce, J., Mostafa, A., Price, S., Ramirez, R., Wenninger, E., 2019. Insecticide resistance in alfalfa weevil and related implications in other alfalfa insect pests. In: Proceedings, 2019 Western Alfalfa and Forage Symposium, Reno, NV, Nov.19-21, 2019. Retrieved from. http://alfalfa.ucdavis.edu.

Saeidi, M., Moharramipour, S., 2017. Physiology of cold hardiness, seasonal fluctuations and cryoprotectant contents in overwintering adults of *Hypera postica* (Coleoptera: Curculionidae). Environ. Entomol. 46, 960–966.

Sanaei, E., Seiedy, M., 2016. Developmental differences of local populations of alfalfa weevil (*Hypera postica*) (Coleoptera: Curculionidae). Turk. J. Zool. 40, 471–479.

Soroka, J., Bennet, A.M.R., Kora, C., Schwarzfeld, M.D., 2020. Distribution of alfalfa weevil (Coleoptera: Curculionidae) and its parasitoids on the Canadian Prairies, with a key to described species of Neartic *Bathyplectes* (Hymenoptera: Ichneumonidae). Can. Entomol. 152, 663–701.

Summers, C.G., 1998. Integrated pest management in forage alfalfa. Integrated Pest Manag. Rev. 3, 127–154.

Wynn-Williams, R.B., Rea, M.B., Purves, R.G., Hawthorne, B.T., 1991. Influence of winter treading on lucerne growth and survival. N. Z. J. Agric. Res. 34, 271–275.

CHAPTER III

Molecular and morphological identification of the alfalfa weevil larval parasitoids *Bathyplectes anura* and *Bathyplectes curculionis* to estimate the rate of parasitism

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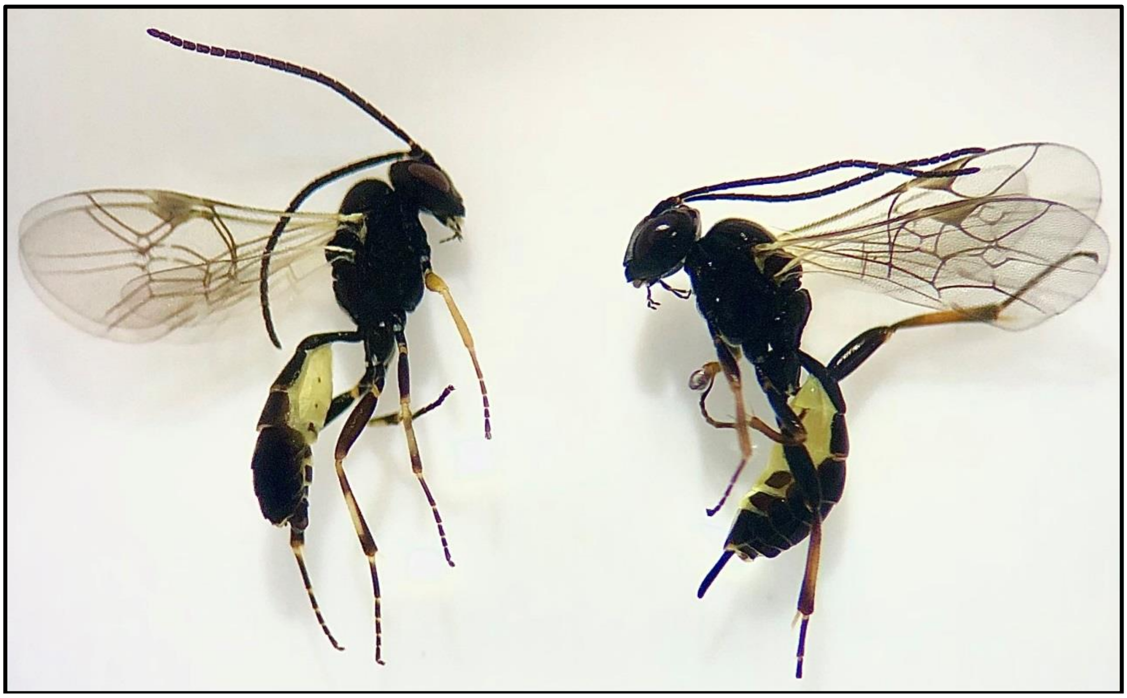

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Molecular and morphological identification of the alfalfa weevil larval parasitoids *Bathyplectes anura* and *Bathyplectes curculionis* to estimate the rate of parasitism

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Abstract The alfalfa weevil (*Hypera postica* Gyllenhal, Coleoptera: Curculionidae) is a major pest of alfalfa crops. Chemical control measures are inefficient, but the larvae are often infested by parasitoid wasps of the genus *Bathyplectes* Förster (Hymenoptera: Ichneumonidae), which offer a potential biological control strategy. The development of effective biological control requires the identification of parasitoid species, but conventional methods involve the rearing of parasitoids to the puparium stage for morphological confirmation. Here we designed a PCR method in which two pairs of primers are used to detect and identify *Bathyplectes curculionis* Thomson and *Bathyplectes anura* Thomson larvae in a faster way. We compared conventional rearing to the new method as a means to determine the parasitism rates caused by each species in Spain during the 2019 and 2020 seasons. In 2019, the PCR method detected five times as many *B. curculionis* events and twice as many *B. anura* events. Similarly, in 2020, the PCR method detected seven times as many *B. curculionis* events and twice as many *B. anura* events. High mortality of *H. postica* larvae was recorded in 2020 due to an epizootic attack by the fungus *Zoophthora phytonomi* Arthur (Zygomycetes: Entomophthorales), explaining the lower overall rate of parasitism and the widespread death of the larvae. Statistical analysis confirmed that the PCR method is more sensitive than conventional rearing for the detection of target parasitoids. However, only rearing can confirm the effective parasitism or the presence of unexpected species. We therefore recommend the use of both methods in parallel when evaluating host–parasitoid systems.

Keywords *Hypera postica* · Cytochrome C oxidase subunit I (COI) · Parasitism rate · Polymerase chain reaction (PCR) · Biological control · *Bathyplectes* sp

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Introduction

Alfalfa (*Medicago sativa* L.) is the most valuable cultivated forage crop in the world (Orloff 1997). In Spain, it is a traditional component of crop rotations covering 250,000 ha, accounting for ~ 20% of the alfalfa land area in Europe (Delgado and Lloveras 2020). Alfalfa is also an important reservoir for pest insects that infest alfalfa as well as surrounding crops (Pons and Nuñez 2020; Madeira et al. 2022).

The alfalfa weevil (*Hypera postica* Gyllenhal; Coleoptera: Curculionidae) is native to Eurasia (Hoffmann 1963) but has spread globally and is now one of the most destructive alfalfa pests (Goosey 2012; Hoff et al. 2002; Pons and Nuñez 2020; Saeidi and

Moharramipour 2017; Soroka et al. 2019). In Spain, the weevil larvae cause serious damage to the first cutting (from March to the end of April). There is little information in the scientific literature about the ecology and pest status of this species in Europe. Natural enemies can reduce alfalfa weevil populations (Soroka et al. 2020). Alfalfa weevil larvae are parasitized by solitary endoparasitoid wasps of the genus *Bathyplectes* (Hymenoptera: Ichneumonidae) which is native to the Old World (Kingsley et al. 1993; Kuhar et al. 1999; Radcliffe and Flanders 1998). *B. anura* and *B. curculionis* were introduced to North America with remarkable success as a weevil control strategy (Radcliffe and Flanders 1998; Rand 2013). Although eight *Bathyplectes* species have been recorded in Spain (Ribes 2012), only *B. anura* Thomson and *B. curculionis* Thomson are associated with alfalfa. The identification of adult wasps is challenging because there are only slight morphological differences between species (Pons and Nuñez 2020), especially in the male (Soroka et al. 2020). The puparia are easier to distinguish because *B. anura* forms a hard, dark-brown puparium with a narrow, raised, white horizontal band, whereas the *B. curculionis* puparium is light brown with a flat, diffuse, white horizontal band (Day 1970; Dysart and Day 1976). For definitive parasitoid identification, each *H. postica* larva must therefore be reared until pupation, which requires optimal environmental conditions and feeding, and the avoidance of other natural factors that cause mortality. Using this approach, the rates of parasitism recorded in Spain are generally low but highly variable (Levi-Mourao et al. 2021; Pons & Nuñez 2020).

In contrast to the delayed results from rearing experiments, DNA analysis allows parasitism to be followed in real time, and does not require the use of captive insects in controlled-environment chambers (Liang et al. 2015, 2018; Wolf et al. 2018; Agustí et al. 2020; Molina et al. 2021). The high sensitivity and fidelity of molecular methods also facilitate detailed studies of trophic interactions that are otherwise inaccessible (Traugott et al. 2013). Such methods require the development of specific molecular probes to detect target organisms. In arthropods, the evolution of mitochondrial genes has been studied in detail, and divergent sequences in related populations provide a source of species-specific PCR primers (Black et al. 1989; Simon et al. 1994). Several studies have used cytochrome C oxidase subunit I (*COI*) mitochondrial DNA fragments as targets to increase the specificity of detection (Agustí et al. 2003a, 2005). Here we report for the first time the development of *COI* primers to detect and identify the main parasitoids of alfalfa weevil (*B. curculionis* and *B. anura*) in order to estimate the rate of parasitism in *H. postica* larvae compared to the classical rearing method.

Materials and methods

Insect rearing

Adult specimens of *H. postica* were collected from a commercial alfalfa field in Lleida, North-East Spain, in spring 2018. They were reared in 2000-ml glass jars covered with mousseline for proper ventilation, and kept at 20 °C, 60–70% relative humidity with an 8–16 (L:D) photoperiod. Fresh alfalfa stems were provided daily for egg laying and feeding. These were placed in a glass vial filled with water and sealed with parafilm to prevent dehydration of the plants and the drowning of adult insects. All non-parasitized *H. postica* larvae individuals used in the experiments were derived from this laboratory population.

For *B. anura* and *B. curculionis* adults obtaining, *H. postica* larvae were collected from the field. They were kept in rearing polyethylene cages of 500 ml capacity (maximum 50 larvae/cage), covered by a mesh to facilitate aeration. Fresh alfalfa was provided in a daily basis. Larvae were maintained in a climatic chamber at 22 °C, 8:16 (L:D) photoperiod and 50% relative humidity until pupation. In the case they were parasitized, parasitoid puparia were used for morphological identification. The resulting *B. anura* and *B. curculionis* adults were frozen at –80 °C for posterior DNA extraction.

Primer design and testing

Fragments of the conserved *COI* gene from 10 individuals of *B. anura* and *B. curculionis* and the host *H. postica* were amplified by PCR using the universal forward primer C1-J-1718 (5′-GGA GGA TTT GGA AAT TGA TTA GTT CC-3′) and the universal reverse primer C1-N-2191 (5′-CCC GGT AAA ATT AAA ATA TAA ACT TC-3′) (Simon et al.

1994). Each 20-µl amplification reaction contained 1 µl (3–50 ng/µl) resuspended individual DNA, 7.5 µl pure water, 9.5 µl 2.0 Taq RED Master Mix kit, 1.5 mM MgCl2 (Apex Bioresearch, Genesee Scientific) and 1 µl of each forward and reverse primer (10 µM). Samples were denatured at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 58 °C (for 30 s, and 72 °C for 1 min, and then a final extension at 72 °C for 3 min. The parasitoid amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Düsseldorf, Germany) and were transferred to the vector pGEM-T easy. *Escherichia coli* DH5α Scompetent cells were transformed with the vector using an adapted heat shock method (Froger and Hall 2007) and cultivated in lysogeny broth (LB) supplemented with 1 mg/ml ampicillin. Transformants were plated on LB agar supplemented with 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 80 mg/ml isopropyl β-D-1-thiogalactopyranoside (IPTG) for blue-white selection. We transferred 50 white colonies, indicating the presence of an insert to 7 ml LB supplemented with 100 µg/ml ampicillin. Plasmid DNA was extracted for PCR with the primers shown above. Thirty DNA samples yielding the anticipated band sizes for *B. anura* (442 bp) and *B. curculionis* (465 bp) were analyzed by Sanger sequencing (Stab vida, Portugal) using SP6 forward primer 5′-ATT TAG GTG ACA CTA TAG-3′ and M13 reverse primer 5′-CAG GAA ACA GCT ATG AC-3′. Sequences were aligned in UGENE for each construct and non-matching nucleotides between sequences were used to design specific primers (following Innis and Gelfand 1990 and Saiki 1990).

DNA was extracted from homogenized non-parasitized *H. postica* larvae and individual field parasitoid adults from each species using the BioSprint 96 DNA Blood Kit (Qiagen) and the samples were stored at− 20 °C. Each 20-µl reaction contained 1 µl (3–50 ng/µl) resuspended larval DNA, 7.5 µl pure water, 9.5 µl 2.0 Taq RED Master Mix kit, 1.5 mM $MgCl_2$ (Apex Bioresearch, Genesee Scientific) and 1 µl of each newly forward and reverse primer (10 µM). Samples were denatured at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 65 °C (for *B. curculionis*) or 60 °C (for *B. anura*) for 30 s, and 72 °C for 1 min, and then a final extension at 72 °C for 5 min. PCR products were separated by 2.0% agarose gel electrophoresis and stained with SYBR safe (Thermo Fisher Scientific, Waltham, MA, USA). We have tested 10 individuals of each species following this protocol.

Sensitivity and primer species specificity

Sensitivity of the assay was determined for both primer pairs by testing serial dilutions of parasitoid DNA. The original parasitoid DNA samples (~3 ng/µl) were serially diluted to 600, 120, 24, 4.8, 0.9 and 0.19 pg/µl. The specificity of each pair of primers was tested on 10 *B. anura* and *B. curculionis* individuals and on 10 *H. postica* larvae as negative controls. 10 *Microctonus* sp. Wesmael (Hymenoptera: Braconidae), an endoparasitoid of *H. postica* adults, were also tested. DNA from seven random *H. postica* larvae was mixed with *B. anura* and *B.* curculionis DNA to ensure efficiently detection. Products were detected using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

Field samples

Samples were collected in the Ebro basin region (north-east Spain), where 60% of Spanish alfalfa is cultivated (Delgado and Lloveras 2020). We selected 35 commercial fields located along four different counties (1-Urgell, 2-Segrià, 3-Baja Cinca and 4-Monegros) during the 2019 and 2020 seasons. All fields were insecticide-free during the study period. Larvae were collected by 20 sweeps, each of 180°, with a 38-cm diameter net (Bio-quip, Rancho Dominguez, CA USA) and were separated into three groups over the next 24 h.

Field rates of parasitism detected by classical rearing

The first set of 20–300 larvae per field (L3–L4) were kept in 500-ml polyethylene cages (maximum 50 larvae per cage) and provided with fresh alfalfa every 2 days. Cages were maintained in a climatic chamber at 22° C, 8:16 (light: dark) photoperiod and 50% relative humidity until pupation. We counted *H. postica* pupae, *Bathyplectes* puparia and dead larvae on a daily basis, and removed them from the cage. *Bathyplectes anura* and *B. curculionis* were distinguished by the characteristics of the puparia. We also recorded (1) the total number of *H. postica* adults that emerged; (2) the number of larvae showing symptoms of infection by *Z. phytonomi* (brown or black color and soft body) according to Los and Allen (1982); and (3) the number of parasitoid adults that emerged from puparia. These puparia were kept under laboratory conditions (24 ± 3 °C) for 12 months. The rate of parasitism and the effective rate (percentage of parasitoid adults that emerged among field parasitized reared *H. postica* larvae) by each species were calculated.

Field rates of parasitism detected by molecular analysis

A second set of 50 larvae per field (L3–L4) was immediately frozen at − 80 °C and used to estimate the rate of parasitism by PCR with the newly designed primers. A third set of 150 larvae in total was used to detect parasitoid DNA in different larval instars. We therefore collected a similar number of L2, L3 and L4 larvae, which were immediately frozen at − 80 °C. Larvae from both sets were 3% bleach rinsed and stored in individual Eppendorf before frozen. Individual larvae were squashed prior to extraction using sterile pestles. Total DNA was extracted from each larva using the DNeasy 96 Blood Kit (Qiagen). PCR was carried out to detect the presence of *B. curculionis* and/or *B. anura* as described above, with three technical replicates per sample. Products were separated by 2% agarose electrophoresis, and analyzed using a ChemiDoc transilluminator (Bio-Rad Laboratories, Hercules, CA, USA). Samples with the anticipated band sizes were recorded as positives. Parasitism rates were calculated by dividing the total number of individuals with anticipated bands by the total number of samples.

Data analysis

The Shapiro–Wilk test was used to determine whether or not the original data was normally distributed. If not, data were arcsin-transformed before analysis. To evaluate differences between results from both methods (classical and molecular), in the case of normally distributed data, a *t*-test was performed. For data that did not satisfy the condition of normality required for parametric tests, a Wilcoxon signed rank test was used. The same analysis was performed to compare the rate of parasitism within each larval instar between *B. anura* and *B. curculionis*. Statistical analysis was carried out using JMP Pro15 (SAS Institute, Cary, NC, USA).

Results

Design of *COI* primers specific for *B. anura* and *B. curculionis*

COI DNA fragments were successfully amplified from total *B. anura* (OM641987), *B. curculionis* (OL413497) and *H. postica* (OL413498) DNA. The fragments were cloned, and the corresponding sequences were confirmed against GenBank. The sequences were aligned in UGENE to identify non-matching bases suitable for the design of unique primers (Table 1). Accordingly, the Bcfw3 forward primer and Bcrv3 reverse primer were designed to amplify a *COI* fragment specific for *B. curculionis*, whereas the 2Bafw2 forward primer and 2Barv2 reverse primer were designed to amplify a *COI* fragment specific for *B. anura* (Fig. 1).

Sensitivity and primer species specificity

Sensitivity of the new PCR method was tested on 1:5 serial dilutions of DNA from each parasitoid species. Successful amplification was achieved using as little as 3.2 pg of *B. curculionis* DNA or 15 pg of *B. anura* DNA, whereas no amplification products were detected when using *H. postica* or *Microctonus*

Table 1 Primer pairs that specifically amplify *COI* gene fragments from *B. anura* and *B. curculionis*

Species	Primer names	Sequence (5′ → 3′)	Product size
B. curculionis	Bcfw3	AGACCTGATATAGCCTTTCCTCG	422 bp
	Bcrv3	ATTGGATCTCCACCCCCAGAA	
B. anura	2Bafw2	AAGAATATCCGCACAACGAA	448 bp
	2Barv2	TCATTGATGACCAATTGATTT	

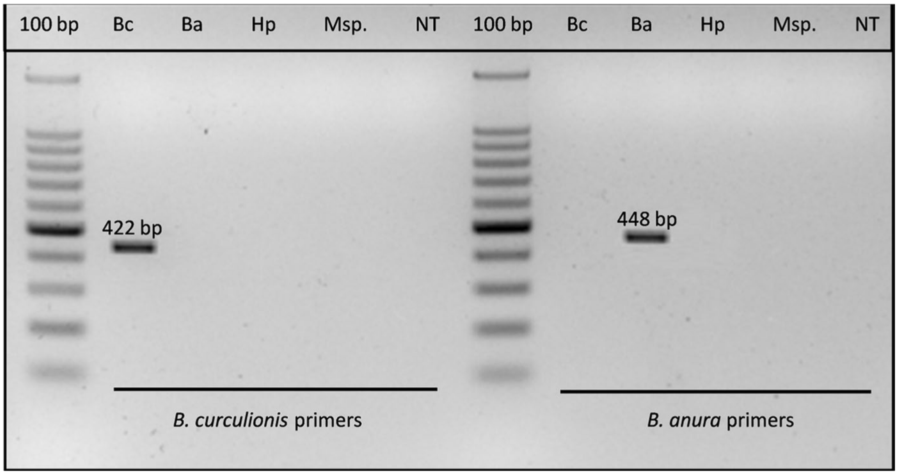

Fig. 1 Analysis of COI amplicons by 2% agarose gel electrophoresis. The primers specific for *B. curculionis* (Bcfw3 and Bcrv3) generate a 422-bp product. The primers specific for *B. anura* (2Bafw2 and 2Barv2) generate a 448-bp product. The left-hand lane in both gels shows 100-bp size markers. Bc, *Bathyplectes curculionis*; Ba, *Bathyplectes anura*; Hp, *Hypera postica* negative control; Msp, *Microctonus* sp. and NT, no template control (without DNA)

sp. DNA. We then mixed DNA from seven random *H. postica* samples with *B. curculionis* or *B. anura* DNA, and found that the parasitoid DNA was still detected efficiently even with a large excess of *H. postica* DNA.

Rates of parasitism detected by classical rearing

The total mortality of *H. postica* larvae varied during the 2019 and 2020 seasons. In 2019, mortality ranged from 0% to almost 30% due to unknown causes, whereas in 2020 up to 100% of the *H. postica* larvae in some fields were killed by the entomopathogenic fungus *Zoophthora phytonomi* (Arthur) (Zygomycetes: Entomophthorales) (Table 2). Yearly parasitism rates per field ranged from less than 2% to more than 30%, with mean values of 15.4 ± 2.3% in 2019 and 5.1 ± 1.6% in 2020 (Table 2). The parasitism rate by *B. anura* was higher in 2019 (12.0 ± 2.6%) than 2020 (4.2 ± 1.3%). Similarly, the parasitism rate by *B. curculionis* was higher in 2019 (3.4 ± 1.6%) than 2020 (0.9 ± 0.3%). In one field during 2019, the parasitism rate exceeded 17 ± 2.6% (Table 2a). The effective parasitism rates in 2019 were 6.9 ± 1.0% for *B. anura* and 3.5 ± 2.6% for *B. curculionis*, but in 2020 both species achieved effective parasitism rates of only 1 ± 0.4%.

Rates of parasitism detected by molecular analysis

A total of 1750 *H. postica* larvae were tested using this protocol. The PCR test detected much greater variability in the parasitism rate per field compared to classical analysis, ranging from 0% to more than 60.4%. The mean parasitism rate due to *B. anura* was 20.7 ± 2.7% in 2019 and 10.7 ± 1.7% in 2020, and the equivalent values for *B. curculionis* were 18.0 ± 5.1% in 2019 and 12.4 ± 2.6% in 2020. Accordingly, *B. curculionis* achieved a slightly lower parasitism rate than *B. anura* in 2019 but the ranking was reversed in 2020 (Table 2b). DNA from both parasitoid species was not detected in the same *H. postica* larvae.

Yearly statistical tests showed significant differences between the classical and molecular methods for both parasitoids. The PCR assay detected a significantly greater rate of parasitism than the classical method in 2019 for *B. anura* ($t = -3.63$, $df = 13$, $p = 0.0031$) and for *B. curculionis* ($z = 3.14$, $p = 0.0016$), and the same was true for 2020 (*B. anura* $z = 3.00$, $p = 0.0026$; *B. curculionis* $z = 3.60$, $p = 0.0003$).

The analysis of parasitized instars revealed that *B. curculionis* and *B. anura* DNA was found at a similar rate in L3 larvae (~ 15% of specimens) and L4 larvae (~ 10% of specimens), but that only *B. curculionis* parasitized L2 larvae (~ 20% of specimens). These results indicate that *B. anura* prefers to parasitize

Table 2 Total number and percentage values for *H. postica* larval mortality factors, emerged *H. postica* adults, parasitism rate and effective parasitism determined using the classical rearing method, and parasitism rates determined by PCR

County	Field	Sampling date	Rearing method															Molecular method		
			Initial # larvae	Mortality				*H. postica* adults		Parasitoid # and parasitism rates						Effective rate		Initial # larvae	Parasitism rates	
				Other cause		Zoophthora				Total		Ba		Bc		Ba	Bc		Ba	Bc
				#	% ± SE	#	% ± SE	#	% ± SE	#	% ± SE	#	% ± SE	#	% ± SE	% ± SE	% ± SE		% ± SE	% ± SE
a) 2019																				
2	1	15/03	80	11	13.8 ± 3.9	0	0.0 ± 0.0	64	80 ± 4.5	5	6.3 ± 2.7	5	6.3 ± 2.7	0	0.0 ± 0.0	6.3 ± 2.7	0.0 ± 0.0	50	18.7 ± 5.5	20.8 ± 5.7
2	2	15/03	88	6	6.8 ± 2.7	0	0.0 ± 0.0	71	81 ± 4.2	11	12.6 ± 3.5	10	11.4 ± 3.4	1	1.2 ± 1.1	11.4 ± 3.4	0.0 ± 0.0	50	18.8 ± 5.5	14.6 ± 4.9
1	3	15/03	82	3	3.7 ± 2.1	0	0.0 ± 0.0	74	90 ± 3.3	5	6.1 ± 2.6	3	3.7 ± 2.1	2	2.4 ± 1.7	3.7 ± 2.0	2.4 ± 1.7	50	29.1 ± 6.4	4.2 ± 2.8
1	4	18/03	90	18	20.0 ± 4.2	0	0.0 ± 0.0	62	69 ± 4.5	9	10.2 ± 3.2	9	10.2 ± 3.2	0	0.0 ± 0.0	8.9 ± 3.0	0.0 ± 0.0	50	12.5 ± 4.68	0.0 ± 0.0
3	5	27/03	200	21	10.5 ± 2.2	0	0.0 ± 0.0	106	53 ± 3.5	76	38.0 ± 3.4	73	36.5 ± 3.4	3	1.5 ± 0.9	13.5 ± 2.4	1.5 ± 0.9	50	27.1 ± 6.29	12.5 ± 4.7
3	6	27/03	300	39	13.0 ± 1.9	0	0.0 ± 0.0	195	65 ± 2.8	61	20.3 ± 2.3	61	20.3 ± 2.3	0	0.0 ± 0.0	9.0 ± 1.7	0.0 ± 0.0	50	25.0 ± 6.1	2.1 ± 2.0
1	7	10/04	300	85	28.3 ± 2.6	0	0.0 ± 0.0	197	66 ± 2.7	18	6.0 ± 1.4	18	6.0 ± 1.4	0	0.0 ± 0.0	4.0 ± 1.1	0.0 ± 0.0	50	20.8 ± 5.7	6.3 ± 3.4
1	8	11/04	259	29	11.2 ± 1.9	0	0.0 ± 0.0	170	66 ± 2.9	65	25.1 ± 2.7	65	25.1 ± 2.7	0	0.0 ± 0.0	12.4 ± 2.0	0.0 ± 0.0	50	27.1 ± 6.3	2.1 ± 2.0
1	9	12/04	270	46	17.0 ± 2.3	0	0.0 ± 0.0	186	69 ± 2.8	35	12.9 ± 2.1	35	12.9 ± 2.0	0	0.0 ± 0.0	7.4 ± 1.6	0.0 ± 0.0	50	43.7 ± 7.0	12.5 ± 4.7
1	10	14/04	250	21	8.4 ± 1.8	0	0.0 ± 0.0	189	76 ± 2.7	40	16.0 ± 2.3	38	15.2 ± 2.3	2	0.8 ± 0.6	6.0 ± 1.5	0.8 ± 0.6	50	25.0 ± 6.1	16.6 ± 5.3
1	11	17/04	250	59	23.6 ± 2.7	0	0.0 ± 0.0	162	65 ± 3.0	29	11.6 ± 2.0	29	11.6 ± 2.0	0	0.0 ± 0.0	7.6 ± 1.7	2.0 ± 0.9	50	16.7 ± 5.3	8.3 ± 3.9
4	12	24/04	300	35	11.7 ± 1.9	0	0.0 ± 0.0	221	74 ± 2.5	44	14.6 ± 2.0	16	5.3 ± 1.3	28	9.3 ± 1.7	2.7 ± 0.9	10.7 ± 1.8	50	6.3 ± 3.4	52.0 ± 7.1
4	13	24/04	160	0	0.0 ± 0.0	81	50.6 ± 3.8	40	25 ± 3.4	26	16.3 ± 2.9	2	1.3 ± 0.8	24	15.0 ± 2.8	1.3 ± 0.9	15.0 ± 2.8	50	4.2 ± 2.8	60.4 ± 6.9
4	14	24/04	210	18	8.6 ± 1.9	0	0.0 ± 0.0	148	70 ± 3.2	41	19.6 ± 2.7	5	2.4 ± 1.2	36	17.2 ± 2.6	2.4 ± 1.0	16.2 ± 2.5	50	14.6 ± 4.9	39.6 ± 6.9
Parasitism overall average rate											15.4 ± 2.3		12 ± 2.6		3.4 ± 1.6	6.9 ± 1.0	3.5 ± 2.3		20.7 ± 2.7	18.0 ± 5.1
b) 2020																				
1	15	24/03	200	27	13.5 ± 2.4	0	0.0 ± 0.0	161	81 ± 2.8	12	6.0 ± 1.7	11	5.5 ± 1.6	1	0.5 ± 0.5	3.5 ± 1.3	0.0 ± 0.0	50	18.8 ± 5.5	14.6 ± 4.9
1	16	24/03	300	0	0.0 ± 0.0	92	30.7 ± 2.7	164	55 ± 2.9	44	14.7 ± 2.0	39	13.0 ± 1.9	5	1.7 ± 0.7	6.0 ± 1.4	0.3 ± 0.3	50	18.8 ± 5.5	4.2 ± 2.8
3	17	27/03	190	0	0.0 ± 0.0	166	87.4 ± 2.4	11	7 ± 1.7	10	5.3 ± 1.6	7	3.7 ± 1.4	3	1.6 ± 0.9	1.1 ± 0.7	0.5 ± 0.5	50	14.6 ± 4.9	2.1 ± 2.0
3	18	27/03	84	0	0.0 ± 0.0	72	85.7 ± 3.8	9	11 ± 3.4	3	3.6 ± 2.0	2	2.8 ± 1.7	1	0.9 ± 1.8	1.2 ± 1.2	0.0 ± 0.0	50	12.5 ± 4.7	0.0 ± 0.0
3	19	28/03	200	0	0.0 ± 0.0	192	96.0 ± 1.4	1	4 ± 0.5	1	0.5 ± 0.5	1	0.5 ± 0.5	0	0.0 ± 0.0	0.5 ± 0.5	0.0 ± 0.0	50	2.1 ± 2.03	2.1 ± 2.0
2	20	29/03	300	0	0.0 ± 0.0	120	40.0 ± 2.8	126	42 ± 2.9	54	18.0 ± 2.2	44	14.7 ± 2.0	10	3.3 ± 1.1	5.3 ± 1.3	0.3 ± 0.3	50	16.6 ± 5.3	10.4 ± 4.3
1	21	06/04	125	0	0.0 ± 0.0	114	91.2 ± 2.5	12	6 ± 2.6	4	3.2 ± 1.6	3	2.4 ± 1.37	1	0.8 ± 0.7	0.8 ± 0.8	0.0 ± 0.0	50	4.2 ± 2.8	0.0 ± 0.0
1	22	06/04	200	0	0.0 ± 0.0	145	72.5 ± 3.2	53	27 ± 3.2	2	1.0 ± 0.7	1	0.5 ± 0.5	1	0.5 ± 0.5	0.5 ± 0.5	0.0 ± 0.0	50	6.3 ± 3.4	2.1 ± 2.0
1	23	08/04	200	0	0.0 ± 0.0	165	82.5 ± 2.7	18	9 ± 2.0	17	8.5 ± 1.9	15	7.5 ± 1.9	2	1.0 ± 0.7	1.0 ± 1.0	4.5 ± 1.5	50	18.8 ± 5.5	8.1 ± 3.9
1	24	08/04	160	0	0.0 ± 0.0	148	92.5 ± 2.1	14	7 ± 2.2	1	0.6 ± 0.5	1	0.6 ± 0.6	0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.6	50	2.1 ± 2.0	12.5 ± 4.7
1	25	08/04	200	0	0.0 ± 0.0	171	85.5 ± 2.5	25	13 ± 2.3	4	2.0 ± 0.9	3	1.5 ± 0.9	1	0.5 ± 0.5	0.5 ± 0.5	1.5 ± 0.9	50	22.9 ± 5.9	22.9 ± 5.9
2	26	09/04	200	0	0.0 ± 0.0	175	87.5 ± 2.3	7	6 ± 1.3	13	6.5 ± 1.7	11	5.5 ± 1.6	2	1.0 ± 0.7	0.5 ± 0.5	3.5 ± 1.3	50	14.6 ± 4.9	6.3 ± 3.4
2	27	09/04	62	0	0.0 ± 0.0	52	83.9 ± 4.7	10	16 ± 4.7	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	50	22.9 ± 5.9	12.5 ± 4.7
2	28	09/04	200	0	0.0 ± 0.0	168	85.3 ± 2.6	28	14 ± 2.5	4	2.0 ± 0.9	2	1.0 ± 0.7	2	1.0 ± 0.7	0.0 ± 0.0	0.5 ± 0.5	50	4.2 ± 2.8	6.3 ± 3.4
2	29	15/04	22	0	0.0 ± 0.0	11	50.0 ± 10.7	5	23 ± 8.9	6	27.3 ± 9.5	5	22.7 ± 8.9	1	4.6 ± 4.4	0.0 ± 0.0	4.5 ± 4.4	50	7.8 ± 3.8	23.2 ± 5.9

Table 2 (continued)

County	Field	Sampling date	Rearing method															Molecular method		
			Initial # larvae	Mortality				*H. postica* adults		Parasitoid # and parasitism rates						Effective rate		Initial # larvae	Parasitism rates	
				Other cause		Zoophthora				Total		Ba		Bc		Ba	Bc		Ba	Bc
				#	% ± SE	#	% ± SE	#	% ± SE	#	% ± SE	#	% ± SE	#	% ± SE	% ± SE	% ± SE		% ± SE	% ± SE
4	30	16/04	23	0	0.0 ± 0.0	22	95.7 ± 4.3	7	4 ± 9.6	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	50	2.1 ± 2.0	44.5 ± 7.0
4	31	16/04	23	0	0.0 ± 0.0	10	43.5 ± 10.3	13	57 ± 10.3	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	50	2.1 ± 2.0	20.8 ± 5.7
4	32	16/04	90	0	0.0 ± 0.0	82	91.1 ± 3.0	0	0 ± 0.0	8	8.9 ± 3.0	6	6.7 ± 2.6	2	2.2 ± 1.5	1.1 ± 1.1	4.4 ± 2.2	50	10.4 ± 4.3	31.3 ± 5.7
1	33	17/04	65	0	0.0 ± 0.0	65	100.0 ± 0.0	0	0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	50	2.1 ± 2.0	22.9 ± 5.9
1	34	17/04	125	0	0.0 ± 0.0	125	100.0 ± 0.0	0	0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	50	2.9 ± 2.4	14.7 ± 5.0
1	35	30/04	49	0	0.0 ± 0.0	49	100.0 ± 0.0	0	0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0	0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	50	18.8 ± 5.5	0.0 ± 0.0
Parasitism overall average rate											5.1 ± 1.6		4.2 ± 1.3		0.9 ± 0.3	1.0 ± 0.4	1.0 ± 0.4		10.7 ± 1.7	12.4 ± 2.6

The mean overall rate of parasitism was calculated for both methods. Part (a) shows data for 2019 and part (b) shows data for 2020. Ba, *B. anura*; Bc, *B. curculionis*. Sampling was performed in 35 fields from 4 different counties (1-Urgell, 2-Segrià, 3-Baja Cinca and 4-Monegros)

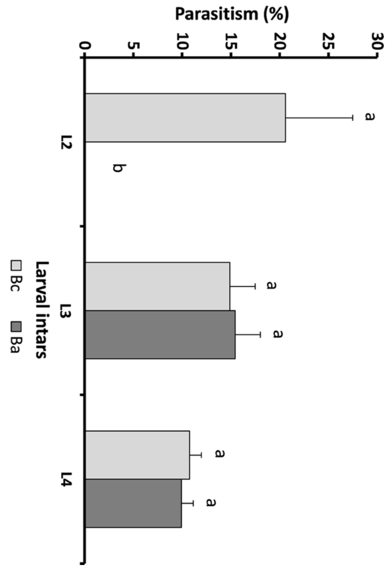

Fig. 2 Rate of parasitism (mean + SE) by *B. curculionis* (Bc) and *B. anura* (Ba) during different stages of *H. postica* larval development (L2, L3 and L4). Different letters in the same instar indicate significant differences at $p < 0.05$

later-instars since no DNA was detected in L2 *H. postica* larvae (Fig. 2).

Discussion

We have developed a new molecular method based on the amplification of the mitochondrial *COI* gene for the quick detection of *B. anura* and *B. curculionis* in *H. postica* larvae. The direct analysis of parasitoid DNA is an alternative to the time-consuming morphological analysis of puparia, which requires the rearing of insects in containment. The mitochondrial *COI* gene has proven useful for the reliable identification of other morphologically similar species (Nanini et al. 2019; Solà et al. 2018; Traugott and Symondson 2008). We therefore designed specific primers that discriminate between the *B. curculionis* and *B. anura* *COI* genes, a strategy that has been successful for other parasitoids and predators (Agustí et al. 2003b, 2005). The new strategy can be used to investigate interactions between *H. postica* and its larval parasitoids in more detail.

The classical technique revealed a larger number of emerging adults for *B. anura* compared to *B. curculionis* and thus a greater rate of parasitism, suggesting that *B. anura* is more prevalent than *B. curculionis* in the study area, as previously reported (Pons and Nuñez 2020). In regions of the New World where the two species are colocalized, *B. anura* is generally dominant over *B. curculionis* and can even displace it completely due to greater reproductive capacity and aggression, and more successful host finding (Harcourt 1990). Our new molecular method detected up to seven times as many parasitism events as the classical technique, indicating greater sensitivity and thus more reliability when estimating the rate of parasitism, as suggested for other species (Agustí et al. 2005; Gariepy et al. 2008; Gomez-Polo et al. 2014). Furthermore, the molecular method suggested for the first time that the rate of parasitism caused by *B. anura* and *B. curculionis* does not differ by so wide a margin as suggested by the classical method (Pons and Nuñez 2020; Levi-Mourao et al. 2021). The mean rate of parasitism was similar for both species, suggesting that the two species coexist in the alfalfa crops of north-east Spain. Moreover, the classical and molecular methods both showed that *B. anura* was the prevalent species in March, when *H. postica* begins to attack alfalfa crops, whereas *B.curculionis* was slightly more prevalent during April. This indicates a succession from one species to the other, as recently reported (Levi-Mourao et al. 2021) and may explain the absence of multiparasitism by both species.

The major and most effective parasitoid of *H. postica* in some regions of North America is thought to be *B. curculionis* (Berberet and Bisges 1998; Radcliffe and Flanders 1998; Soroka et al. 2019). However, its effectiveness is often comprised by the encapsulation of the parasitoid egg by hemocytes in the host hemocoel (Berberet et al. 2003; Salt and van den Bosch 1967; Shoubu et al. 2005). *H. postica* L1 larvae have little defense against parasitism, but 30–50% of the L2–L4 instars survive as a result of encapsulation (Berberet et al. 1987; van Den Bosch and Dietrick 1959). This may explain why we detected a larger number of *B. curculionis* parasitism events by PCR compared to conventional rearing. PCR-based methods can overestimate the rate of parasitism because they detect parasitoids that are already neutralized by the host immune system, whereas the classical method allows the direct measurement of parasitoid survival (Traugott et al. 2006). On the other hand, classical rearing techniques are influenced by the mortality of parasitoids under laboratory conditions, which can result in partial data loss (Tilmon et al. 2000). Furthermore, the puparia of each species have different environmental requirements to complete their life cycle, which can also influence the results. For example, *B. curculionis* can extend its diapause up to 10–12 months in an unfavorable environment (Radcliffe and Flanders 1998), but high emergence rates were achieved by placing the puparia in a refrigerator for 4–6 months before transfer to an environment set at 21 °C with a 12-h photoperiod (Jacob and Evans 2000; 2004). The low effective rate of parasitism we observed in the case of *B. curculionis* may be due to the maintenance of the puparia in rearing cages under laboratory conditions. In contrast to *B. curculionis*, *B. anura* eggs are almost never encapsulated by *H. postica* (Maund and Hsiao 1991; Puttler 1967). This may explain the correlation between the two methods during 2019, when there was no additional mortality caused by *Z. phytonomi*.

Our results also showed that *B. anura* females prefers L3 *H. postica* larvae because no DNA was found in L2 larvae, agreeing with previous findings (Bartell and Pass 1980; Dowell and Horn 1977). In contrast, *B. curculionis* targeted L2 larvae, concurring with reports showing that this species favors early-instar *H. postica* larvae and that this is strictly related to high parasitoid larval survival (Duodu and Davis 1974; Barney et al. 1978). The differences in larval instar preference probably reflects the length of the ovipositor, which is longer for *B. curculionis* and facilitates the utilization of early instars still hidden in unfolded leaves and buds (Dowell and Horn 1977). Our results also suggest that *B. anura* has a shorter larval development phase than *B. curculionis*.

Given the differences in parasitoid occurrence, development, and host instar preferences, alfalfa crop management during the first cutting could be optimized to enhance the survival and development of *B. anura* and *B. curculionis*. *Bathyplectes anura* can survive and complete its development during alfalfa weevil infestation because it appears earlier in the field, favors late-instar host larvae and develops more quickly (Levi-Mourao et al. 2021). In contrast, the survival of *B. curculionis* can be seriously compromised by the timing of first cutting because it appears later in the field (mainly during the second half of April, when most *H. postica* larvae have nearly completed development) and favors young larvae which are increasingly scarce by this time point (Levi-Mourao et al. 2021; Levi et al. in preparation). The commercial cutting of alfalfa at the end of April eliminates almost the entire *H. postica* population, so bringing this forward to reduce yield losses could severely limit the availability of hosts for *B. curculionis*. A delay in this practice can help to the survival of this parasitoid species. Beside this, a recent study in the Ebro Basin on the efficacy of a winter alfalfa cutting (Levi-Mourao et al. 2022) to reduce the egg population and to prevent the development of larvae of *H. postica* at the first alfalfa commercial cutting, shows that the larval density was significantly reduced, whereas the rate of parasitism increased, especially *B. anura*, the prevalent species at the beginning of the spring. Furthermore, the reduction of alfalfa weevil larvae below the economic thresholds, enhanced by the winter cutting, would allow delaying the date of the first commercial cutting and, in turn, *B. curculionis* survival.

Parasitism rates in 2020 were lower than in 2019 due to the presence of *Z. phytonomi.* Epizooties of this fungus occur in years with high rainfall (Barney and Armbrust 1981; Kuhar et al. 1999). This was the case in 2020 but not in 2019, which featured a dry winter. Although, *Z. phytonomi* kills *H. postica* larvae and is considered an important biological control agent (Harcourt and Guppy, 1991; Giles and Obricky, 1997), it also kills parasitoid larvae (Giles et al. 1994; Kuhar et al. 1999). Our results show that epizootic infections of this fungus disrupt the alfalfa weevil–parasitoid system under Spanish crop conditions. The lower *B. anura* parasitism rate in 2020 suggests that this species was probably the most affected by the fungus. *B. curculionis* appears later in the study area and therefore has an advantage over *B. anura* because the environmental conditions no longer favor the spread of the disease, increasing the likelihood of host survival until pupation and thus the survival of the parasitoid.

Parasitism rates in 2019 varied at the field level, with maximum values of 37% for *B. anura* and 17% for *B. curculionis*. This agrees with other studies in the same area, where variable rates were reported with a maximum of 30% (Pons and Nuñez 2020). These parasitoids were most effective when introduced into North America to control the alfalfa weevil. The rate of parasitism with *B. curculionis* tended to be high, at times exceeding 90% in the mild San Francisco Bay and Pleasanton areas, but this approach was much less effective in the hotter San Joaquin Valley (Radcliffe and Flanders 1998). Rearing studies conducted in south-western Canada revealed *B. curculionis* parasitism rates of up to 17% (Soroka et al. 2020). This suggests that environmental conditions play a key role in the success of parasitism, with hotter temperatures inhibiting parasitoid performance, and can explain our lower rates recorded in our study area. In other regions of North America, where *B. anura* tends to be the prevalent species (as is the case in Spain), the rate of parasitism was similar to our findings (Harcourt 1990; Berberet and Bisges 1998).

In conclusion, our new molecular strategy provides information about the ecology of *B. anura* and *B. curculionis*, reveals the prevalence of both species, and contributes to the development of biological control strategies in Europe. Our results show that specific primers can be used to detect and identify both endoparasitoid wasps in alfalfa weevil specimens, and provided an alternative way to estimate the rate of parasitism in the field. One drawback of the new method is its tendency to overestimate the rate of parasitism by counting unsuccessful events. Accordingly, we recommend that DNA analysis should be combined with conventional rearing to determine the effective rate of parasitism and also to accommodate interactions with other species that are not specifically targeted by the molecular assay. In spite of the potential of *Bathyplectes* sp. as a biological control agent for *H. postica*, it seems that the alfalfa crop management system currently performed in Spain may be unfavorable to their control capacity. However, the incorporation of a winter cutting and the delay of the first spring alfalfa cutting, which increase the rates of parasitism of *B. anura* and can help to the survival of *B. curculionis*, respectively, are tools that can be included in integrated pest management strategies in Spain and, potentially, in other Mediterranean countries.

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Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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References

Agustí N, Shayler SP, Harwood JD, Vaughan IP, Sunderland KD, Symondson WOC (2003a) Collembola as alternative prey sustaining spiders in arable ecosystems: Prey detection within predators using molecular markers. Mol Ecol 12:3467–3475

Agustí N, Unruh TR, Welter SC (2003b) Detecting *Cacopsylla pyricola* (Hemiptera: Psyllidae) in predator guts using COI mitochondrial markers. Bull Entomol Res 93:179–185

Agustí N, Bourguet D, Spataro T, Delos M, Eychenne N, Folcher L, Arditi R (2005) Detection, identification and geographical distribution of European corn borer larval parasitoids using molecular markers. Mol Ecol 14:3267–3274

Agustí N, Castañé C, Fraile I, Alomar O (2020) Development of a PCR-based method to monitor arthropod dispersal in agroecosystems: *Macrolophus pygmaeus* (Hemiptera: Miridae) from banker plants to tomato crops. Insect Sci 27:1125–1134

Barney RJ, Armbrust EJ (1981) List of the insect predators of the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae). Bull Entomol Soc Am 27:241–244

Barney RJ, Armbrust EJ, Bartell DP, Goodrich MA (1978) Frequency occurrence of *Bathyplectes curculionis* within instars of *Hypera postica* larvae in Illinois. Environ Entomol 7:241–245

Bartell DP, Pass BC (1980) Morphology, development, and behavior of the immature stages of the parasite *Bathyplectes anurus* (Hymenoptera: Ichneumonidae). Can Entomol 112:481–487

Berberet RC, Bisges AD (1998) Potential for competition among natural enemies of larvae of *Hypera postica* (Coleoptera: Curculionidae) in the southern plains. Environ Entomol 27:743–751

Berberet RC, Willson LJ, Odejar M (1987) Probabilities for encapsulation of eggs of *Bathyplectes curculionis* (Hymenoptera: Ichneumonidae) by larvae of *Hypera postica* (Coleoptera: Curculionidae) and resulting reduction in effective parasitism. Ann Entomol Soc Am 80:483–485

Berberet RC, Zarrabi AA, Payton ME, Bisges AD (2003) Reduction in effective parasitism of *Hypera postica* (Coleoptera: Curculionidae) by *Bathyplectes curculionis* (Hymenoptera: Ichneumonidae) due to encapsulation. Environ Entomol 32:1123–1130

Black W, McLain D, Rai K (1989) Patterns of variation in the rDNA cistron within and among world populations of a mosquito, *Aedes albopictus* (Skuse). Genetics 121:539–550

Day WH (1970) The survival value of its jumping cocoons to *Bathyplectes anurus*, a parasite of the alfalfa weevil. J Econ Entomol 63:586–589

Delgado I, Lloveras J (2020) Historia y distribución de la alfalfa. In: Lloveras J, Delgado I, Chocarro C (eds) La alfalfa, agronomía y utilización, 1st edn. Edicions de la Universitat de Lleida, Lleida, Spain, pp 17–32

Dowell RV, Horn DJ (1977) Adaptive strategies of larval parasitoids of the alfalfa weevil (Coleoptera: Curculionidae). Can Entomol 109:641–648

Duodu YA, Davis DW (1974) Selection of alfalfa weevil larval instars by, and mortality due to, the parasite *Bathyplectes curculionis* (Thomson). Environ Entomol 3:549–552

Dysart RJ, Day WH (1976) Release and recovery of introduced parasites of the alfalfa weevil in Eastern North America, 1st edn. Agricultural Research Service - United States Department of Agriculture, Washington

Froger A, Hall JE (2007) Transformation of Plasmid DNA into E. coli using the heat shock method. J vis Exp 6:253

Gariepy T, Kuhlmann U, Gillott C, Erlandson M (2008) A large-scale comparison of conventional and molecular methods for the evaluation of host-parasitoid associations in non-target risk-assessment studies. J Appl Ecol 45:708–715

Giles KL, Obrycki JJ (1997) Reduced insecticide rates and strip-harvesting effects on alfalfa weevil (Coleoptera: Curculionidae) larval populations and prevalence of

Zoophthora phytonomi (Entomophthorales: Entomophthoraceae). J Econ Entomol 90:933–944

Giles KL, Obrycki JJ, Degoover TA (1994) Prevalence of predators associated with *Acyrthosiphon pisum* (Homoptera: Aphididae) and *Hypera postica* Gyllenhal (Coleoptera: Curculionidae) during growth of the first crop of alfalfa. Biol Control 4:170–177

Gomez-Polo P, Traugott M, Alomar O, Castañé C, Rojo S, Agustí N (2014) Identification of the most common predatory hoverflies of Mediterranean vegetable crops and their parasitism using multiplex PCR. J Pest Sci 87:371–378

Goosey HB (2012) A degree-day model of sheep grazing influence on alfalfa weevil and crop characteristics. J Econ Entomol 105:102–112

Harcourt DG (1990) Displacement of *Bathyplectes curculionis* (Thoms.) (Hymenoptera: Ichneumonidae) by *B. anurus* (Thoms.) in eastern Ontario populations of the alfalfa weevil, *Hypera postica* (Gyll.) (Coleoptera: Curculionidae). Can Entomol 122:641–645

Harcourt DG, Guppy JC (1991) Numerical analysis of an outbreak of the alfalfa weevil (Coleoptera: Curculionidae) in eastern Ontario. Environ Entomol 20:217–223

Hoff KM, Brewer MJ, Blodgett SL (2002) Alfalfa weevil (Coleoptera: Curculionidae) larval sampling: comparison of shake-bucket and sweep-net methods and effect of training. J Econ Entomol 95:748–753

Hoffman A (1963) Sous famille des Curculionidae. Tribu des Hyperini. Les Hypera (Syn Phytonomus). In: Balachowsky A (ed) Entomologe appliquée a l'agriculture Tome I Coleoptères, second volume. París, Masson et Cie, pp 984–989

Innis M, Gelfand D (1990) Optimization of PCRs. In: Innis M, Gelfand D, Sninsky J, White T (eds) PCR protocols. Academic Press, San Diego, pp 3–12

Jacob HS, Evans EW (2000) Influence of carbohydrate foods and mating on longevity of the parasitoid *Bathyplectes curculionis* (Hymenoptera: Ichneumonidae). Environ Entomol 29:1088–1095

Jacob HS, Evans EW (2004) Influence of different sugars on the longevity of *Bathyplectes curculionis* (Hym., Ichneumonidae). J Appl Entomol 128:316–320

JMP, version *Pro* 15. SAS Institute Inc, Cary, NC, 1989–2021

Kingsley PC, Bryan MD, Day WH, Burger TL, Dysart RJ, Schwalbe CP (1993) Alfalfa weevil (Coleoptera: Curculionidae) biological control: spreading the benefits. Environ Entomol 22:1234–1250

Kuhar TP, Youngman RR, Laub CA (1999) Alfalfa weevil (Coleoptera: Curculionidae) pest status and incidence of *Bathyplectes* spp. (Hymenoptera: Ichneumonidae) and *Zoophthora phytonomi* (Zygomycetes: Entomophthorales) in Virginia. J Econ Entomol 92:1184–1189

Levi-Mourao A, Núñez E, García A, Meseguer R, Pons X (2022) Alfalfa winter cutting: Effectiveness against the alfalfa weevil, Hypera postica (Gyllenhal) (Coleoptera: Curculionidae) and effect on its rate of parasitism due to Bathyplectes spp. (Hymenoptera: Ichneumonidae). Crop Prot 152:105858

Levi-Mourao A, Meseguer R, Pons X (2021) Parasitism of the alfalfa weevil *Hypera postica* Gyllenhal by *Bathyplectes* sp in Spain, in Proceedings of the 1st International Electronic Conference on Entomology. 10505

Liang GH, Jang EB, Heller WP, Chang CL, Chen JH, Zhang FP, Geib SM (2015) A qPCR-based method for detecting parasitism of *Fopius arisanus* (Sonan) in oriental fruit flies, *Bactrocera dorsalis* (Hendel). Pest Manag Sci 71:1666–1674

Liang GH, Fu LQ, Zheng JX, Lin HY, Lin JH, Sim SB, Jang BJ, Heller WP, Geib SM (2018) Molecular characterization of interspecific competition of *Diachasmimorpha longicaudata* (Ashmead) and *Fopius arisanus* (Sonan) parasitizing the oriental fruit fly, *Bactrocera dorsalis* (Hendel). Biol Control 118:10–15

Los LM, Allen WA (1982) Visual technique for determining presence and stage of *Zoophthora phytonomi* in dead alfalfa weevil larvae. J Econ Entomol 75:375–377

Madeira F, Clemente-Orta G, Alomar O, Batuecas I, Sossai S, Albajes R (2022) Land use alters the abundance of herbivore and predatory insects on crops: the case of alfalfa. J Pest Sci 95:473–491

Maund CM, Hsiao TH (1991) Differential encapsulation of two *Bathyplectes* parasitoids among alfalfa weevil strains, *Hypera postica* (Gyllenhal). Can Entomol 123:197–203

Molina P, Martínez-Ferrer MT, Campos-Rivela JM, Riudavets J, Agustí N (2021) Development of a PCR-based method for the screening of potential predators of the African citrus psyllid *Trioza erytreae* (Del Guercio). Biol Control 160:104661

Nanini F, Maggio DH, Ferronato P, Rugno G, Yamamoto PT, Corrêa AS (2019) Molecular marker to identify *Diaphorina citri* (Hemiptera: Liviidae) DNA in gut content of predators. Neotrop Entomol 48:927–933

Orloff SB (1997) Introduction. In: Orloff SB, Karlson HL, Tauber LR (eds) Intermountain alfalfa management. University of California. Division of Agriculture and Natural Resources, Oakland, pp 2–3

Pons X, Nuñez E (2020) Plagas da la alfalfa: Importancia, daños y estrategias de control. In: Lloveras J, Delgado I, Chocarro C (eds) La alfalfa, agronomía y utilización, 1st edn. Edicions de la Universitat de Lleida, Lleida, Spain, pp 167–202

Puttler B (1967) Interrelationship of *Hypera postica* (Coleoptera: Curculionidae) and *Bathyplectes curculionis* (Hymenoptera: Ichneumonidae) in the Eastern United States with particular reference to encapsulation of the parasite eggs by the weevil larvae. Ann Entomol Soc Am 60:1031–1038

Radcliffe EB, Flanders KL (1998) Biological control of alfalfa weevil in North America. Integr Pest Manag Rev 3:225–242

Rand TA (2013) Host density drives spatial variation of the alfalfa weevil, *Hypera postica*, across dryland and irrigated alfalfa cropping systems. Environ Entomol 42:116–122

Ribes A (2012) Himenòpters de Ponent. http://ponent.atspace.org/fauna/ins/index.htm. Accessed 3 May 2021

Saeidi M, Moharramipour S (2017) Physiology of cold hardiness, seasonal fluctuations, and cryoprotectant contents in overwintering adults of *Hypera postica* (Coleoptera: Curculionidae). Environ Entomol 46:960–966

Saiki R (1990) Amplification of genomic DNA. In: Innis M, Gelfand D, Sninsky J, White T (eds) PCR protocols. Academic Press, San Diego, pp 13–20

Salt G, van den Bosch R (1967) The defense reactions of three species of *Hypera* (Coleoptera: Curculionidae) to an ichneumon wasp. J Invertebr Pathol 9:164–177

Shoubu M, Okumura M, Shiraishi A, Kimura H, Takagi M, Ueno T (2005) Establishment of *Bathyplectes anurus* (Hymenoptera: Ichneumonidae), a larval parasitoid of the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae) in Japan. Biol Control 34:144–151

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann Entomol Soc Am 87:651–701

Solà M, Riudavets J, Agustí N (2018) Detection and identification of five common internal grain insect pests by multiplex PCR. Food Control 84:246–254

Soroka J, Grenkow L, Cárcamo H, Meers S, Barkley S, Gavloski J (2019) An assessment of degree-day models to predict the phenology of alfalfa weevil (Coleoptera: Curculionidae) on the Canadian Prairies. Can Entomol 152:110–129

Soroka J, Bennett AMR, Kora C, Schwarzfeld MD (2020) Distribution of alfalfa weevil (Coleoptera: Curculionidae) and its parasitoids on the Canadian Prairies, with a key to described species of Nearctic *Bathyplectes* (Hymenoptera: Ichneumonidae). Can Entomol 152:663–701

Tilmon KJ, Danforth BN, Day WH, Hoffmann MP (2000) Determining parasitoid species composition in a host population: a molecular approach. Ann Entomol Soc Am 93:640–647

Traugott M, Symondson WOC (2008) Molecular analysis of predation on parasitized hosts. Bull Entomol Res 98:223–231

Traugott M, Zangerl P, Juen A, Schallhart N, Pfiffner L (2006) Detecting key parasitoids of lepidopteran pests by multiplex PCR. Biol Control 39:39–46

Traugott M, Kamenova S, Ruess L, Ruess L, Seeber J, Plantegenest M (2013) Empirically characterising trophic networks: what emerging DNA-based methods, stable isotope and fatty acid analyses can offer. Adv Ecol Res 49:177–224

Van Den Bosch R, Dietrick EJ (1959) The interrelationships of *Hypera brunneipennis* (Coleoptera: Curculionidae) and *Bathyplectes curculionis* (Hymenoptera: Ichneumonidae) in Southern California. Ann Entomol Soc Am 52:609–616

Wolf S, Zeisler C, Sint D, Romeis J, Traugott M, Collatz J (2018) A simple and cost-effective molecular method to track predation on *Drosophila suzukii* in the field. J Pest Sci 91:927–935

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CHAPTER IV

Influence of landscape composition on the abundance of the alfalfa weevil (*Hypera postica*) and its natural enemies in alfalfa crops of Northeast Iberian Peninsula

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Influence of landscape composition on the abundance of the alfalfa weevil (*Hypera postica*) and its natural enemies in alfalfa crops of Northeast Iberian Peninsula

ABSTRACT

The alfalfa weevil, *Hypera postica* Gyllenhal (Coleoptera: Curculionidae) is one of the most destructive pests of alfalfa distributed worldwide. As a perennial crop, alfalfa offers a stable habitat for many predators and parasitoids. Pest outbreaks and its natural biological control not only depend on local field conditions and have strong links to landscape structure patterns. The present study provides, for the first time, evidences of the effect of landscape composition on the abundance of the alfalfa weevil and its main natural enemies. A total of 65 alfalfa commercial fields, located along the Ebro Basin, were sampled during three years. Landscape composition was recorded at buffer radii of 250 m, 500 m and 1000 m from the centre of each field. *H. postica* abundance mostly depended on the characteristics of the own alfalfa field, since very few relation were observed at a landscape scale. Landscape composition variables explained very few of *Bathyplectes* spp. abundance and nothing about its parasitism rate. On the contrary, only landscape variables, as the proportion of winter cereal cover area, contributed positively to the abundance of *C. septempunctata*, an occasional predator of *H. postica* larvae.

KEY WORDS: Landscape structure; alfalfa pest; parasitism rate; *Bathyplectes* spp.; *Coccinella septempunctata*; local variables; landscape variables.

INTRODUCTION

Economically, alfalfa (*Medicago sativa* L.) is one of the most important forage crop worldwide (Michaud et al. 1988; Frame et al. 1998). In Spain, it covers an area of 250,000 ha, accounting for approximately of the 20% of the European cultivated surface. In the Ebro basin, alfalfa is a common crop in irrigated areas and represents ~60% of Spanish total alfalfa cultivated surface (Delgado and Lloveras 2020).

One of the most destructive specific pests of alfalfa, is the alfalfa weevil *Hypera postica* Gyllenhal (Coleoptera: Curculionidae) (Goosey 2012; Saeidi and Moharramipour 2017; Pons and Nuñez 2020). Although nowadays has spread worldwide, the weevil is native from Eurasia region (Hoffmann 1963). Despite this, there is little information on scientific literature about its ecology and control in Europe. In Spain, damages are mainly produced during the first alfalfa cutting in spring (March-April), but sometimes it can also affect the second one (Hoffmann 1963; Domínguez 1989; Alfaro 2005; Pons and Nuñez 2020). Females starts their oviposition in October, after a summer estivation (Levi-Mourao et al. 2021), and lay clusters of eggs inside the alfalfa stems (Hoffmann 1963; Levi-Mourao et al. 2022b). Both adults and larvae feed on alfalfa, but larvae cause most of the damage, resulting this in economic losses since they reduce plant growth and cause a significant biomass loss (Berberet and McNew 1986; Alfaro 2005).

As a perennial crop and one of the traditional components of crop rotations in Spain, alfalfa offers a favourable stable habitat for many beneficial arthropods, including predators and parasitoids (Núñez 2002; Pellissier et al. 2017). It is known that this natural enemy complex contributes to minimize primary and secondary pest outbreaks not only for itself, but also to its surrounding crops (Summers 1998; Madeira et al. 2019, 2021). One of the most frequent natural enemy of the alfalfa weevil larvae are the solitary larval endoparasitoid wasps of the genus *Bathyplectes* spp. (Hymenoptera: Ichneumonidae) (Flanders et al. 1994; Radcliffe and Flanders 1998; Pellissier et al. 2017). Although Ribes (2012) reported the presence of eight species in the Iberian peninsula, only two are known to be associated with the alfalfa weevil, *B. anura* and *B. curculionis* (Thomson) (Pons and Nuñez 2020; Levi-Mourao et al. 2022a). Besides these parasitoids, some predatory species that coincide in time with the alfalfa weevil during the first alfalfa cutting in Spain can also contribute reducing this pest population in the field when their natural prey is scarce (Lavallee and Shaw 1969; Hussain 1975; Ouayogode and Davis 1981; Richards and Evans 1998; Pellissier et al. 2017; Rand 2017; Shrestha et al. 2021). Recent studies in the area suggests that the aphidophagous *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), uses *H. postica* larvae as an alternative source of food when pea aphid populations are low (Meseguer et al. 2021). Hence, the abundance of these natural enemies can prevent alfalfa weevil outbreaks.

Pest outbreaks and pest control by its natural enemies do not depend only on local field conditions and have strong links to landscape patterns (With et al. 2002). Most of the literature on landscape composition and insect abundance is related to the natural enemies of insect pests with the main goal of cost-effective control (Symondson et al. 2003; Bianchi et al. 2006; Tscharntke et al. 2012; Rusch et al. 2016). Since most pests and natural enemies need to move over the landscape to search for resources (change of host, feeding or mating), up-scaling from field to landscape appears necessary (Landis et al. 2003; Rand et al. 2006; Tscharntke et al. 2012; Bianchi et al. 2013). Contrarily to other landscape types, agricultural landscape are one of the most dynamics (Petit 2009) since most crops are subjected to a high frequency of disturbance caused either by agronomic practises, changes in crop phenology, harvest or crop rotation that makes them periodically unsuitable. The impact on landscape features on pest abundance and pest control by natural enemies is thus expected to change within year and between years (Menalled et al. 2003; Bianchi et al. 2006). As a pluriannual crop, in spite of crop management (several cuttings during the vegetative growing season), alfalfa offers a much more stable habitat than other extensive field crops such as winter cereals, maize, sunflower or ryegrass. This allows many arthropods to use alfalfa as a habitat for feeding, development, reproduction, and shelter (Summers 1998; Núñez 2002; Pons and Nuñez 2020).

When considering a specialist pest and the specific crop it attacks, a positive relation is expected between field pest abundance and the surface of that specific cultivated crop at a landscape scale. However, there are reasons for which this relationship could be inexistent or even negative. Östman et al. (2001) and Thies et al. (2005) reported that some pests have several alternative hosts and may live during an important part of their life cycle outside the crops; Ricci et al. (2009) observed that the abundance of some pests in the fields may depend on the presence of non-disturbed elements in the landscape, such as non-treated areas, margins, and woody areas.

In the case of natural enemies, it is recognized that its abundance in the fields largely depends on the amount of alternative habitats at landscape scale (Langellotto and Denno 2004; Bianchi et al. 2006; Tscharntke et al. 2007). Increasing biodiversity in crops is generally associated with increases in natural enemy abundance and/or diversity (Schmidt et al. 2008; Drapela et al. 2008; Werling and Gratton 2008; Gardiner et al. 2009; Chaplin-Kramer et al. 2011). These increases in natural enemy abundance and diversity are no guarantee for pest control (Bianchi et al. 2006; Straub et al. 2008; Tscharntke et al. 2016; Karp et al. 2018), but in some cases suggests that there is a tendency for pest enemy communities to better control pests arthropods (Letourneau et al. 2009). Several authors along these last years documented positive relationships between landscape complexity and rates of parasitism or predation (Costamagna et al. 2004; Bianchi et al. 2006; Thies et al. 2008; Boccaccio and Petacchi 2009; Rusch et al. 2016).

So far, alfalfa has been described mainly as a unit independent of its environment analysing the pests and the control methodologies that can be applied at field scale. Recognizing the need for up-scaling, some studies in the Ebro Basin have reported the relationships between landscape characteristics and insect abundance in different crops, such as maize and alfalfa (Madeira et al. 2014, 2021; di Lascio et al. 2016; Clemente-Orta et al. 2020). However, none of these studies focused on the alfalfa weevil and its natural enemies. Little is known about the movement capacity of alfalfa weevil adults, and how the abundance of this pest may be affected by other alfalfa fields or by the surrounding landscape components. Some other studies revealed the importance of field margins providing overwintering and summer aestivation sites for this pest (Manglitz 1958; Dennis and Fry 1992; Holland and Fahrig 2000).

The objective of the present study is to understand the influence of the surrounding landscape composition on the alfalfa weevil abundance, a specific pest of alfalfa; the abundance and parasitism rate of *Bathyplectes* spp., its specific parasitoid; and the abundance of *C. septempunctata*, an aphidophagous predator, which can use *H. postica* larvae as an alternative source of food. In base of this objective, we present three hypotheses: 1) since the alfalfa weevil is a specific pest of this crop, a positive relationship is expected between its abundance and the surface of cultivated alfalfa at a landscape scale; 2) the alfalfa weevil presents low mobility and therefore it will be more influenced by crop characteristics and management than by the surrounding landscape; 3) the abundance of its natural enemies will be more related with the surrounding landscape, and not only with the alfalfa itself.

MATERIALS AND METHODS

Study area

The study was performed in the Ebro basin region (Figure 1). It is a geographic region of Northeast of the Iberian Peninsula, western Mediterranean area (Figure 1b). Mean temperatures range from 1 °C in winter to 30 °C in summer. Annual rainfall is variable and ranges from 200 to 800 mm, and is mainly concentrated in spring and autumn. Mean altitude is 200 m (asl). In this area, agricultural landscapes are traditionally dominated by arable crops that are managed by the rotation of winter cereals, such as wheat and barley, summer cereals, mainly maize, and alfalfa. Winter cereals occupy the field from October to June and maize is present from the end of March/April to October, although the current tendency is to sow it earlier during March in non-rotation or in single crop-rotation systems (Cantero-Martínez et al. 2006; Albajes et al. 2022). Besides this, non-cultivated areas, formed by forests and woody areas, older fallows, natural habitats, margins, roads, and irrigation canals can be found inserted in these agricultural landscape mosaics. Forests are mainly compound by repopulated *Pinus halepensis* (Mill.) and Mediterranean bushes.

A total of 65 commercial fields of alfalfa located in four different counties of the region (Urgell, Segrià, Baja Cinca, and Monegros) (Figure 1b) were selected during 2018, 2019, and 2020 (Table S1 to see field characteristics and location). The fields in each county were separated by at least 2 km to avoid potential spatial autocorrelation. Selection was made based in a landscape gradient composition, ranging from landscapes with more predominance of alfalfa to others with less predominance (Table S2). The selected alfalfa fields were 2, 3 or 4 years old, and were sown with the Aragon variety. This commercial variety, obtained from the ecotype Aragon, has been cultivated in the Ebro Valley for decades. In addition to tolerating temperatures below - 15 ◦C, it has a short dormancy period, fast development in spring and after cutting regrowth, and it may be cut 5–6 times per season under irrigation (Delgado 2020; Delgado and Lloveras 2020). No insect resistance traits are known for this variety. The field size ranged from 1 to 17 ha, a common range in this area, and were sprinkler or blanket irrigated. During the study period no pesticides were applied.

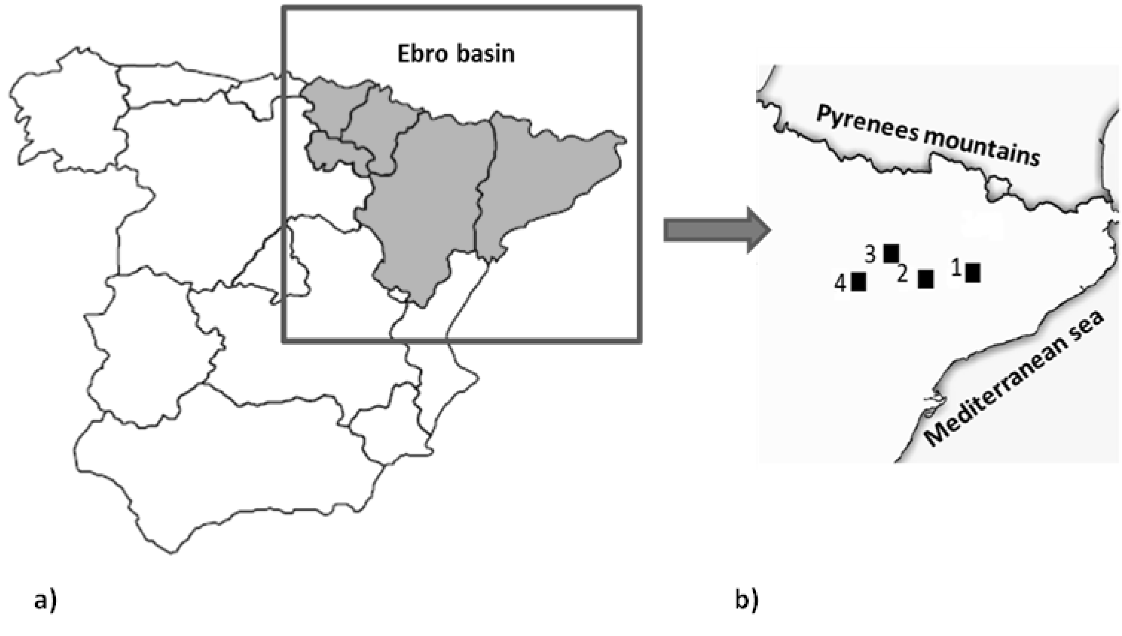

Figure 1 a) Localization of the Ebro Basin region in Spain. **b)** The four field counties were the 65 fields were selected during 2018, 2019 and, 2020: (1) Urgell (n= 21); (2) Segrià (n= 13); (3) Baja Cinca (n= 10); and (4) Monegros (n= 7).

Data records

For each alfalfa field selected, crop management and field characteristics were recorded. We also recorded the landscape composition at buffer radii of 250 m, 500 m and 1000 m. from the centre of the sampled field. Each field was sampled for insects once in winter (eggs) and twice (larva and adult) during the first alfalfa intercut (the period of alfalfa lasted from the beginning of the vegetative grow period to the first crop cutting, see Pons et al. (2005)) in March and April. When possible, sampling was repeated in the same fields in the three study years, but others due to crop rotations, remained only one or two years in the study.

Local field variables

As local field variables we included the perimeter of each alfalfa sampled field, area, altitude, alfalfa age, irrigation system, the abundance of the alfalfa weevil (eggs, larvae and adults), the total abundance of the predator *C. septempunctata*, and the total parasitism rate by both parasitoid species: *B. anura* and *B. curculionis* (Table 1). The perimeter and the area of each selected alfalfa field was calculated using ArcGIS software 10.3.3 (ESRI 2018).

Landscape variables (cover types)

ArcGIS software was used to quantify land cover types. Landscape composition was obtained by: 1) direct field inspections during the first alfalfa cutting (March-April); 2) using orthophotos from the Plan Nacional de Ortografía Aérea (PNO, https://pno.ign.es/); 3) using geographical information maps of the Instituto Geográfico Nacional de España (https://www.ign.es); and 4) consulting Declaració Agrària (DUN) from the Departament d'Acció Climàtica, Alimentació i Agenda Rural (http://agricultura.gencat.cat/ declaracio-unica-agraria/) (The DUN is an annual declaration that must be submitted by the person in charge of the holding, defining the crop and its cultivated area). Land cover characterizations were performed during the first alfalfa intercut (March-April), during the three years of the study. The landscape elements initially identified within the field inspections were categorized for each of the three different buffer radii, being the same at 250 and 500m, and more grouped at 1000m (see table 1).

Insect sampling

H. postica egg abundances were obtained by collecting 200 stems per field (from middle December to the second fortnight of January), excising them from the plant crown with a scissors. Stems were then brought to the entomology laboratory of the University of Lleida, kept in a refrigerator at 5 °C, and dissected during the following 7 days.

H. postica larvae and adult, *C. septempunctata* (considering adults and larvae together) and *Bathyplectes* spp. adult abundances were obtained by conducting 180 ° sweeping with a 38 cm diameter net. The sampling pattern for each field consisted on 24 total samples, each one consisting on five sweeps (each field was divided in 4 sectors, 6 samples for each). Collected field samples were transported to the laboratory and frozen at - 20 °C until processing.

In addition to the sampling pattern described, on the same sampling date, we collected an additional sample consisting of 20 sweeps per field (1 sample for each sector) for larval parasitism estimation. Within the next few hours, 50 larvae per field (L2–L4) were immediately frozen at −80 °C and used to estimate the total rate of parasitism by PCR, using *B. anura* and *B. curculionis* specific primers and protocol (Levi-Mourao et al. 2022a) .

Data analysis

Field and landscape variables considered in the analysis are listed and described in Table 1.

The cover-types identified in the landscape, and included in the model analysis, were grouped into ten categories at the 250 and 500m radii buffers as: alfalfa, winter cereals, rye-grass, forest and/ or woody natural habitat, orchard, margins, non-crop habitats, unproductive and/ or gardens, and roads (Table 1). Fruit orchards were grouped into the same category, although they were initially categorized by species, due to its high variability between sampled areas; to that the most prevalent ones belonged to the same family (Rosaceae); and that in a great proportion of landscape characterized field areas were nearly absent (Monegros, Baja Cinca).

Besides these initial categories, six alternative categories were defined at the 1000m buffer radii, derived from the aggrupation of the previous ones, resulting in: alfalfa, arable crops other than alfalfa, forests, orchards, margins and/or non-crop habitats, and roads (Table 1).

Autocorrelation can be a problem for classical statistical tests, such as a regression, which rely on independently distributed errors, as it may lead to erroneous conclusions regarding the significance of covariates in studies of species-environment relationships (Legendre 1993; Wagner and Fortin 2005). Therefore, in this study, correlation degrees between landscape composition and field variables were tested using Spearman rank correlations (Dormann et al. 2013) (Table S3). Following Schmidt et al. (2019), variables were only discarded from the models if strongly or very strongly correlated (Spearman's rho >0.6) (Campbell and Swinscow 2009), and when it occurred, only one variable was included in the model. Spatial correlation among fields was tested based on the mean captures using Moran's I statistic (Paradis 2019) (Table S4). Cover landscape variables with less than 1% of surface were also discarded from the model analysis (see Table S2).

Table 1 Local field and landscape composition surrounding the sampled fields within the three different buffers radii (250, 500 and 1000m) included in the model analysis.

Categories	Variables 250 and 500m	Variables 1000m	Description
Field	Perimeter		Sampled alfalfa field perimeter (m)
	Altitude		Sampled alfalfa field altitude (m)
	Age		Number of years of alfalfa in the field
	Irrigation		Alfalfa watering system: blanket or sprinkler
	Pest/ parasitoid		Abundance of larvae and adults of *H. postica* , and abundance of *Bathyplectes* spp. adults
Landscape	Alfalfa	Alfalfa	Alfalfa proportion
	Winter cereal	Arable crops	Winter crops, mainly wheat, and barley; rye-grass; pea and/or vetch; and maize proportions
	Rye-grass		
	Forest and/ or woody natural habitat	Forest	Natural forest proportion, mainly from the genus *Pinus* sp.
	Orchard	Orchard	Fruit orchard proportion, mainly peach, pear and apple. In few cases apricot, olive, cherry, fig, almond, and walnut trees
	Margin	Margins and non-crop habitats	Margin strips and patches, unproductive areas, older fallows, natural habitats, gardens and wetlands proportion
	Non-crop habitats		
	Unproductive and/ or gardens		
	Roads	Roads	Roads and paths proportion

To analyse the effects of the landscape composition and the local variables on abundances of the alfalfa weevil abundance, *C. septempunctata* (considering adults and larvae), and adult parasitoid, and parasitism rates, a linear mixed-effects model was used, where year was considered a random factor using the 'nlme' package (Pinheiro et al. 2021) in R software (R Development Core Team 2018). For each field and year, the insect abundances of the two samplings carried out during the first alfalfa cutting were averaged. The Shapiro-Wilk test was used to determine whether or not the original data were normally distributed. Mean insect captures were log-transformed [log10(x + 1)], and parasitism rates were arcsin-transformed, in order to achieve as normal a distribution of the model residuals as possible. Landscape metrics was standardized, for each model, using 'caret' package (Max et al. 2021). A multimodel inference approach was used to obtain a robust parameter estimate ('MuMIn' package, Barton 2020). The 'dredge' functions of the R package 'MuMin' was used to fit all possible combinations of models, describe the effects of independent variables on each dependent variable, and to calculate their associated corrected Akaike's Information Criteria (AICc). AICc were used for models selection, with the values of the full models. Model averaging was performed on the model set with ΔAiCc<2 (Burnham and Anderson 2004) using the 'model.avg' function in the 'MuMIn' package. The model residuals were graphically analysed using qqplot and histogram graphics to ensure no violation of normality and homoscedasticity assumptions (Zuur et al. 2010). Finally, to represent the effects in partial residual plots, 'effects' package (Fox et al. 2020) was used.

RESULTS

Insect abundances

For *H. postica*, a total of 25024 eggs, 171808 larvae, and 1952 adults were collected in the 65 sampled alfalfa fields during the 2018, 2019, and 2020 campaigns. In the case of its natural enemies, 863 adult *Bathyplectes* spp. and 1149 *C. septempunctata* were collected.

Effects of field variables

The significant effects of alfalfa field variables on *H. postica*, *Bathyplectes* spp. (adults and parasitism rate) and *C. septempunctata* are shown in Table 2.

The abundance of *H. postica* eggs showed to be associated to the irrigation system and it was higher when the field was sprinkler irrigated. The field perimeter was positively correlated with the abundance of H. postica eggs, but only when included the landscape variables at 500 m radius buffer (Figure 2). The field perimeter also positively determined the abundance of alfalfa weevil larvae at the three different radii (Figure 2). The abundance of *H. postica* adults was positively associated to that of adult abundance of *Bathyplectes* spp. at all the landscape buffers considered (Figure 2), and the reciprocal relationship also occurred. The alfalfa age and the altitude also positively affected the abundance of *Bathyplectes* spp. adults but only when included the landscape variables at 250 m (Figure 2).

Parasitism rate due to *Bathyplectes* spp. was negatively correlated with the abundance of larvae of *H. postica* (Figure 2). No effects of field variables on the abundance of *C. septempunctata* were found.

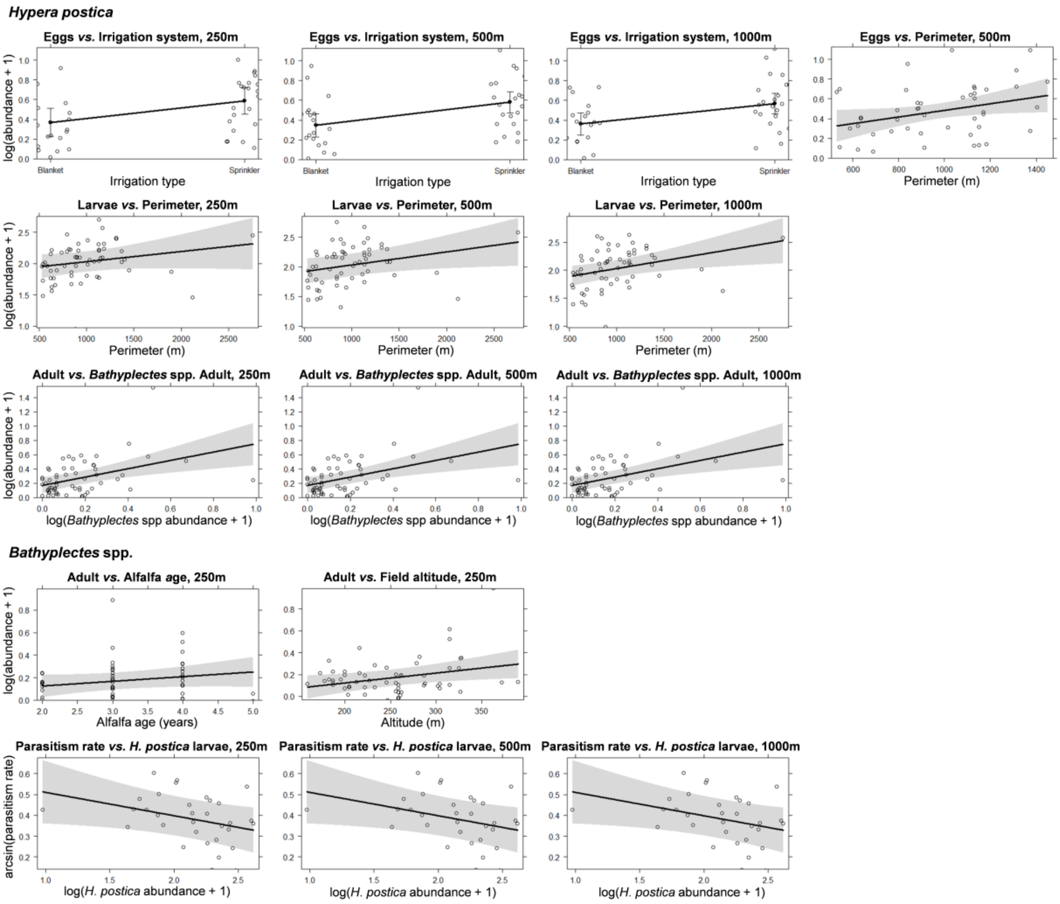

Figure 2 Effects of field variables on the abundance of *H. postica* (eggs, larvae, and adults), and *Bathyplectes* spp. (adults) and parasitism rate by *Bathyplectes* spp.

Effects of landscape variables

The significant effects of alfalfa landscape variables on *H. postica*, *Bathyplectes* spp. (adults and parasitism rate) and *C. septempunctata* are shown in Table 2.

The landscape composition had a weak effect on the abundance of *H. postica*. Non-crop habitats were positively associated to the abundance of eggs at 500 and 1000 m radii buffers. The proportion of winter cereal cover affected negatively to the abundance of larvae at 250 and 500 m (Figure 3) radii buffer. No landscape variable effects were observed in relation with *H. postica* adult abundance.

No effects of landscape composition on the abundance of *Bathyplectes* spp. was found at 250 m, but there were positive effects of the proportion of winter cereals at 500 m, and arable crops (including winter cereals) at 1000m (Figure 3). At this last radius buffer, the proportion of alfalfa resulted in a negative association with *Bathyplectes* spp. adult abundance (Figure 3). In the case of parasitism rates, no effect of the landscape composition was observed.

The proportion of winter cereals at 250 and 500 m radii buffer contributed positively to the abundance of *C. septempunctata* in alfalfa fields (Figure 3). This abundance was also fostered by the proportion of arable crops at 1000 m buffer, where winter cereals category was included (Figure 3).

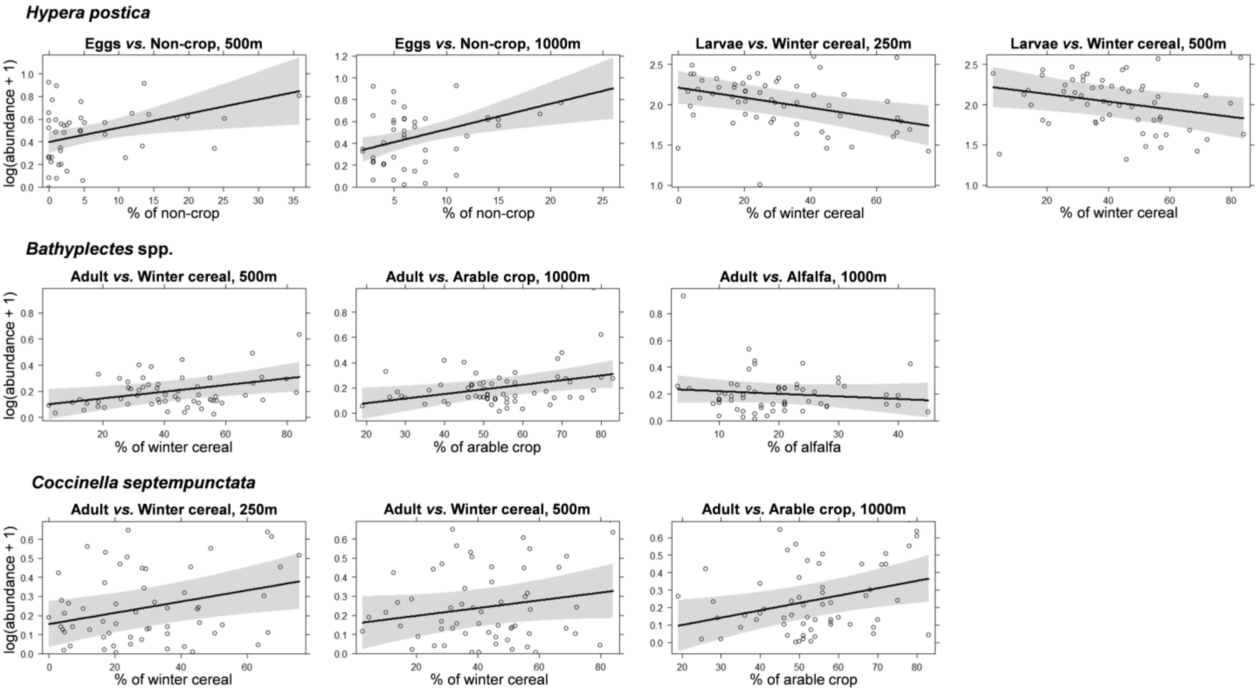

Figure 3 Effects of landscape composition variables on the abundance of *H. postica* (eggs, larvae, and adults), *Bathyplectes* spp. (adults) and its parasitism rate, and *C. septempunctata* (adults + larvae).

Table 2 Significant variables ($p \leq 0.05$) in the best models (ΔAiCc<2) relating *H. postica* (eggs, larvae, and adults), *Bathyplectes* spp. (adults, and parasitism rate), and *C. septempunctata* (adults + larvae) abundances with field and landscape variables, respectively. Different tables represent the three radii, 250, 500, and 1000m used in this study. Variables were mean-centred and scaled. Relative importance is the sum of Akaike's weight associated with the variables in the best models.

250m

Insects (Variables)		**Variables best Model**	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr (>|z|)**	**Relative importance**	**N containing models**
H. postica	Eggs	*(Intercept)*	0.71162	0.15099	0.15546	4.577	4.70E-06		
		Irrigation System	0.70856	0.20881	0.21488	3.297	**0.000976**	1	18
	Larvae	*(Intercept)*	4.65973	0.16428	0.16809	27.722	< 2e-16		
		Perimeter	0.54452	0.15276	0.15628	3.484	**0.000493**	1	19
		Cereal	-0.34156	0.09273	0.09483	3.602	**0.000316**	1	19
	Adults	*(Intercept)*	0.38855	0.12041	0.12312	3.156	0.001601		
		Bathyplectes adults	0.58323	0.17186	0.17572	3.319	**0.000903**	1	9
Bathyplectes spp.	Adults	*(Intercept)*	0.39151	0.08108	0.08295	4.72	0.0000024		
		H. postica adults	0.24069	0.07876	0.0806	2.986	**0.002824**	1	4
		Age	0.1165	0.05343	0.05463	2.133	**0.0329**	0.82	6
		Altitude	0.10383	0.04734	0.04842	2.144	**0.032**	1	8
	Parasitism rate	*(Intercept)*	0.56486	0.14127	0.14519	3.89	1.00E-04		
		H. postica larvae	-0.04704	0.02274	0.02378	1.979	**0.0479**	0.81	10
C. septempunctata	Adults + larvae	*(Intercept)*	-0.05643	0.41322	0.41995	0.134	0.8931		
		Cereal	0.18076	0.06076	0.06202	2.914	**0.00356**	1	8

Table 2 (continued)

500m

Insects (Variables)		**Variables best Model**	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr (>|z|)**	**Relative importance**	**N containing models**
H. postica	Eggs	*(Intercept)*	0.79425	0.13761	0.14187	5.598	< 2e-16		
		Irrigation system	0.55072	0.18896	0.19478	2.827	**0.00469**	1	10
		Perimeter	0.21839	0.10098	0.104	2.1	**0.03573**	1	10
		Non-crop	0.23934	0.09403	0.09687	2.471	**0.01349**	1	10
	Larvae	*(Intercept)*	4.66939	0.17905	0.18334	25.468	< 2e-16		
		Perimeter	0.54749	0.15897	0.16262	3.367	**0.00076**	1	11
		Cereal	-0.24527	0.09898	0.10096	2.429	**0.01512**	1	11
	Adults	*(Intercept)*	0.38331	0.12611	0.12894	2.973	0.002951		
		Bathyplectes adults	0.59892	0.17388	0.17775	3.369	**0.000753**	1	7
Bathyplectes spp.	Adults	*(Intercept)*	0.3975	0.11076	0.11333	3.508	0.000452		
		H. postica adult	0.23906	0.0795	0.08135	2.939	**0.0033**	1	38
		Cereal	0.11537	0.05305	0.05411	2.132	**0.033006**	0.72	35
	Parasitism rate	*(Intercept)*	0.57064	0.13932	0.14339	3.98	6.90E-05		
		H. postica larvae	-0.04661	0.0227	0.02373	1.964	**0.0495**	0.83	8
C. septempunctata	Adults + larvae	*(Intercept)*	0.49853	0.21935	0.22216	2.244	0.0248		
		Cereal	0.13181	0.06571	0.06697	1.968	**0.0491**	0.56	19

Table 2 (continued)

1000m

Insects (Variables)		**Variables best Model**	**Estimate**	**Std. Error**	**Adjusted SE**	**z value**	**Pr (>|z|)**	**Relative importance**	**N containing models**
H. postica	Eggs	*(Intercept)*	0.86489	0.15902	0.16210	5.336	1.00E-07		
		Sprinkler irrigation	0.48321	0.19765	0.20293	2.381	**0.01726**	0.86	18
		Non-crop	0.28665	0.09466	0.09738	2.944	**0.00324**	1	22
	Larvae	*(Intercept)*	4.66491	0.17351	0.17749	26.283	< 2e-16		
		Perimeter	0.57917	0.16925	0.17307	3.346	**0.000818**	1	15
	Adults	*(Intercept)*	0.38843	0.12436	0.12713	3.055	0.002248		
		Bathyplectes adults	0.58588	0.17229	0.17612	3.327	**0.000879**	1	3
Bathyplectes spp.	Adults	*(Intercept)*	0.38719	0.10292	0.10521	3.68	0.000233		
		H. postica adult	0.2361	0.07925	0.08104	2.913	**0.00358**	1	16
		Alfalfa	-0.11414	0.04851	0.04958	2.302	**0.021328**	0.36	8
		Arable crop	0.19152	0.08646	0.08763	2.186	**0.028847**	0.73	18
	Parasitism rate	*(Intercept)*	0.58902	0.13464	0.13899	4.238	2.26E-05		
		H. postica larvae	-0.04814	0.02263	0.02367	2.034	**0.0419**	0.88	8
C. septempunctata	Adults + larvae	*(Intercept)*	0.39551	0.31401	0.31744	1.246	0.2128		
		Arable crop	0.15338	0.06255	0.06378	2.405	**0.0162**	1	7

DISCUSSION

Several studies, developed in the Ebro Basin during the last decades, have described the composition, ecological role and abundance of insects that live in alfalfa, concluding that it can act as an important reservoir of natural enemies not only for itself, but also for the other neighbouring crops, whereas pests are rather specific of this crop (Núñez 2002; Pons et al. 2005; Madeira et al. 2014, 2019; Di Lascio et al. 2016; Batuecas et al. 2022). These studies have focused at field and farm scales. Recently, some studies showed that landscape composition can modulate the influence of alfalfa on the abundance of herbivores and its natural enemies in some neighbour crops located within a landscape buffer of 500 m radii (Clemente-Orta et al. 2020; Madeira et al. 2021). However, these studies did not focus on how the effect of landscape composition or even field characteristics affects the main pests of alfalfa.

The alfalfa weevil is a highly worldwide destructive pest in most of the areas where alfalfa is cultivated, including Spain (Goosey 2012; Saeidi and Moharramipour 2017; Soroka et al. 2019; Levi-Mourao et al. 2022b). Despite this, little is known about the factors that regulate the abundance of this pest in Europe. The present study reports, for the first time, the effects of landscape composition on alfalfa weevil and its natural enemies in Mediterranean alfalfa crop conditions.

As initially hypothesized in our study, and since the alfalfa weevil is a specific pest of alfalfa, we expected a positive relationship between its abundance and the alfalfa surface in the surrounding landscape. Nevertheless, very few habitats of landscape affected its abundance at any buffer radii, and none was directly related with the proportion of alfalfa itself. Beside this, and according to our second hypothesis, the abundance of *H. postica* mostly depended on the characteristics of the own alfalfa field.

In the particular case of *H. postica* eggs, there was an effect on the abundance caused by the irrigation system, and more eggs were found in fields irrigated with sprinklers. This fact can be explained because sprinkler supply piper, whose bases hinder the cutting of alfalfa more accurately, offers constant plant availability for adults to oviposit, working as a possible field refugee, as it was also suggested in Levi-Mourao et al. (2022b). The field perimeter length was also positively related with *H. postica* egg and larval abundances. Field perimeter may be considered as a proxy of the amount of margin surrounding a field. Field margins and non-crop habitats surrounding crops, can act as natural refugees for alfalfa herbivores and some natural enemies, enhancing their abundance (Landis et al. 2003; Madeira et al. 2021). In the case of the alfalfa weevil, field margins represent an essential element for its life cycle accomplishment. Several authors have described that summer aestivating adult weevils were considerably more concentrated in the border of fields, resulting in a quicker field re-infestation for feeding and reproduction in autumn (Prokopy and Gyrisco 1965; Prokopy et al. 1967; Saeidi and Moharramipour 2017). This effect of autumn adult field re-infestation is indirectly suggested by the abundance of eggs and larvae recorded in spring.

The *H. postica* adult abundance and those of *Bathyplectes* spp. were positively related. This association can be explained since a greater abundance of weevil adults is directly related to the fact of a previously greater abundance of larvae, which have given rise to these adults. In turn, this could result in a greater number of adult parasitoid probably due to a greater number of parasitism events.

The only landscape variables related with the *H. postica* abundance were the proportion of non-crop and cereal covers. The proportion of non-crop cover positively affected the abundance of eggs at 500 and 1000 buffer radii. This kind of cover may also play a role of refuge for adults in a similar way to that of field margin. The proportion of cereal cover negatively affected the abundance of *H.postica* larvae at 250 m and 500 m buffer radii. This fact can be explained because the surface of cereal and alfalfa within a buffer are complementary and when more cereal less alfalfa proportion.

Contrarily to what we hypothesized, landscape composition variables explain very few of *Bathyplectes* spp. abundance and nothing about its parasitism rate. In the case of local field variables, alfalfa age was positively related with the wasp abundance. As alfalfa is a pluriannual crop, a higher density of weeds can be observed as the alfalfa becomes overexploited or mostly aged (Taberner 2020). Most of these weeds are spring emergence species and belong to the Asteraceae family. *Bathyplectes* spp. visit plants in the field to feed on floral and extra floral nectar (van Emden 1963; Maingay et al. 1991; Jervis et al. 1993; Jacob and Evans 2000), increasing this its longevity and reproductive performance. Jacob and Evans (2000) observed a positive influence effect on these life parameters when *Bathyplectes* spp. females were feed on *Taraxacum officinale* Weber. This can explain, somehow, this positive relationship between the parasitoid abundance and field age. No explanation can be suggested in relation to field altitude at a 250 m buffer scale related with parasitoid adult abundance, and can be considered as a statistical artefact when included in the model analysis.

Only the proportion of winter cereal cover at 500 m radius buffer and those of arable crops and alfalfa at 1000 m, showed significant relationship with the abundance of adults of *Bathyplectes* spp. A somewhat unexpected result was that the correlation with the proportion of alfalfa was negative. There are two possible non-exclusive explanations for this result. Either this could be the result of a dilution effect whereby a given number of individuals is distributed at a lower density over a larger area (Veddeler et al. 2006; Zaller et al. 2008), or it could result from a metapopulation dynamics where only a few alfalfa fields hosted high wasp populations, while others were unfavourable temporal habitats due to agronomic practices, or even some biotic factors that maintained populations at lower densities (Ricci et al. 2009).

These findings suggest that *Bathyplectes* spp. is linked to agricultural crop cover rather than natural or non-agricultural covers. This has also been observed by several authors in cereal aphid parasitoids (Thies et al. 2005, 2008; Roschewitz et al. 2005). The rate of parasitism was negatively related to the *H. postica* larval abundance. This negative density dependence has already been reported by Rand (2013), who argue that the reasons for this to happen are unclear, but it may occur through hindering the host selection or by increasing the host handling time.

Our data showed that the abundance of *C. septempunctata* was affected by winter cereal proportion at 250 m, 500 m and the proportion of arable crops (which includes winter cereal) at 1000 m. Indeed, *C. septempunctata* has a great ability to disperse and to explore the most favourable habitat in the landscape, and its capacity of movement between crops has been already reported (Pons et al. 2005; Gardiner et al. 2009; Maisonhaute et al. 2010; Madeira et al. 2014; Di Lascio et al. 2016). This ladybeetle is an active aphidophagous predator that when it leaves overwintering sites, needs to feed for reproduction. Cereals are the main crop for breeding *C. septempunctata* populations because of its large acreage, frequently high aphid abundance, prey quality, convenient microclimate, absence of insecticide treatments, and late harvest date (Honěk 1989; Bianchi et al. 2007). Moreover, it has also been reported the capacity of C. *septempunctata* to predate *H. postica* larvae (Richard and Evans, 1998) and numerical responses between this predator, aphids and *H. postica* larvae have been also found in a recent study (Meseguer et al. 2021).

Pests, natural enemies and biological control services not always respond to the landscape diversity (Rusch et al. 2016; Tscharntke et al. 2016; Karp et al. 2018). Our study provides, for the first time, evidences that alfalfa weevil and its parasitoid *Bathyplectes* spp. rather respond to field conditions than landscape composition whereas its occasional predator *C. septempunctata* mainly responds to landscape composition. However, more detailed analysis, which includes landscape configuration parameters, will be needed to better understand how landscape may affect the abundance of the alfalfa weevil and its related natural enemies.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPLEMENTARY

Table S1 Specific sampled field information: county, year, area, altitude, age, irrigation, and coordinates.

FIELD	County	Year	Field area (ha)	Field altitude (m)	Field age (years)	Irrigation type	Latitude	Longitud
1	Segrià	2018	3.11	227	4	Sprinkler	41° 37' 0.11'' N	0° 27' 51.24'' E
2	Segrià	2018	2.94	159	3	Blanket	41° 38' 53.11'' N	0° 40' 48.76'' E
3	Urgell	2019	3.68	302	2	Blanket	41°41’22.36’’N	1°3’58.83’’E
4	Urgell	2020	3.68	302	3	Blanket	41°41’22.36’’N	1°3’58.83’’E
5	Urgell	2018	3.21	256	2	Blanket	41° 45' 34.36'' N	0° 54' 43.16'' E
6	Urgell	2018	3.76	222	3	Blanket	41° 38' 16.99'' N	0° 50' 19.51'' E
7	Urgell	2018	1.74	204	3	Blanket	41° 38' 18.73'' N	0° 47' 56.00'' E
8	Urgell	2018	2.41	187	2	Blanket	41°38’58.94’’N	0°45’6.98’’E
9	Urgell	2019	2.41	187	3	Blanket	41°38’58.94’’N	0°45’6.98’’E
10	Urgell	2020	2.41	187	4	Blanket	41°38’58.94’’N	0°45’6.98’’E
11	Urgell	2019	4.82	183	3	Blanket	41° 39' 9.58'' N	0° 44' 15.74'' E
12	Urgell	2020	4.82	183	4	Blanket	41° 39' 9.58'' N	0° 44' 15.74'' E
13	Urgell	2018	2.77	196	2	Blanket	41° 40' 29.2'' N	0° 48' 25.7'' E
14	Urgell	2018	1.86	200	3	Blanket	41° 41' 48.1'' N	0° 49' 21.2'' E
15	Urgell	2019	4.98	200	3	Blanket	41°39’42.59’’N	0°48’9.19’’E
16	Urgell	2020	4.98	200	4	Blanket	41°39’42.59’’N	0°48’9.19’’E
17	Urgell	2019	2.79	205	3	Blanket	41°40’59.75’’N	0°49’44.68’’E
18	Urgell	2020	2.79	205	4	Blanket	41°40’59.75’’N	0°49’44.68’’E
19	Monegros	2020	1.63	327	4	Sprinkler	41° 29' 24.12'' N	0° 6' 6.60'' W
20	Monegros	2019	3.89	325	2	Sprinkler	41°29’0.41’’N	0°6’11.15’’E
21	Monegros	2020	5.13	282	3	Sprinkler	41°29’2.29’’N	0°5’46.44’’E
22	Monegros	2019	2.05	372	4	Sprinkler	41°32’25.74’’N	0°9’42.93’’E
23	Monegros	2019	4.39	315	3	Sprinkler	41° 28' 12.75'' N	0° 4' 27.75'' W
24	Monegros	2020	4.39	315	4	Sprinkler	41° 28' 12.75'' N	0° 4' 27.75'' W
25	Monegros	2019	2.65	363	3	Sprinkler	41°31’0.66’’N	0°10’33.68’’E
26	Urgell	2019	4.04	262	3	Blanket	41°38’13.42’’N	0°57’3.90’’E
27	Urgell	2020	4.04	262	4	Blanket	41°38’13.42’’N	0°57’3.90’’E
28	Segrià	2018	1.18	259	2	Sprinkler	41°47’30.82’’N	0°16’52.37’’E
29	Segrià	2019	1.18	259	3	Sprinkler	41°47’30.82’’N	0°16’52.37’’E
30	Segrià	2020	1.18	259	4	Sprinkler	41°47’30.82’’N	0°16’52.37’’E
31	Segrià	2018	1.58	259	4	Sprinkler	41°47’54.43’’N	0°17’25.43’’E
32	Segrià	2019	1.58	259	5	Sprinkler	41°47’54.43’’N	0°17’25.43’’E
33	Segrià	2020	1.58	259	5	Sprinkler	41°47’54.43’’N	0°17’25.43’’E
34	Segrià	2018	4.51	247	4	Blanket	41° 38' 50.53'' N	0° 21' 49.49'' E
35	Segrià	2018	8.62	213	2	Sprinkler	41°38'23.3"N	0°32'34.8"E
36	Urgell	2019	6.64	256	3	Blanket	41°42’20.05’’N	0°58’50.76’’E
37	Urgell	2020	6.64	256	4	Blanket	41°42’20.05’’N	0°58’50.76’’E
38	Urgell	2018	3.82	233	3	Sprinkler	41°44’10.03’’N	0°53’24.9’’E

Table S1 (continued)

FIELD	County	Year	Field area (ha)	Field altitude (m)	Field age (years)	Irrigation type	Latitude	Longitud
39	Baja Cinca	2018	8.4	261	3	Sprinkler	41°54’16.3’’N	0°07’31.7’’E
40	Baja Cinca	2019	7.6	328	4	Sprinkler	41°53’27.92’’N	0°2’9.58’’E
41	Baja Cinca	2020	7.6	328	4	Sprinkler	41°53’27.92’’N	0°2’9.58’’E
42	Baja Cinca	2019	3.92	304	2	Sprinkler	41°54’33.19’’N	0°3’11.14’’E
43	Baja Cinca	2020	3.92	304	3	Sprinkler	41°54’33.19’’N	0°3’11.14’’E
44	Baja Cinca	2018	3.39	279	2	Sprinkler	41°48’28.1’’N	0°2’23.14’’E
45	Baja Cinca	2019	4.5	315	2	Sprinkler	41° 57' 17.82'' N	0° 3' 14.55'' E
46	Baja Cinca	2020	4.5	315	3	Sprinkler	41° 57' 17.82'' N	0° 3' 14.55'' E
47	Baja Cinca	2018	17.8	327	3	Sprinkler	41° 49' 27.72'' N	0° 01' 55.00'' W
48	Baja Cinca	2018	7.87	390	2	Sprinkler	41° 54' 07.72'' N	0° 04' 10.8'' W
49	Segrià	2018	4.55	173	3	Sprinkler	41° 42' 3.71'' N	0° 21' 39.10'' E
50	Segrià	2018	20.4	233	3	Sprinkler	41° 35' 13.4'' N	0° 25' 59.5'' E
51	Segrià	2019	2.52	287	2	Sprinkler	41° 46' 49.35'' N	0° 31' 12.15'' E
52	Segrià	2020	2.52	287	3	Sprinkler	41° 46' 49.35'' N	0° 31' 12.15'' E
53	Segrià	2018	2.26	261	3	Blanket	41° 47' 59.9'' N	0° 30' 27.7'' E
54	Segrià	2019	2.62	187	2	Sprinkler	41° 43' 42.73'' N	0° 20' 54.59'' E
55	Segrià	2020	2.62	187	2	Sprinkler	41° 43' 42.73'' N	0° 20' 54.59'' E
56	Segrià	2019	5.76	178	2	Sprinkler	41° 42' 46.44'' N	0° 20' 53.08'' E
57	Segrià	2020	5.76	178	2	Sprinkler	41° 42' 46.44'' N	0° 20' 53.08'' E
58	Urgell	2018	5.12	288	4	Blanket	41°42'01.8'' N	0°26'13.0''E
59	Segrià	2019	4.58	216	3	Blanket	41°44’9.59’’N	0°47’17.84’’E
60	Segrià	2020	4.58	216	4	Blanket	41°44’9.59’’N	0°47’17.84’’E
61	Urgell	2019	7.06	222	3	Blanket	41°35’5.45’’N	0°50’12.09’’E
62	Urgell	2020	7.06	222	4	Blanket	41°35’5.45’’N	0°50’12.09’’E
63	Urgell	2019	10.81	246	2	Blanket	41°39’16.48’’N	0°55’15.53’’E
64	Urgell	2020	10.81	246	3	Blanket	41°39’16.48’’N	0°55’15.53’’E
65	Urgell	2018	2.66	256	3	Blanket	41°39’51.2’’N	0°56’35.00’’E

Table S2 Mean, ±SE, minimum, and maximum of landscape composition proportion and local variables (perimeter, area, altitude, and alfalfa age) within the three different radii's (250, 500, and 100m), during 2018, 2019 and 2020 seasons.

Buffer (m)	**Variables**	**Mean**	**(±) SE**	**Min.**	**Max.**
	Perimeter (m)	991.41	47.02	531.63	2754.65
	Area (ha)	4.93	0.55	1.18	28.40
	Alfalfa age (years)	3.00	0.10	2.00	5.00
	Altitude (m)	252.63	6.73	159.00	390.00
250	Alfalfa (%)	43.30	2.38	8.31	95.84
	Winter cereal (%)	29.16	2.43	0.00	75.80
	Rye-grass (%)	2.63	0.77	0.00	25.80
	Pea and vetch (%)	0.36	0.16	0.00	7.94
	Maize (%)	0.28	0.19	0.00	10.94
	Forest (%)	2.50	0.81	0.00	37.95
	Orchard (%)	7.09	1.27	0.00	38.69
	Margin (%)	2.51	0.36	0.00	15.44
	Non-crop habitats (%)	6.24	1.22	0.00	39.89
	Unproductive or gardens (%)	1.56	0.29	0.00	10.87
	Water areas (%)	0.72	0.19	0.00	5.31
	Roads (%)	2.70	0.36	0.00	15.81
	Buildings and urban areas (%)	0.48	0.12	0.00	6.64
500	Alfalfa (%)	25.47	1.80	5.41	86.65
	Winter cereal (%)	41.91	2.39	2.50	83.95
	Rye-grass (%)	2.93	0.61	0.00	26.41
	Pea and vetch (%)	0.82	0.31	0.00	13.32
	Maize (%)	0.14	0.09	0.00	4.57
	Forest (%)	3.00	0.84	0.00	48.35
	Orchard (%)	9.98	1.42	0.00	43.83
	Margin (%)	2.71	0.27	0.00	9.00
	Non-crop habitats (%)	6.26	1.01	0.00	35.86
	Unproductive or gardens (%)	1.77	0.28	0.00	8.21
	Water areas (%)	0.69	0.11	0.00	4.55
	Roads (%)	3.09	0.35	0.30	15.68
	Buildings and urban areas (%)	1.48	0.34	0.00	18.43
1000	Alfalfa (%)	19.72	1.18	2.74	44.55
	Arable crops (winter crops) (%)	53.88	1.80	19.34	82.67
	Forest (%)	1.88	0.60	0.00	29.54
	Orchard (%)	11.14	1.58	0.00	48.00
	Margins and non-crop habitats (%)	7.67	0.62	1.71	26.25
	Water areas (%)	0.65	0.06	0.00	1.98
	Roads (%)	2.62	0.19	1.07	8.39
	Buildings and urban areas (%)	2.16	0.43	0.02	14.29

Table S3 Spearman rank correlation coefficients (Spearman's rho) among landscape composition and local variables around sampled alfalfa fields at 250 (a), 500 (b) and 1000 m (c) buffer radii. Significant at: * p < 0.05; ** p < 0.01; ***p < 0.001.

a)

Variables	Perimeter	Area	Alfalfa	Winter cereal	Forest and/or woody natural habitat	Unproductive and/or gardens	Margin	Non-crop habitats	Rye grass	Roads	Orchards
Perimeter	1										
Area	**0.806*****	1									
Alfalfa	**0.365****	**0.497*****	1								
Winter cereal	**-0.323****	**-0.408*****	**-0.598*****	1							
Forest/ woody natural habitat	-0.013	-0.046	-0.106	0.085	1						
Unproductive/ gardens	0.019	-0.104	-0.031	-0.065	0.036	1					
Margin	-0.182	-0.038	0.091	-0.064	-0.112	0.037	1				
Non-crop habitats	**-0.362****	**-0.416*****	-0.226	**-0.255***	-0.011	-0.021	0.189	1			
Rye grass	0.072	0.104	-0.136	0.052	0.098	0.032	-0.117	-0.111	1		
Roads	0.044	0.083	-0.089	-0.113	0.035	0.175	**0.418*****	0.185	-0.100	1	
Orchards	0.101	-0.146	-0.200	-0.228	-0.224	0.170	-0.141	**0.316****	0.041	-0.118	1

b)

Variables	Perimeter	Area	Alfalfa	Winter cereal	Forest and/or woody natural habitat	Unproductive and/or gardens	Margin	Non-crop habitats	Rye grass	Roads	Orchards
Perimeter	1										
Area	__0.806***__	1									
Alfalfa	0.013	0.093	1								
Winter cereal	-0.143	-0.127	__-0.524***__	1							
Forest/ woody natural habitat	-0.075	-0.034	-0.156	0.011	1						
Unproductive/ gardens	0.146	0.036	-0.014	-0.127	0.146	1					
Margin	-0.231	-0.070	0.156	-0.084	-0.192	__-0.246*__	1				
Non-crop habitats	__-0.246*__	__-0.285*__	-0.026	__-0.492***__	0.138	0.118	0.157	1			
Rye grass	-0.046	-0.085	0.156	__-0.284*__	-0.100	-0.052	0.044	0.149	1		
Roads	0.021	0.009	-0.092	-0.116	0.035	0.015	__0.438***__	__0.290*__	-0.116	1	
Orchards	0.225	-0.027	-0.006	__-0.438***__	__-0.299*__	0.088	-0.179	__0.265*__	0.206	-0.002	1

c)

Variables	Perimeter	Area	Arable crop	Alfalfa	Forest	Non-crop	Orchard	Roads
Perimeter	1							
Area	__0.806***__	1						
Arable crops	-0.236	-0.067	1					
Alfalfa	-0.144	-0.168	__-0.259*__	1				
Forest	__0.277*__	__0.257*__	-0.114	-0.156	1			
Margins and non-crop habitats	0.067	0.081	-0.119	-0.216	0.029	1		
Orchard	__0.262*__	0.063	__-0.725***__	-0.169	-0.084	-0.034	1	
Roads	0.184	0.135	-0.020	-0.230	0.190	__0.343**__	0.015	1

Table S4 Moran's Index (correlation coefficient) calculated for *H. postica* (Eggs, Larvae and Adults), *Bathyplectes* spp. (total adults and total parasitism rate), and *C. septempunctata* (total) collected in the 65 alfalfa fields during the 3 years of study.

Insects (variables)		**Moran's Index (observed)**	***p*-value**
H.postica	Eggs	0,031	0,163
	Larvae	-0,064	0,269
	Adults	0,022	0,152
Bathyplectes spp.	Total adults	-0,020	0,917
	Total parasitism rate	-0,020	0,623
C. septempunctata	Total	-0,014	0,787

REFERENCES

Albajes, R., Pons, X., Cantero-Martínez, C., Achón, M.A., López, C., Eizaguirre, M., 2022. Intensificación de las rotaciones de cereales en la Cuenca del Ebro: efecto en la incidencia de plagas y virosis. Phytoma España 335, 36–42.

Alfaro, A., 2005. Entomología Agraria. Los parásitos animales de las plantas cultivadas. Publicaciones de la Excma. Diputación de Soria.

Bartoń, K., 2020. MuMIn: title multi-model inference. R package version: 1.43.6. MuMIn.pdf (r-project.org)

Batuecas, I., Alomar, O., Castañé, C., Gallardo-Montoya, L., Agustí, N., 2022. Disentangling arthropod and plant resources consumed by *Orius* spp. in peach and alfalfa crops by metagenomic analysis. https://doi.org/10.21203/rs.3.rs-1474466/v1

Berberet, R.C., McNew, R.W., 1986. Reduction in yield and quality of leaf and stem components of alfalfa forage due to damage by larvae of *Hypera postica* (Coleoptera: Curculionidae). J. Econ. Entomol. 79, 212–218.

Bianchi, F.J.J.A., Booij, C.J.H., Tscharntke, T., 2006. Sustainable pest regulation in agricultural landscapes: a review on landscape composition, biodiversity and natural pest control. Proc. R. Soc. B Biol. Sci. 273, 1715–1727.

Bianchi, F.J.J.A., Honěk, A., Van Der Werf, W., 2007. Changes in agricultural land use can explain population decline in a ladybeetle species in the Czech Republic: Evidence from a process-based spatially explicit model. Landsc. Ecol. 22, 1541–1554.

Bianchi, F.J.J.A., Schellhorn, N.A., Cunningham, S.A., 2013. Habitat functionality for the ecosystem service of pest control: reproduction and feeding sites of pests and natural enemies. Agric. For. Entomol. 15, 12–23.

Boccaccio, L., Petacchi, R., 2009. Landscape effects on the complex of Bactrocera oleae parasitoids and implications for conservation biological control. BioControl 54, 607–616.

Burnham K.P., Anderson D.R., 2004. Multimodel Inference: Understanding AIC and BIC in Model Selection. Soc. Methods Res. 33:261–304.

Campbell, M.J., Swinscow, T.D. V., 2009. Statistics at square one, 11th ed. Wiley-Blackewll, Chichester.

Cantero-Martínez, C., Santiveri, P., Lloveras, J., Chocarro, C., 2006. Agronomy of field crops, in: Estany, J. (Ed.), Agriculture and Agri-Food Production in Perspective. Profile of the Sector in Catalonia. Universitat de Lleida, Lleida, Spain.

Chaplin-Kramer, R., O'Rourke, M.E., Blitzer, E.J., Kremen, C., 2011. A meta-analysis of crop pest and natural enemy response to landscape complexity. Ecol. Lett. 14, 922–932.

Clemente-Orta, G., Madeira, F., Batuecas, I., Sossai, S., Juárez-Escario, A., Albajes, R., 2020. Changes in landscape composition influence the abundance of insects on maize: The role of fruit orchards and alfalfa crops. Agric. Ecosyst. Environ. 291, 106805.

Costamagna, A.C., Menalled, F.D., Landis, D.A., 2004. Host density influences parasitism of the armyworm Pseudaletia unipuncta in agricultural landscapes. Basic Appl. Ecol. 5, 347–355.

Delgado, I., 2020. Botánica, in: Lloveras, J., Delgado, Ignacio, Chocarro, C. (Eds.), La Alfalfa -Agronomia y Utilización-. Edicions de la Universitat de Lleida. Centro de Investigación y Tecnología Agroalimentaria de Aragón. Lleida- Zaragoza, Spain, pp. 33–42.

Delgado, I., Lloveras, J., 2020. Historia y distribución de la alfalfa, in: Lloveras, J., Delgado, I., Chocarro, C. (Eds.), La Alfalfa, Agronomía y Utilización. Edicions de la Universitat de Lleida. Centro de Investigación y Tecnología Agroalimentaria de Aragón. Lleida-Zaragoza Spain, pp. 17–32.

Dennis, P., Fry, G.L.A., 1992. Field margins: can they enhance natural enemy population densities and general arthropod diversity on farmland? Agric. Ecosyst. Environ. 40, 95–115.

di Lascio, A., Madeira, F., Costantini, M.L., Rossi, L., Pons, X., 2016. Movement of three aphidophagous ladybird species between alfalfa and maize revealed by carbon and nitrogen stable isotope analysis. BioControl 61, 35–46.

Domínguez, F., 1989. Plagas y enfermedades de las plantas cultivadas, 8th ed. Mundi Prensa, Madrid, Spain.

Dormann, C.F., Elith, J., Bacher, S., Buchmann, C., Carl, G., Carré, G., Marquéz, J.R.G., Gruber, B., Lafourcade, B., Leitão, P.J., Münkemüller, T., Mcclean, C., Osborne, P.E., Reineking, B., Schröder, B., Skidmore, A.K., Zurell, D., Lautenbach, S., 2013. Collinearity: a review of methods to deal with it and a simulation study evaluating their performance. Ecography (Cop.). 36, 27–46.

Drapela, Thomas, Moser, Dietmar, Zaller, Johann G, Frank, Thomas, Drapela, T, Moser, D, Zaller, J G, Frank, T, 2008. Spider assemblages in winter oilseed rape affected by landscape and site factors. Ecography (Cop.). 31, 254–262.

ESRI, 2015. Arcgis Desktop Version 10.3.1. Environmental Systems Research Institute, Redlands, CA, USA.

Flanders, K.L., Radcliffe, E.B., Krueger, C.A., 1994. Natural enemies of alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae), in Minnesota. Gt. Lakes Entomol. 27, 7–18.

Frame, J., Charlton, J., Laidlaw, A., 1998. Temperate forrage legumes. CAB international. London, UK.

Fox J., Weisberg S., Price B., Friendly M., Hong J., Anderson R., Firth D., Taylor S., 2020. Effects: effect displays for linear, generalized linear, and other models. R Package Version: 4.2–0. https://cran.r-project.org/web/packages/effects/effects.pdf

Gardiner, M.M., Landis, D.A., Gratton, C., DiFonzo, C.D., O'Neal, M., Chacon, J.M., Wayo, M.T., Schmidt, N.P., Mueller, E.E., Heimpel, G.E., 2009. Landscape diversity enhances biological control of an introduced crop pest in the north-central USA. Ecol. Appl. 19, 143–154.

Gardiner, M.M., Landis, D.A., Gratton, C., Schmidt, N., O'Neal, M., Mueller, E., Chacon, G.E., Heimpel, G.E., DiFonzo, C.D., 2009. Landscape composition influences patterns of native and exotic lady beetle abundance. Divers. Distrib. 15, 554–564.

Goosey, H.B., 2012. A degree-day model of sheep grazing influence on alfalfa weevil and crop characteristics. J. Econ. Entomol. 105, 102–112.

Hoffmann, A., 1963. Sous-famille des Curculionidae, Tribu des Hyperini, Les Hypera (syn: Phytonomus), in: Balachowsky, A.S. (Ed.), Entomologie Appliquée a l'agriculture. Tome I. Coléoptères. Second Volume. Masson et Cíe., Paris, Francia, pp. 984–989.

Holland, J., Fahrig, L., 2000. Effect of woody borders on insect density and diversity in crop fields: a landscape-scale analysis. Agric. Ecosyst. Environ. 78, 115–122.

Honěk, A., 1989. Overwintering and annual changes of abundance of *Coccinella septempunctata* in Czechoslovakia (Coleoptera, Coccinellidae). Acta Entomol. Bohemoslov.

Hussain, M., 1975. Predators of the alfalfa weevil, *Hypera postica* in Western Nevada: a greenhouse study. (Coleoptera: Curculionidae). J. New York Entomol. Soc. 83, 226–228.

Jacob, H.S., Evans, E.W., 2000. Influence of carbohydrate foods and mating on longevity of the parasitoid *Bathyplectes curculionis* (Hymenoptera: Ichneumonidae). Environ. Entomol. 29, 1088–1095.

Jervis, M.A., Kidd, N.A.C., Fitton, M.G., Huddleston, T., Dawah, H.A., 1993. Flower-visiting by hymenopteran parasitoids. J. Nat. Hist. 27, 67–105.

Karp, D.S., Chaplin-Kramer, R., Meehan, T.D., Martin, E.A., DeClerck, F., Grab, H., Gratton, C., Hunt, L., Larsen, A.E., Martínez-Salinas, A., O'Rourke, M.E., Rusch, A., Poveda, K., Jonsson, M., Rosenheim, J.A., Schellhorn, N.A., Tscharntke, T., Wratten, S.D., Zhang, W., Iverson, A.L., Adler, L.S., Albrecht, M., Alignier, A., Angelella, G.M., Anjum, M.Z., Avelino, J., Batáry, P., Baveco, J.M., Bianchi, F.J.J.A., Birkhofer, K., Bohnenblust, E.W., Bommarco, R., Brewer, M.J., Caballero-López, B., Carrière, Y., Carvalheiro, L.G., Cayuela, L., Centrella, M., Ćetković, A., Henri, D.C., Chabert, A., Costamagna, A.C., De la Mora, A., de Kraker, J., Desneux, N., Diehl, E., Diekötter, T., Dormann, C.F., Eckberg, J.O., Entling, M.H., Fiedler, D., Franck, P., van Veen, F.J.F., Frank, T., Gagic, V., Garratt, M.P.D., Getachew, A., Gonthier, D.J., Goodell, P.B., Graziosi, I., Groves, R.L., Gurr, G.M., Hajian-Forooshani, Z., Heimpel, G.E., Herrmann, J.D., Huseth, A.S., Inclán, D.J., Ingrao, A.J., Iv, P., Jacot, K., Johnson, G.A., Jones, L., Kaiser, M., Kaser, J.M., Keasar, T., Kim, T.N., Kishinevsky, M., Landis, D.A., Lavandero, B., Lavigne, C., Le Ralec, A., Lemessa, D., Letourneau, D.K., Liere, H., Lu, Y., Lubin, Y., Luttermoser, T., Maas, B., Mace, K., Madeira, F., Mader, V., Cortesero, A.M., Marini, L., Martinez, E., Martinson, H.M., Menozzi, P., Mitchell, M.G.E., Miyashita, T., Molina, G.A.R., Molina-Montenegro, M.A., O'Neal, M.E., Opatovsky, I., Ortiz-Martinez, S., Nash, M., Östman, Ö., Ouin, A., Pak, D., Paredes, D., Parsa, S., Parry, H., Perez-Alvarez, R., Perović, D.J., Peterson, J.A., Petit, S., Philpott, S.M., Plantegenest, M., Plećas, M., Pluess, T., Pons, X., Potts, S.G., Pywell, R.F., Ragsdale, D.W., Rand, T.A., Raymond, L., Ricci, B., Sargent, C., Sarthou, J.P., Saulais, J., Schäckermann, J., Schmidt, N.P., Schneider, G., Schüepp, C., Sivakoff, F.S., Smith, H.G., Whitney, K.S., Stutz, S., Szendrei, Z., Takada, M.B., Taki, H., Tamburini, G., Thomson, L.J., Tricault, Y., Tsafack, N., Tschumi, M., Valantin-Morison, M., van Trinh, M., van der Werf, W., Vierling, K.T., Werling, B.P., Wickens, J.B., Wickens, V.J., Woodcock, B.A., Wyckhuys, K., Xiao, H., Yasuda, M., Yoshioka, A., Zou, Y., 2018. Crop pests and predators exhibit inconsistent responses to surrounding landscape composition. Proc. Natl. Acad. Sci. 115, E7863-E7870.

Landis, D.A., Wratten, S.D., Gurr, G.M., 2003. Habitat management to conserve natural enemies of arthropod pests in agriculture. 45, 175–201.

Langellotto, G.A., Denno, R.F., 2004. Responses of invertebrate natural enemies to complex-structured habitats: A meta-analytical synthesis. Oecologia 139, 1–10.

Lavallee, A.G., Shaw, F.R., 1969. Preferences of golden-eye lacewing larvae for pea aphids, leafhopper and plant bug nymphs, and alfalfa weevil larvae. J. Econ. Entomol. 62, 1228–1229.

Legendre, P., 1993. Spatial Autocorrelation: Trouble or New Paradigm? Ecology 74, 1659–1673.

Letourneau, D.K., Jedlicka, J.A., Bothwell, S.G., Moreno, C.R., 2009. Effects of Natural Enemy Biodiversity on the Suppression of Arthropod Herbivores in Terrestrial Ecosystems. Annu. Rev. Ecol. Evol. Syst. 40, 573–592.

Levi-Mourao, A., Madeira, F., Meseguer, R., García, A., Pons, X., 2021. Effects of temperature and relative humidity on the embryonic development of *Hypera postica* Gyllenhal (Col.: Curculionidae). Insects 12, 3, 250.

Levi-Mourao, A., Muñoz, P., Cerda-Bennasser, P., Meseguer, R., Pons, X., 2022a. Molecular and morphological identification of the alfalfa weevil larval parasitoids Bathyplectes anura and Bathyplectes curculionis to estimate the rate of parasitism. BioControl 2022 1–12.

Levi-Mourao, A., Núñez, E., García, A., Meseguer, R., Pons, X., 2022b. Alfalfa winter cutting: Effectiveness against the alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) and effect on its rate of parasitism due to *Bathyplectes* spp. (Hymenoptera: Ichneumonidae). Crop Prot. 152, 105858.

Madeira, F., Clemente-Orta, G., Alomar, O., Batuecas, I., Sossai, S., Albajes, R., 2021. Land use alters the abundance of herbivore and predatory insects on crops: the case of alfalfa. J. Pest Sci. 2021 1, 1–19.

Madeira, F., di Lascio, A., Carlino, P., Costantini, M.L., Rossi, L., Pons, X., 2014. Stable carbon and nitrogen isotope signatures to determine predator dispersal between alfalfa and maize. Biol. Control 77, 66–75.

Madeira, F., di Lascio, A., Costantini, M.L., Rossi, L., Rösch, V., Pons, X., 2019. Intercrop movement of heteropteran predators between alfalfa and maize examined by stable isotope analysis. J. Pest Sci. (2004). 92, 757–767.

Maisonhaute, J.E., Peres-Neto, P., Lucas, E., 2010. Influence of agronomic practices, local environment and landscape structure on predatory beetle assemblage. Agric. Ecosyst. Environ. 139, 500–507.

Maingay, H.M., Bugg, R.L., Carlson, R.W., Davidson, N.A., 1991. Predatory and parasitic wasps (Hymenoptera) feeding at flowers of sweet fennel (*Foeniculum vulgare* Miller var. *dulce* Battandier & Trabut, Apiaceae) and spearmint (*Mentha spicata* L., Lamiaceae) in Massachusetts. Biol. Agric. Hortic. 7, 363–383.

Manglitz, G.R., 1958. Aestivation of the alfalfa weevil. J. Econ. Entomol. 51, 506–508.

Max K., Wing J., Weston S., Williams A., Keefer C., Engelhardt A., Cooper T., 2021. Caret: Title classification and regression training. R Package Version: 6.0–84. https://cran.r-project.org/web/packa ges/caret/caret.pdf

Menalled, F.D., Costamagna, A.C., Marino, P.C., Landis, D.A., 2003. Temporal variation in the response of parasitoids to agricultural landscape structure. Agric. Ecosyst. Environ. 96, 29–35.

Meseguer, R., Levi-Mourao, A., Pons, X., 2021. Species Complex and Temporal Associations between Coccinellids and Aphids in Alfalfa Stands in Spain. Insects https://doi.org/10.3390/INSECTS12110971

Michaud, R., Lehman, W.F., Rumbaugh, M.D., 1988. World distribution and historical development, in: Hanson, A.A., Barnes, D.K., Hill, R.R. (Eds.), Alfalfa and Alfalfa Improvement. Agronomy 29. American Society of America, Agronomy, Crop Science Society of America, Soil Science Society of America, Madison,WI., USA, pp. 25–91.

Núñez, E., 2002. La alfalfa como reservorio de enemigos naturales. PhD thesis. Universitat de Lleida.

Östman, Ö., Ekbom, B., Bengtsson, J., 2001. Landscape heterogeneity and farming practice influence biological control. Basic Appl. Ecol. 2, 365–371.

Ouayogode, B. V., Davis, D.W., 1981. Feeding by selected predators on alfalfa weevil larvae. Environ. Entomol. 10, 62–64.

Paradis, E., 2019. Ape : Analyses of phylogenetics and evolution. R Package Version 5.3. http://cran.r-project.org/web/package/ape/ape.pdf

Pellissier, M.E., Nelson, Z., Jabbour, R., 2017. Ecology and management of the alfalfa weevil (Coleoptera: Curculionidae) in Western United States alfalfa. J. Integr. Pest Manag. 8, 5.

Petit, S., 2009. The dimensions of land use change in rural landscapes: Lessons learnt from the GB Countryside Surveys. J. Environ. Manage. 90, 2851–2856.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., R Core Team, 2021. nlme: linear and nonlinear mixed efects models. R package version 3.1–153. https://cran.r-project.org/web/packages/nlme/nlme.pdf

Pons, X., Nuñez, E., 2020. Plagas da la alfalfa: Importancia, daños y estrategias de control., in: Lloveras, J., Delgado, I., Chocarro, C. (Eds.), La Alfalfa, Agronomía y Utilización. Edicions de la Universitat de Lleida, Lleida, Spain, pp. 167–202.

Pons, X., Núñez, E., Lumbierres, B., Albajes, R., 2005. Epigeal aphidophagous predators and the role of alfalfa as a reservoir of aphid predators for arable crops. Eur. J. Entomol. 519–525.

Prokopy, R.J., Armbrust, E.J., Cothran, W.R., Gyrisco, G.G., 1967. Migration of the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae), to and from estivation sites. Ann. Entomol. Soc. Am. 60, 26–31.

Prokopy, R.J., Gyrisco, G.G., 1965. Summer migration of the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae). Ann. Entomol. Soc. Am. 58, 630–641.

R Development Core Team, 2018. R: A language and environment for statistical computing. R Foundation for statistical computing. Vienna, Austria. Available online at http://www.R-project.org/

Radcliffe, E.B., Flanders, K.L., 1998. Biological control of alfalfa weevil in North America. Integr. Pest Manag. Rev. 3, 225–242.

Rand, T.A., 2017. Assessing the role of generalist predators in the biological control of alfalfa weevil (Coleoptera: Curculionidae). Can. Entomol. 149, 525–533.

Rand, T.A., 2013. Host density drives spatial variation in parasitism of the alfalfa weevil, *Hypera postica*, across dryland and irrigated alfalfa cropping systems. Environ. Entomol. 42, 116–122.

Rand, T.A., Tylianakis, J.M., Tscharntke, T., 2006. Spillover edge effects: the dispersal of agriculturally subsidized insect natural enemies into adjacent natural habitats. Ecol. Lett. 9, 603–614.

Ribes, A., 2012. Himenòpters de Ponent. http://ponent.atspace.org/fauna/ins/index.htm (accessed 5/3/21).

Ricci, B., Franck, P., Toubon, J.F., Bouvier, J.C., Sauphanor, B., Lavigne, C., 2009. The influence of landscape on insect pest dynamics: A case study in southeastern France. Landsc. Ecol. 24, 337–349.

Richards, D.R., Evans, E.W., 1998. Reproductive responses of aphidophagous lady beetles (Coleoptera: Coccinellidae) to nonaphid diets: an example from alfalfa. Ann. Entomol. Soc. Am. 91, 632–640.

Roschewitz, I., Hücker, M., Tscharntke, T., Thies, C., 2005. The influence of landscape context and farming practices on parasitism of cereal aphids. Agric. Ecosyst. Environ. 108, 218–227.

Rusch, A., Chaplin-Kramer, R., Gardiner, M.M., Hawro, V., Holland, J., Landis, D., Thies, C., Tscharntke, T., Weisser, W.W., Winqvist, C., Woltz, M., Bommarco, R., 2016. Agricultural landscape simplification reduces natural pest control: A quantitative synthesis. Agric. Ecosyst. Environ. 221, 198–204.

Saeidi, M., Moharramipour, S., 2017. Physiology of cold hardiness, seasonal fluctuations, and cryoprotectant contents in overwintering adults of *Hypera postica* (Coleoptera: Curculionidae). Environ. Entomol. 46, 960–966.

Schmidt, J.M., Whitehouse, T.S., Green, K., Krehenwinkel, H., Schmidt-Jeffris, R., Sial, A.A., 2019. Local and landscape-scale heterogeneity shape spotted wing drosophila (*Drosophila suzukii*) activity and natural enemy abundance: Implications for trophic interactions. Agric. Ecosyst. Environ. 272, 86–94.

Schmidt, M.H., Thies, C., Nentwig, W., Tscharntke, T., 2008. Contrasting responses of arable spiders to the landscape matrix at different spatial scales. J. Biogeogr. 35, 157–166.

Shrestha, G., Rijal, J.P., Reddy, G.V.P., 2021. Characterization of the spatial distribution of alfalfa weevil, Hypera postica, and its natural enemies, using geospatial models. Pest Manag. Sci. 77, 906–918.

Soroka, J., Grenkow, L., Cárcamo, H., Meers, S., Barkley, S., Gavloski, J., 2019. An assessment of degree-day models to predict the phenology of alfalfa weevil (Coleoptera: Curculionidae) on the Canadian Prairies. Can. Entomol. 152, 110–129.

Straub, C.S., Finke, D.L., Snyder, W.E., 2008. Are the conservation of natural enemy biodiversity and biological control compatible goals? Biol. Control 45, 225–237.

Summers, C.G., 1998. Integrated pest management in forage alfalfa. Integr. Pest Manag. 3, 127–154.

Symondson, W.O.C., Sunderland, K.D., Greenstone, M.H., 2003. Can Generalist Predators be Effective Biocontrol Agents? Annu. Rev. Entomol. 47, 561–594.

Taberner, A., 2020. Control de malas hierbas, in: Lloveras, J., Delgado, I., Chocarro, C. (Eds.), La Alfalfa, Agronomía y Utilización. Edicions de la Universitat de Lleida. Centro de Investigación y Tecnología Agroalimentaria de Aragón, Lleida- Zaragoza, Spain, pp. 253–272.

Thies, C., Roschewitz, I., Tscharntke, T., 2005. The landscape context of cereal aphidparasitoid interactions. Proc. R. Soc. B Biol. Sci. 272, 203–210.

Thies, C., Steffan-Dewenter, I., Tscharntke, T., 2008. Interannual landscape changes influence plant–herbivore–parasitoid interactions. Agric. Ecosyst. Environ. 125, 266–268.

Tscharntke, T., Bommarco, R., Clough, Y., Crist, T.O., Kleijn, D., Rand, T.A., Tylianakis, J.M., Van Nouhuys, S., Vidal, S., 2007. Conservation biological control and enemy diversity on a landscape scale. Biol. Control 294–309.

Tscharntke, T., Karp, D.S., Chaplin-Kramer, R., Batáry, P., DeClerck, F., Gratton, C., Hunt, L., Ives, A., Jonsson, M., Larsen, A., Martin, E.A., Martínez-Salinas, A., Meehan, T.D., O'Rourke, M., Poveda, K., Rosenheim, J.A., Rusch, A., Schellhorn, N., Wanger, T.C., Wratten, S., Zhang, W., 2016. When natural habitat fails to enhance biological pest control – Five hypotheses. Biol. Conserv. 204, 449–458.

Tscharntke, T., Tylianakis, J.M., Rand, T.A., Didham, R.K., Fahrig, L., Batáry, P., Bengtsson, J., Clough, Y., Crist, T.O., Dormann, C.F., Ewers, R.M., Fründ, J., Holt, R.D., Holzschuh, A., Klein, A.M., Kleijn, D., Kremen, C., Landis, D.A., Laurance, W., Lindenmayer, D., Scherber, C., Sodhi, N., Steffan-Dewenter, I., Thies, C., van der Putten, W.H., Westphal, C., 2012. Landscape moderation of biodiversity patterns and processes - eight hypotheses. Biol. Rev. 87, 661–685.

van Emden, H.F., 1963. Observations on the effect of flowers on the activity of parasitic Hymenoptera. Entomol. Mon. Mag. 265–270.

Veddeler, D., Klein, A.M., Tscharntke, T., 2006. Contrasting responses of bee communities to coffee flowering at different spatial scales. Oikos 112, 594–601.

Wagner, H.H., Fortin, M.J., 2005. Spatial analysis of landscapes: concepts and statistics. Ecology 86, 1975–1987.

Werling, B.P., Gratton, C., 2008. Influence of field margins and landscape context on ground beetle diversity in Wisconsin (USA) potato fields. Agric. Ecosyst. Environ. 128, 104–108.

With, K.A., Pavuk, D.M., Worchuck, J.L., Oates, R.K., Fisher, J.L., 2002. Threshold effects of landscape structure on biological control in agroecosystems. Ecol. Appl. 12, 52–65.

Zaller, J.G., Moser, D., Drapela, T., Schmöger, C., Frank, T., 2008. Effect of within-field and landscape factors on insect damage in winter oilseed rape. Agric. Ecosyst. Environ. 123, 233–238.

Zuur, A.F., Ieno, E.N., Elpicks, C.S., 2010. A protocol for data exploration to avoid common statistical problems. Methods Ecol. Evol. 1, 3-14.

GENERAL DISCUSSION

GENERAL DISCUSSION

One of the most voracious leaf feeders that affect alfalfa is the alfalfa weevil. In recent years this insect has become the most concerning pest of this crop in the Ebro Basin (Pons and Nuñez 2020), and one of the most important in the entire world (Hoff et al. 2002; Goosey 2012; Zahiri et al. 2014; Saeidi and Moharramipour 2017; Soroka et al. 2019). Although *H. postica* has origins in Eurasia (Hoffmann 1963), there is little information in scientific literature about its ecology, pest status and control strategies in Europe. Accordingly, it is unclear how abiotic factors, such as temperature, agronomic and cultural practices, natural enemies and the surrounding landscape affect its activity and population growth in European crops. The studies developed in this thesis allow a better knowledge of the biology, ecology, and control of this pest and contributes fulfilling this gap of scientific information. In the present dissertation it has been studied: 1) the effect of temperature on the fitness of *H. postica*, which has allowed establishing the life cycle in the Ebro basin. 2) The usefulness of a winter alfalfa cutting in preventing the development of spring populations. 3) The occurrence and effectiveness of the larval parasitoids of the genus *Bathyplectes* spp. 4) finally, the effects of field and landscape characteristics on the abundance of *H. postica* and its main natural enemies. Results obtained are of interest for a more sustainable management of this pest in the Ebro Basin, Spain and western Mediterranean regions.

1. Effect of temperature on the fitness of *H. postica*

Although the effect of temperature on *H. postica* has been studied in North American and Middle East Asian populations (Sweetman and Wedemeyer 1933; Zahiri et al. 2010a, b), no reports with European populations exist in spite of being an Eurasian species. In **CHAPTER I** (objective 1), the fitness of Spanish alfalfa weevil populations raised at constant different temperatures was studied, using life tables and models describing the temperature-dependent development rate. Temperature significantly affected the fitness of this pest, influencing individual parameters such as survival, developmental time and reproduction. The effect of temperature on the postembryonic survival of the alfalfa weevil was similar to the one reported on populations from other different geographical locations (Sweetman and Wedemeyer 1933; Guppy and Mukerji 1974; Zahiri et al. 2010a; Soroka et al. 2019). The increasing developmental rate from 8 to 32 °C in our experiments was consistent with the one observed with North American and Asian Middle East populations (Sweetman and Wedemeyer 1933; Koehler and Gyrisco 1961; Guppy and Mukerji 1974; Zahiri et al. 2010a). The linear and three non-linear (Lactin-1; Brière 1 and Brière-2) models fitted well with the temperature-dependent developmental rate of the *H. postica* population from the Ebro basin. In this study, the minimum developmental thresholds (t_{min}) predicted by the linear and Lactin-2 models were very similar for post-embryonic and total development. A higher t_{min} was reported for all immature developmental stages in Iranian populations (Zahiri et al. 2010a), supporting these the presumption that higher latitudes correspond to lower t_{min} values (Hodek and Honěk 1996). Accordingly to a previous report (Zahiri et al. 2010b), our results show that the survival and longevity of *H. postica* adults declined at higher temperatures, suggesting that constant high temperatures are detrimental. We also found that female adult pre-oviposition period (APOP) and ovipositional periods decreased significantly as the temperature increased from 12 to 24 °C. Fecundity was also temperature dependent, and females reared at 8, 32 and 36 °C did not lay eggs. In turn, population parameters such as the intrinsic rate of increase and the generation time were also affected by temperature. In our study, *r* increased within the range 12–28 °C and decreased at higher temperatures. Some of our results agreed with some others developed in different world regions, but some other differs noticeably (Parks 1914; Sweetman and Wedemeyer 1933; Litsinger and Apple 1973; Hsieh and Armbrust 1974; Zahiri et al. 2010b), suggesting that Spanish *H. postica* populations are less well-adapted to higher temperatures than those of warmer regions, highlighting the differences between geographically separated populations.

Recent studies in the area (Pons and Nuñez 2020; Levi-Mourao et al. 2021a) have suggested that *H. postica* produces more than one generation under the conditions in the Ebro basin, thus affecting both the first and second alfalfa intercuts. Following several studies (Jalali et al. 2014, 2018; Davídková and Doležal 2019; Azrag et al. 2020), we predicted the occurrence in the field of *H. postica* at different developmental stages, using the estimated values of t_{min} and *K* from the linear model. According to based temperature prediction, the day-length occurrence and the effect on adult reproductive behaviour (Huggans and Blickenstaff 1964; Rosenthal and Koehler 1968; Latheef et al. 1979; Ohto 1996), and the field phenology of the *H. postica*, we have determined that only two generation of this pest can occur, being the second one much less important due to the spring alfalfa cutting management. The first alfalfa cutting is usually performed by the end of April or the beginning of May, removing most of the larvae and pupae present on the foliage. Further cuttings would also help to limit the growth of weevil populations. Even, with the warming temperature scenario caused by the climatic change, no extra generations would occur.

2. Usefulness of a winter alfalfa cutting in preventing the development of spring populations

In the **CHAPTER II** (objective 2) of this dissertation, we evaluated the effectiveness of a winter alfalfa cutting to reduce spring populations of the alfalfa weevil larvae, and how this winter cutting affected the parasitism rates due to its parasitoid, *Bathyplectes* spp. The interest and efficacy of the winter cutting was previously reported for some areas of the region (Núñez et al. 2015; Pons and Nuñez 2020), but the results presented in this study include a greater number of fields, located along the main Spanish alfalfa growing area and cutting was performed with a conventional mower, which is available in most of the farms Although the natural abundance of the alfalfa weevil greatly varied among fields , the results of the comparison between the cut and the uncut section of each field revealed the effectiveness of this method in reducing an average of 50% of the spring larval populations of this pest. For these results, we assume that the applicability could be extended to other Spanish alfalfa crop conditions and potentially to other Mediterranean regions. However, in this study the efficacy varied across counties, a 50% in 2019 was reported. A significant correlation between efficacy and mowing method was observed. Laser mower cuts and collect nearly all stems, removing from the field plant pieces that can infest new alfalfa stems, even during winter (Summers 1998; Blodgett et al. 2000; Blodgett and Lenssen 2004).

Our results also showed that the efficacy of the winter cutting may be negatively mediated by the occurrence of epizooties of the entomophathogen fungus *Zoophthora phytonomi*. Epizootics of *Z. phytonomy* have been reported in Europe (Papierok et al. 1986), including in

Spain (Pons and Nuñez 2020). The infection is exacerbated in years with high rainfall during the winter and the beginning of spring (Radcliffe and Flanders 1998), as occurred in 2020. These results indicate that in epizootic years, the effects of winter cutting management may be less evident. Additionally, the rate of infected larvae was higher in uncut than in cut plots in several fields, suggesting that the epizootic was more severe where the density of alfalfa weevil larvae was higher (Los and Allen 1983).

Contrary to our hypothesis, winter cutting management did not negatively affect the rate of parasitism by *B. anura*. The rates of parasitism obtained were higher in cut than in uncut plots, being in concordance with that reported by Rand (2013) who found higher rates of parasitism in fields with lower alfalfa weevil densities. The reasons for this are unclear, but it has been suggested that an excess of larvae may hinder the host selection or increasing the host handling time. These results further underline the value of winter cutting and suggest that this method may enhance conservation biological control of *B. anura*. Our results showed that *B. anura* and *Z. phytonomi* are able to coexist in in our region, and such was also observed in the USA (Parr et al. 1993). Although *Z. phytonomi* causes high mortality of *H. postica* larvae in wet seasons it is considered an important biological control agent (Harcourt and Guppy 1991; Giles and Obrycki 1997). Results show that fungal epizooties can negatively affect the role of *B. anura* by reducing the rate of parasitism, and causing a disruption in the alfalfa weevil-parasitoid system (Giles et al. 1994; Kuhar et al. 1999).

3. Identification of *Bathyplectes* spp. and estimation of its parasitism rates on *H. postica* larvae

For definitive parasitoid identification, each *H. postica* larva must therefore be reared until pupation, which requires optimal environmental conditions and feeding, and the avoidance of other natural factors that cause mortality. Using this classical approach, the rates of parasitism recorded in Spain are generally low but highly variable among years (Pons and Nuñez 2020; Levi-Mourao et al. 2021b). In the **CHAPTER III** (objective 3) of this thesis, we have reported the development of specific *COI* mitochondrial primers to detect and identify the two main alfalfa weevil parasitoid species, *B. anura* and *B. curculionis*, in order to estimate the parasitism rates at field conditions and to compare with classical rearing method. Several recent studies have proven the use of the mitochondrial COI gene for the reliable identification of other parasitoids and morphologically similar species (Agustí et al. 2005; Traugott and Symondson 2008; Solà et al. 2018; Nanini et al. 2019).

The classical methodology suggested that *B. anura* was predominant over *B. curculionis*, which has been previously reported in Spain recently (Pons and Nuñez 2020; Levi-Mourao et al. 2021b) and in other world's regions (Harcourt 1990). Our newly designed primers detected up to seven times as many parasitism events as the classical rearing technique, indicating greater sensitivity and thus more reliability when estimating the rate of parasitism, as suggested for other species (Agustí et al. 2005; Gariepy et al. 2008; Gomez-Polo et al. 2014). Furthermore, this technique suggested, for the first time, that the rate of parasitism caused by both parasitoids does not differ by so wide a margin as showed by the classical method (Pons and Nuñez 2020; Levi-Mourao et al. 2021b). The mean rate of parasitism was similar for both species, suggesting that the two species coexist in the alfalfa crops of north-east Spain and would have a similar potential of parasitization. Despite this, *B. curculionis* effectiveness can be often comprised by the encapsulation of the parasitoid egg by hemocytes in the host hemocoel (Salt and van den Bosch 1967; Berberet et al. 2003; Shoubu et al. 2005). This may explain why we detected a larger number of *B. curculionis* parasitism events by PCR compared to conventional rearing. PCR-based methods can overestimate the rate of parasitism because they detect parasitoids that are already neutralized by the host immune system, whereas the classical method allows the direct measurement of parasitoid survival (Traugott et al. 2006). On the other hand, classical rearing techniques are influenced by the mortality of parasitoids under laboratory conditions, which can result in partial data loss (Tilmon et al. 2000), such as it was observed in 2020 due to an epizootic event of the entomopathogenic fungus *Z. phytonomy*.

Our results also showed that a *B. anura* and *B. curculionis* females prefer to parasitize different *H. postica* larvae instars, agreeing with previous findings (Duodu and Davis 1974; Dowell and Horn 1977; Barney et al. 1978; Bartell and Pass 1980). The differences in larval instar preference probably reflects the length of the ovipositor, which is longer for *B. curculionis* and facilitates the utilization of early instars still hidden in unfolded leaves and buds (Dowell and Horn 1977). Classical parasitism rates in 2020 were lower than in 2019 due to the presence of *Z. phytonomi*. The lower *B. anura* parasitism rate in 2020 suggests that this species was probably the most affected by the fungus. Since *B. curculionis* appears later in the study area, and has an advantage over *B. anura* because environmental conditions no longer favour the spread of the fungal disease, increasing the likelihood of host survival until pupation and thus the survival of the parasitoid.

Parasitism rates in 2019 varied at the field level between 17 and 37%, for both *B. curculionis* and *B. anura*, respectively. This concurs with Pons and Nuñez (2020) and Levi-Mourao et al. (2021b) where variable rates were reported with a maximum of 30%. These parasitoids were most effective when introduced into North America to control the alfalfa weevil (Radcliffe and Flanders 1998) where rates of parasitism exceeded 90% in the mild San Francisco Bay and Pleasanton areas, but much less effective in the hotter San Joaquin Valley (Radcliffe and Flanders 1998). Rearing studies conducted in south-western Canada revealed *B. curculionis* parasitism rates of up to 17% (Soroka et al. 2020). This suggests that environmental conditions play a key role in the success of parasitism, with hotter temperatures inhibiting parasitoid performance, and can explain our lower rates recorded in our study area. In other regions of North America, where *B. anura* tends to be the prevalent species (as is the case in Spain), the rate of parasitism was similar to our findings (Harcourt 1990; Berberet and Bisges 1998).

The molecular strategy developed in this thesis provides information on the ecology of *B. anura* and *B. curculionis* and reveals a similar potential prevalence for both species, although the actual effective parasitism may be overestimated. Thus, DNA analysis should be combined with conventional rearing techniques to determine the effective rate of parasitism and to accommodate interactions with other species that are not specifically targeted by the molecular assay. In spite of the potential of *Bathyplectes* spp. as biological control agent, the alfalfa crop management currently performed in the Ebro Basin may be unfavorable to their control capacity. A winter cutting and a delay of the first spring cutting are tools that may be included in integrated pest management.

4. Landscape composition influence on *H. postica* abundance and its natural enemies

Recent studies showed that landscape composition can modulate the influence of alfalfa on the abundance of herbivores and its natural enemies in neighbour crops (Clemente-Orta et al. 2020; Madeira et al. 2021). However, none of these works focused on how the effect of landscape composition can affect the main pests of alfalfa. In the **CHAPTER IV (objective 4)** of this dissertation we reported, the first time, the effects of landscape composition on alfalfa weevil and its natural enemies in the Ebro basin alfalfa crop conditions.

In our initial hypothesis we expected a positive relationship between the alfalfa weevil abundance and the alfalfa surface in the surrounding landscape, since this pest is specific of this crop. Though, very few effects of landscape composition affected its abundance at any buffer radii, and none was directly related with the proportion of alfalfa itself. Moreover, and according to our second hypothesis, the abundance of *H. postica* mostly depended on the characteristics of the own alfalfa field. In the particular case of *H. postica* eggs, there was an effect on the abundance caused by the irrigation system, and more eggs were found in fields irrigated with sprinklers. Somehow this can be justified since sprinkler supply pipers offers constant plant availability for adults to oviposit, working as a possible field refugee (Levi-Mourao et al. 2022). Another field variable related with *H. postica* eggs was field perimeter length. Field perimeter can be related to the amount of margin surrounding a field, and it represents an essential element for *H. postica* life cycle accomplishment. Several studies have described this important role, since summer aestivating adult weevils were considerably more concentrated in the border of fields, resulting in a quicker field re-infestation for feeding and reproduction in autumn (Prokopy and Gyrisco 1965; Prokopy et al. 1967; Saeidi and Moharramipour 2017). This effect of autumn adult field re-infestation is indirectly suggested by the abundance of eggs and larvae recorded in spring. The *H. postica* adult abundance and those of *Bathyplectes* spp. were positively related. This association can be explained since a greater abundance of weevil adults is directly related to the fact of a previously greater abundance of larvae, which have given rise to these adults. In turn, this could result in a greater number of adult parasitoid probably due to a greater number of parasitism events. When related to landscape variables, only the proportion of non-crop and winter cereal affected the *H. postica* abundance. In the case of non-crop, *H. postica* eggs abundance was positively affected at 500 and 1000 buffer radii. This relation may be explained as previously, were non-crop acts as a refuge for adults in a similar way to that of field margin.

In relation with alfalfa natural enemies, and contrarily to what we initially hypothesized, landscape composition variables little explain about *Bathyplectes* spp. abundance and nothing about its parasitism rate. Regarding local field variables, alfalfa age was positively related with the wasp abundance, and this suggests that as older the field, more density of weeds can be expected (Taberner 2020). It is well known that flower food sources increased *Bathyplectes* spp. adult female's longevity and reproductive performance (van Emden 1963; Maingay et al. 1991; Jervis et al. 1993; Jacob and Evans 2000).

In the case of landscape composition covers, alfalfa showed significant negative relationship with the abundance of adults of *Bathyplectes* spp., and this was unexpected somehow. Either this could be the result of a dilution effect (Veddeler et al. 2006; Drapela et al. 2008), or it could result from a metapopulation dynamics in the field (Ricci et al. 2009). The rate of parasitism was negatively related to the *H. postica* larval abundance. Rand (2013) reported that this may occur through hindering the host selection or by increasing the host handling time. These findings suggest that *Bathyplectes* spp. is linked to agricultural crop cover rather than natural or non-agricultural covers.

Several studies suggested that the aphidophagous *C. septempunctata* uses *H. postica* larvae as an alternative source of food when pea aphid populations are low (Richards and Evans 1998; Meseguer et al. 2021). Our data showed that the abundance of *C. septempunctata* was affected by winter cereal and arable crop proportions at the three buffer radii. Indeed, this ladybeetle is an active aphidophagous predator that when it leaves overwintering sites, needs to feed for reproduction. Cereals are the main crop for breeding *C. septempunctata* populations because of its large acreage, frequently high aphid abundance, prey quality, convenient microclimate, absence of insecticide treatments, and late harvest date (Honěk 1989; Bianchi et al. 2006), and these justifies our recent findings.

Pests, natural enemies and biological control services not always respond to the landscape diversity (Rusch et al. 2016; Tscharntke et al. 2016; Karp et al. 2018). Our study provided, for the first time, evidences that alfalfa weevil and its parasitoid *Bathyplectes* spp. rather respond to field conditions than landscape composition whereas its occasional predator *C. septempunctata* mainly responds to landscape composition. However, more detailed analysis, which includes landscape configuration parameters, will be needed to better understand how landscape may affect the abundance of the alfalfa weevil and its related natural enemies.

REFERENCES

Agustí, N., Bourguet, D., Spataro, T., Delos, M., Eychenne, N., Folcher, L., Arditi, R., 2005. Detection, identification and geographical distribution of European corn borer larval parasitoids using molecular markers. Mol. Ecol. 14, 3267–3274.

Azrag, A.G.A., Yusuf, A.A., Pirk, C.W.W., Niassy, S., Mbugua, K.K., Babin, R., 2020. Temperature-dependent development and survival of immature stages of the coffee berry borer *Hypothenemus hampei* (Coleoptera: Curculionidae). Bull. Entomol. Res. 110, 207–218.

Barney, R.J., Armbrust, E.J., Bartell, D.P., Goodrich, M.A., 1978. Frequency occurrence of *Bathyplectes curculionis* within instars of *Hypera postica* larvae in Illinois. Environ. Entomol. 7, 241–245.

Bartell, D.P., Pass, B.C., 1980. Morphology, development, and behavior of the immature stages of the parasite *Bathyplectes anurus* (Hymenoptera: Ichneumonidae). Can. Entomol. 112, 481–487.

Berberet, R.C., Bisges, A.D., 1998. Potential for competition among natural enemies of larvae of *Hypera postica* (Coleoptera: Curculionidae) in the southern plains. Environ. Entomol. 27, 743–751.

Berberet, R.C., Zarrabi, A.A., Payton, M.E., Bisges, A.D., 2003. Reduction in effective parasitism of *Hypera postica* (Coleoptera: Curculionidae) by *Bathyplectes curculionis* (Hymenoptera: Ichneumonidae) due to encapsulation. Environ. Entomol. 32, 1123–1130.

Bianchi, F.J.J.A., Booij, C.J.H., Tscharntke, T., 2006. Sustainable pest regulation in agricultural landscapes: a review on landscape composition, biodiversity and natural pest control. Proc. R. Soc. B Biol. Sci. 273, 1715–1727.

Blodgett, S.L., Lenssen, A.W., 2004. Distribution of alfalfa weevil (Coleoptera: Curculionidae) larvae among postcutting locations. J. Econ. Entomol. 97, 1319–1322.

Blodgett, S.L., Lenssen, A.W., Cash, S.D., 2000. Harvest with raking for control of alfalfa weevil (Coleoptera: Curculionidae). J. Entomol. Sci. 35, 129–135.

Clemente-Orta, G., Madeira, F., Batuecas, I., Sossai, S., Juárez-Escario, A., Albajes, R., 2020. Changes in landscape composition influence the abundance of insects on maize: The role of fruit orchards and alfalfa crops. Agric. Ecosyst. Environ. 291, 106805.

Davídková, M., Doležal, P., 2019. Temperature-dependent development of the double-spined spruce bark beetle *Ips duplicatus* (Sahlberg, 1836) (Coleoptera; Curculionidae). Agric. For. Entomol. 21, 388–395.

Dowell, R. V., Horn, D.J., 1977. Adaptive strategies of larval parasitoids of the alfalfa weevil (Coleoptera: Curculionidae). Can. Entomol. 109, 641–648.

Drapela, T., Moser, D., Zaller, J. G., Frank, T., 2008. Spider assemblages in winter oilseed rape affected by landscape and site factors. Ecography 31, 254–262.

Duodu, Y.A., Davis, D.W., 1974. Selection of alfalfa weevil larval instars by, and mortality due to, the parasite *Bathyplectes curculionis* (Thomson). Environ. Entomol. 3, 549–552.

Gariepy, T., Kuhlmann, U., Gillott, C., Erlandson, M., 2008. A large-scale comparison of conventional and molecular methods for the evaluation of host-parasitoid associations in non-target risk-assessment studies. J. Appl. Ecol. 45, 708–715.

Giles, K.L., Obrycki, J.J., 1997. Reduced insecticide rates and strip-harvesting effects on alfalfa weevil (Coleoptera: Curculionidae) larval populations and prevalence of *Zoophthora phytonomi* (Entomophthorales: Entomophthoraceae). J. Econ. Entomol. 90, 933–944.

Giles, K.L., Obrycki, J.J., Degooyer, T.A., Orr, C.J., 1994. Seasonal occurrence and impact of natural enemies of *Hypera postica* (Coleoptera: Curculionidae) larvae in Iowa. Environ. Entomol. 23, 167–176.

Gomez-Polo, P., Traugott, M., Alomar, O., Castañé, C., Rojo, S., Agustí, N., 2014. Identification of the most common predatory hoverflies of Mediterranean vegetable crops and their parasitism using multiplex PCR. J. Pest Sci. 87, 371–378.

Goosey, H.B., 2012. A degree-day model of sheep grazing influence on alfalfa weevil and crop characteristics. J. Econ. Entomol. 105, 102–112.

Guppy, J.C., Mukerji, M.K., 1974. Effects of temperature on developmental rate of the immature stages of the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae). Can. Entomol. 106, 93–100.

Harcourt, D.G., 1990. Displacement of *Bathyplectes curculionis* (Thoms.) (Hymenoptera: Ichneumonidae) by *B. anurus* (Thoms.) in eastern ontario populations of the alfalfa weevil, *Hypera postica* (Gyll.) (Coleoptera: Curculionidae). Can. Entomol. 122, 641–645.

Harcourt, D.G., Guppy, J.C., 1991. Numerical analysis of an outbreak of the alfalfa weevil (Coleoptera: Curculionidae) in eastern Ontario. Environ. Entomol. 20, 217–223.

Hodek, I., Honěk, A., 1996. Ecology of *coccinellidae*. Kluwer academics, Dordrecht.

Hoff, K.M., Brewer, M.J., Blodgett, S.L., 2002. Alfalfa weevil (Coleoptera: Curculionidae) larval sampling: comparison of shake-bucket and sweep-net methods and effect of training. J. Econ. Entomol. 95, 748–753.

Hoffmann, A., 1963. Sous-famille des Curculionidae, Tribu des Hyperini, Les Hypera (syn: Phytonomus), in: Balachowsky, A.S. (Ed.), Entomologie Appliquée a l'agriculture. Tome I. Coléoptères. Second Volume. Masson et Cíe., Paris, Francia, pp. 984–989.

Honěk, A., 1989. Overwintering and annual changes of abundance of *Coccinella septempunctata* in Czechoslovakia (Coleoptera, Coccinellidae). Acta Entomol. Bohemoslov.

Hsieh, F., Armbrust, E.J., 1974. Temperature limits of alfalfa weevil oviposition and egg density in Illinois. J. Econ. Entomol. 67, 203–206.

Huggans, J.L., Blickenstaff, C.C., 1964. Effects of photoperiod on sexual development in alfalfa weevil. J. Econ. Entomol. 57, 167–169.

Jacob, H.S., Evans, E.W., 2000. Influence of carbohydrate foods and mating on longevity of the parasitoid *Bathyplectes curculionis* (Hymenoptera: Ichneumonidae). Environ. Entomol. 29, 1088–1095.

Jalali, M., Mehrnejad, M.R., Kontodimas, D.C., 2014. Temperature-dependent development of the five psyllophagous ladybird predators of *Agonoscena pistaciae* (Hemiptera: Psyllidae). Ann. Entomol. Soc. Am. 107, 445–452.

Jalali, M., Sakaki, S., Ziaaddini, M., Daane, K.M., 2018. Temperature-dependent development of *Oenopia conglobata* (Col.: Coccinellidae) fed on *Aphis gossypii* (Hem.: Aphididae). Int. J. Trop. Insect Sci. 38, 410–417.

Jervis, M.A., Kidd, N.A.C., Fitton, M.G., Huddleston, T., Dawah, H.A., 1993. Flower-visiting by hymenopteran parasitoids. J. Nat. Hist. 27, 67–105.

Karp, D.S., Chaplin-Kramer, R., Meehan, T.D., Martin, E.A., DeClerck, F., Grab, H., Gratton, C., Hunt, L., Larsen, A.E., Martínez-Salinas, A., O'Rourke, M.E., Rusch, A., Poveda, K., Jonsson, M., Rosenheim, J.A., Schellhorn, N.A., Tscharntke, T., Wratten, S.D., Zhang, W., Iverson, A.L., Adler, L.S., Albrecht, M., Alignier, A., Angelella, G.M., Anjum, M.Z., Avelino, J., Batáry, P., Baveco, J.M., Bianchi, F.J.J.A., Birkhofer, K., Bohnenblust, E.W., Bommarco, R., Brewer, M.J., Caballero-López, B., Carrière, Y., Carvalheiro, L.G., Cayuela, L., Centrella, M., Ćetković, A., Henri, D.C., Chabert, A., Costamagna, A.C., De la Mora, A., de Kraker, J., Desneux, N., Diehl, E., Diekötter, T., Dormann, C.F., Eckberg, J.O., Entling, M.H., Fiedler, D., Franck, P., van Veen, F.J.F., Frank, T., Gagic, V., Garratt, M.P.D., Getachew, A., Gonthier, D.J., Goodell, P.B., Graziosi, I., Groves, R.L., Gurr, G.M., Hajian-Forooshani, Z., Heimpel, G.E., Herrmann, J.D., Huseth, A.S., Inclán, D.J., Ingrao, A.J., Iv, P., Jacot, K., Johnson, G.A., Jones, L., Kaiser, M., Kaser, J.M., Keasar, T., Kim, T.N., Kishinevsky, M., Landis, D.A., Lavandero, B., Lavigne, C., Le Ralec, A., Lemessa, D., Letourneau, D.K., Liere, H., Lu, Y., Lubin, Y., Luttermoser, T., Maas, B., Mace, K., Madeira, F., Mader, V., Cortesero, A.M., Marini, L., Martinez, E., Martinson, H.M., Menozzi, P., Mitchell, M.G.E., Miyashita, T., Molina, G.A.R., Molina-Montenegro, M.A., O'Neal, M.E., Opatovsky, I., Ortiz-Martinez, S., Nash, M., Östman, Ö., Ouin, A., Pak, D., Paredes, D., Parsa, S., Parry, H., Perez-Alvarez, R., Perović, D.J., Peterson, J.A., Petit, S., Philpott, S.M., Plantegenest, M., Plećas, M., Pluess, T., Pons, X., Potts, S.G., Pywell, R.F., Ragsdale, D.W., Rand, T.A., Raymond, L., Ricci, B., Sargent, C., Sarthou, J.P., Saulais, J., Schäckermann, J., Schmidt, N.P., Schneider, G., Schüepp, C., Sivakoff, F.S., Smith, H.G., Whitney, K.S., Stutz, S., Szendrei, Z., Takada, M.B., Taki, H., Tamburini, G., Thomson, L.J., Tricault, Y., Tsafack, N., Tschumi, M., Valantin-Morison, M., van Trinh, M., van der Werf, W., Vierling, K.T., Werling, B.P., Wickens, J.B., Wickens, V.J., Woodcock, B.A., Wyckhuys, K., Xiao, H., Yasuda, M., Yoshioka, A., Zou, Y., 2018. Crop pests and predators exhibit inconsistent responses to surrounding landscape composition. Proc. Natl. Acad. Sci. 115, E7863–E7870.

Koehler, C.S., Gyrisco, G.G., 1961. Responses of the alfalfa weevil, *Hypera postica*, to controlled environments. J. Econ. Entomol. 54, 625–627.

Kuhar, T.P., Youngman, R.R., Laub, C.A., 1999. Alfalfa weevil (Coleoptera: Curculionidae) pest status and incidence of *Bathyplectes* spp. (Hymenoptera: Ichneumonidae) and *Zoophthora phytonomi* (Zygomycetes: Entomophthorales) in Virginia. J. Econ. Entomol. 92, 1184–1189.

Latheef, M.A., Parr, J.C., Pass, B.C., 1979. Factors affecting survival of Kentucky populations of the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae). Environ. Entomol. 8, 1032–1036.

Levi-Mourao, A., Madeira, F., Meseguer, R., García, A., Pons, X., 2021a. Effects of temperature and relative humidity on the embryonic development of *Hypera postica* Gyllenhal (Col.: Curculionidae). Insects 12, 3, 250.

Levi-Mourao, A., Meseguer, R., Pons, X., 2021b. Parasitism of the alfalfa weevil *Hypera postica* Gyllenhal by *Bathyplectes* sp. in Spain, in Proceedings of the 1st International Electronic Conference on Entomology 10505. https://doi.org/10.3390/IECE-10505

Levi-Mourao, A., Núñez, E., García, A., Meseguer, R., Pons, X., 2022. Alfalfa winter cutting: Effectiveness against the alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) and effect on its rate of parasitism due to *Bathyplectes* spp. (Hymenoptera: Ichneumonidae). Crop Prot. 152, 105858.

Litsinger, J.A., Apple, J.W., 1973. Thermal requirements for embryonic and larval development of the alfalfa weevil in Winsconsin. J. Econ. Entomol. 66, 309–311.

Los, L.M., Allen, W.A., 1983. Incidence of *Zoophthora phytonomi* (Zygomycetes: Entomophthorales) in *Hypera postica* (Coleoptera: Curculionidae) larvae in Virginia. Environ. Entomol. 12, 1318–1321.

Madeira, F., Clemente-Orta, G., Alomar, O., Batuecas, I., Sossai, S., Albajes, R., 2021. Land use alters the abundance of herbivore and predatory insects on crops: the case of alfalfa. J. Pest Sci. 2021 1, 1–19.

Maingay, H.M., Bugg, R.L., Carlson, R.W., Davidson, N.A., 1991. Predatory and parasitic wasps (Hymenoptera) feeding at flowers of sweet fennel (*Foeniculum vulgare* Miller var. *dulce* Battandier & Trabut, Apiaceae) and spearmint (*Mentha spicata* L., Lamiaceae) in Massachusetts. Biol. Agric. Hortic. 7, 363–383.

Meseguer, R., Levi-Mourao, A., Pons, X., 2021. Species complex and temporal associations between coccinellids and aphids in alfalfa stands in Spain. Insects 12, 971.

Nanini, F., Maggio, D.H., Ferronato, P., Rugno, G., Yamamoto, P.T., Corrêa, A.S., 2019. Molecular marker to identify *Diaphorina citri* (Hemiptera: Liviidae) DNA in gut content of predators. Neotrop. Entomol. 2019 486 48, 927–933.

Núñez, E., Taberner, A., Muñoz, F., Delgado, I., 2015. Efecto del corte de la alfalfa en parada invernal en el control de *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae). Valencia.

Ohto, K., 1996. Effects of photoperiod on the adult diapause in the alfalfa weevil, *Hypera postica* (Gyllenhal). Res. Bull. Plant Prot. Japan 32.

Papierok, B., Aeschlimann, J.P., Loan, C., 1986. Two entomophthoralean fungi occurring on *Hypera postica* in southern France. J. Invertebr. Pathol. 48, 377–380.

Parks, T.H., 1914. Effect of temperature upon the oviposition of the alfalfa weevil (*Phytonomus posticus* Gyllenhal). J. Econ. Entomol. 7, 417–421.

Parr, J.C., Pass, B.C., Nordin, G.L., 1993. Compatibility of *Zoophthora phytonomi* (Entomophthorales : Entomophthoraceae) and *Bathyplectes anurus* (Hymenoptera: Ichneumonidae) in Kentucky alfalfa fields. Biol. Control 22, 674–678.

Pons, X., Nuñez, E., 2020. Plagas da la alfalfa: Importancia, daños y estrategias de control., in: Lloveras, J., Delgado, I., Chocarro, C. (Eds.), La Alfalfa, Agronomía y Utilización. Edicions de la Universitat de Lleida, Centro de Investigación y Tecnología Agroalimentaria de Aragón, Lleida- Zaragoza, Spain, pp. 167–202.

Prokopy, R.J., Armbrust, E.J., Cothran, W.R., Gyrisco, G.G., 1967. Migration of the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae), to and from estivation sites. Ann. Entomol. Soc. Am. 60, 26–31.

Prokopy, R.J., Gyrisco, G.G., 1965. Summer migration of the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae). Ann. Entomol. Soc. Am. 58, 630–641.

Radcliffe, E.B., Flanders, K.L., 1998. Biological control of alfalfa weevil in North America. Integr. Pest Manag. Rev. 3, 225–242.

Rand, T.A., 2013. Host density drives spatial variation in parasitism of the alfalfa weevil, *Hypera postica*, across dryland and irrigated alfalfa cropping systems. Environ. Entomol. 42, 116–122.

Ricci, B., Franck, P., Toubon, J.F., Bouvier, J.C., Sauphanor, B., Lavigne, C., 2009. The influence of landscape on insect pest dynamics: A case study in southeastern France. Landsc. Ecol. 24, 337–349.

Richards, D.R., Evans, E.W., 1998. Reproductive responses of aphidophagous lady beetles (Coleoptera: Coccinellidae) to nonaphid diets: an example from alfalfa. Ann. Entomol. Soc. Am. 91, 632–640.

Rosenthal, S., Koehler, C.S., 1968. Photoperiod in relation to diapause in *Hypera postica* from California. Ann. Entomol. Soc. Am. 61, 531-536.

Rusch, A., Chaplin-Kramer, R., Gardiner, M.M., Hawro, V., Holland, J., Landis, D., Thies, C., Tscharntke, T., Weisser, W.W., Winqvist, C., Woltz, M., Bommarco, R., 2016. Agricultural landscape simplification reduces natural pest control: A quantitative synthesis. Agric. Ecosyst. Environ. 221, 198–204.

Saeidi, M., Moharramipour, S., 2017. Physiology of cold hardiness, seasonal fluctuations, and cryoprotectant contents in overwintering adults of *Hypera postica* (Coleoptera: Curculionidae). Environ. Entomol. 46, 960–966.

Salt, G., van den Bosch, R., 1967. The defense reactions of three species of *Hypera* (Coleoptera, curculionidae) to an ichneumon wasp. J. Invertebr. Pathol. 9, 164–177.

Shoubu, M., Okumura, M., Shiraishi, A., Kimura, H., Takagi, M., Ueno, T., 2005. Establishment of *Bathyplectes anurus* (Hymenoptera: Ichneumonidae), a larval parasitoid of the alfalfa weevil, *Hypera postica* (Coleoptera: Curculionidae) in Japan. Biol. Control 34, 144–151.

Solà, M., Riudavets, J., Agustí, N., 2018. Detection and identification of five common internal grain insect pests by multiplex PCR. Food Control 84, 246–254.

Soroka, J., Bennett, A.M.R., Kora, C., Schwarzfeld, M.D., 2020. Distribution of alfalfa weevil (Coleoptera: Curculionidae) and its parasitoids on the Canadian Prairies, with a key to described species of Nearctic *Bathyplectes* (Hymenoptera: Ichneumonidae). Can. Entomol. 152, 663–701.

Soroka, J., Grenkow, L., Cárcamo, H., Meers, S., Barkley, S., Gavloski, J., 2019. An assessment of degree-day models to predict the phenology of alfalfa weevil (Coleoptera: Curculionidae) on the Canadian Prairies. Can. Entomol. 152, 110–129.

Summers, C.G., 1998. Integrated pest management in forage alfalfa. Integr. Pest. Manag. 3, 127–154.

Sweetman, H.L., Wedemeyer, J., 1933. Further studies of the physical ecology of the alfafa weevil, *Hypera postica* (Gyllenhal). Ecology 14, 46–60.

Taberner, A., 2020. Control de malas hierbas, in: Lloveras, J., Delgado, I., Chocarro, C. (Eds.), La Alfalfa, Agronomía y Utilización. Edicions de la Universitat de Lleida, Centro de Investigación y Tecnología Agroalimentaria de Aragón. Lleida- Zaragoza, Spain, pp. 253–

272.

Tilmon, K.J., Danforth, B.N., Day, W.H., Hoffmann, M.P., 2000. Determining parasitoid species composition in a host population: a molecular approach. Ann. Entomol. Soc. Am 93, 640–647.

Traugott, M., Symondson, W.O.C., 2008. Molecular analysis of predation on parasitized hosts. Bull. Entomol. Res. 98, 223–231.

Traugott, M., Zangerl, P., Juen, A., Schallhart, N., Pfiffner, L., 2006. Detecting key parasitoids of lepidopteran pests by multiplex PCR. Biol. Control 39, 39–46.

Tscharntke, T., Karp, D.S., Chaplin-Kramer, R., Batáry, P., DeClerck, F., Gratton, C., Hunt, L., Ives, A., Jonsson, M., Larsen, A., Martin, E.A., Martínez-Salinas, A., Meehan, T.D., O'Rourke, M., Poveda, K., Rosenheim, J.A., Rusch, A., Schellhorn, N., Wanger, T.C., Wratten, S., Zhang, W., 2016. When natural habitat fails to enhance biological pest control – Five hypotheses. Biol. Conserv. 204, 449–458.

van Emden, H.F., 1963. Observations on the effect of flowers on the activity of parasitic Hymenoptera. Entomol. Mon. Mag. 265–270.

Veddeler, D., Klein, A.M., Tscharntke, T., 2006. Contrasting responses of bee communities to coffee flowering at different spatial scales. Oikos 112, 594–601.

Zahiri, B., Fathipour, Y., Khanjani, M., Moharramipour, S., Zalucki, M.P., 2014. Alternatives to key factor analyses for assessing the population dynamics of *Hypera postica* (Coleoptera: Curculionidae). Popul. Ecol. 56, 185–194.

Zahiri, B., Fathipour, Y., Khanjani, M., Moharramipour, S., Zalucki, M.P., 2010a. Preimaginal development response to constant temperatures in *Hypera postica* (Coleoptera: Curculionidae): Picking the best model. Environ. Entomol. 39, 177–189.

Zahiri, B., Fathipour, Y., Khanjani, M., Moharramipour, S., Zalucki, M.P., 2010b. Modeling demographic response to constant temperature in *Hypera postica* (Coleoptera: Curculionidae). J. Econ. Entomol. 103, 292–301.

GENERAL CONCLUSIONS

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CHAPTER I: Effects of temperature on the fitness of the alfalfa weevil *(Hypera postica)*

- Two-sex life table and developmental rate models can help predicting the effect of temperature on the fitness of this pest.
- The use of two-sex life tables provides a more realistic data on whole-life performance.
- The minimum developmental temperature is around 6.5 °C.
- The thermal requirement for complete postembryonic development and total development are 358 DD and 484 DD, respectively.
- The optimal temperature, for Spanish populations, resides between 20-24 °C.
- Prediction was validated with collected field data, revealing that no more than two generations are possible within an annual cycle.
- In a hypothetical increase of the average temperatures due to global warming, the number of generations would not change since photoperiod plays an important role inducing diapause.

CHAPTER II: Alfalfa winter cutting: effectiveness against the alfalfa weevil, *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae) and effect on its rate of parasitism due to *Bathyplectes* spp. (Hymenoptera: Ichneumonidae)

- A winter cutting significantly reduces the larval abundances in spring, with an effectiveness of about 50%.
- The winter cutting does not affect negatively the rate of parasitism by *Bathyplectes* spp. but enhances that of *B. anura.*
- By killing the *H. postica* larvae, the occurrence of epizooties of the entomophthoral fungus *Z. phytonomy*, hinder the effectiveness of the winter cutting and negatively affect rate of parasitism causing a disruption in the alfalfa weevil-parasitoid system.
- A winter cutting can be a useful cultural method as a component of an IPM program in Spain and other European regions.

CHAPTER III: Molecular and morphological identification of the alfalfa weevil larval parasitoids *Bathyplectes anura* and *Bathyplectes curculionis* to estimate the rate of parasitism

- Two specific *COI* primers, for *B. anura* and *B. curculionis*, have been successfully designed and their accuracy and utility for species identification positively tested.
- This new molecular strategy provides newly information about the ecology of *B. anura* and *B. curculionis*, revealing the prevalence of both species in the Ebro basin field conditions.
- Molecular method can overestimate the parasitism rate, especially in the case of *B. curculionis*.
- DNA analysis method should be combined with classical approaches in order to determine effective parasitism rates and accommodate interactions with other species that are not specially targeted by the molecular assay.

- The alfalfa crop management currently performed in the Ebro Basin may be unfavorable to their control capacity. A winter cutting and a delay of the first spring cutting are tools that may be included in integrated pest management.

CHAPTER IV: Influence of landscape composition on the abundance of the alfalfa weevil (*Hypera postica*) and its natural enemies in alfalfa crops of Northeast Iberian Peninsula

- The abundance of the alfalfa weevil was more influenced by field local variables like field characteristics and crop management than by landscape variables.
- The alfalfa fields irrigated by sprinkler and the length of the field perimeter favorized the abundance of eggs of this pest.
- The abundance of adults of *Bathyplectes* spp. responded positively to some field characteristics but only winter cereal crop cover explained something about its abundance. These findings suggest that *Bathyplectes* spp. is linked to agricultural crop cover rather than natural or non-agricultural covers.
- The rate of parasitism of *H. postica* larvae due to *Bathyplectes* spp., was negatively associated with the *H. postica* larval abundance.
- The alfalfa weevil and its larval parasitoid *Bathyplectes* spp. rather respond to field than landscape composition characteristics whereas its occasional predator *C. septempunctata* mainly responds to landscape composition characteristics, in this particular case of winter cereals.