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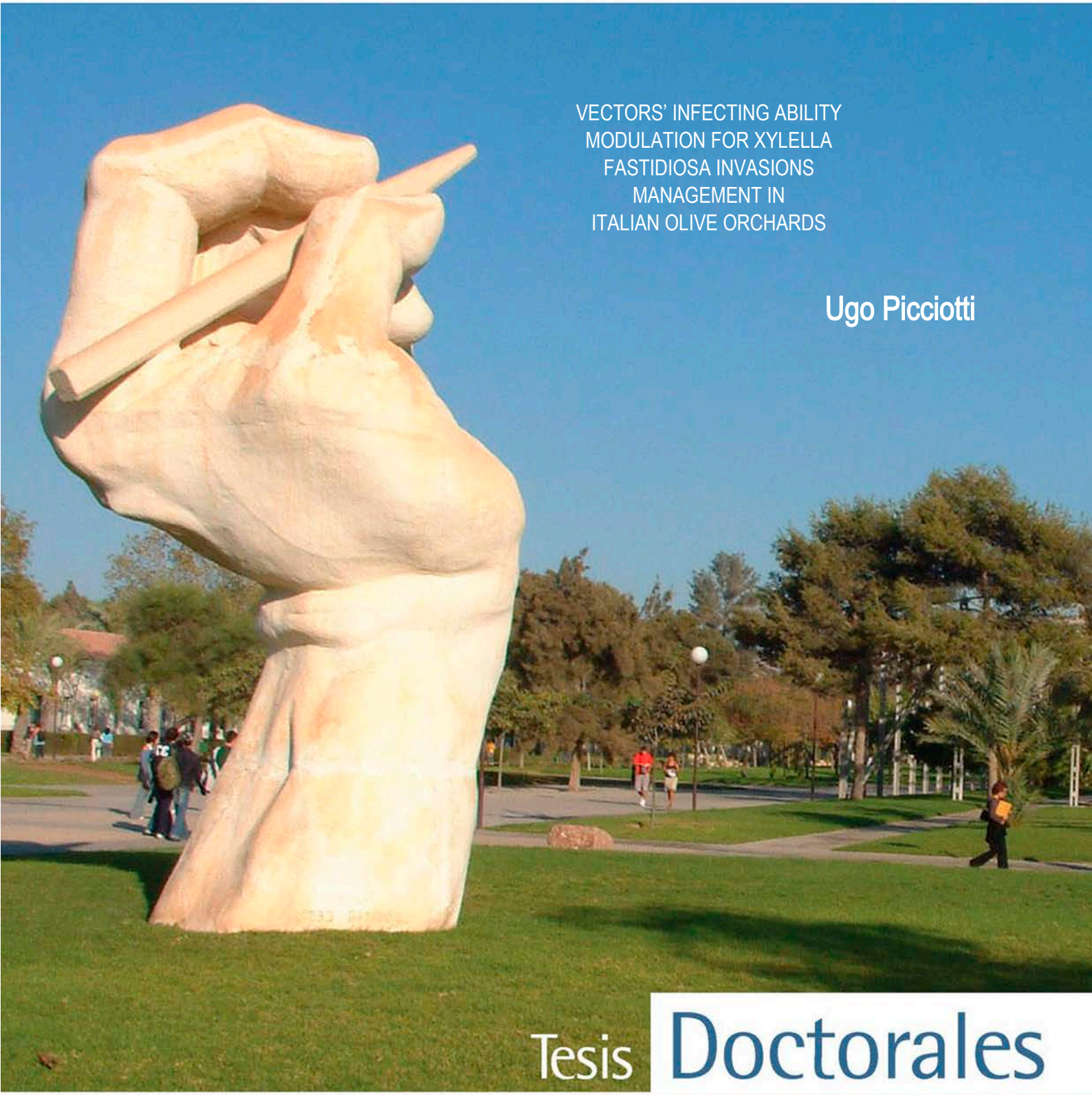
VECTORS' INFECTING ABILITY MODULATION FOR XYLELLA FASTIDIOSA INVASIONS MANAGEMENT IN ITALIAN OLIVE ORCHARDS

Ugo Picciotti



Tesis Doctorales

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VECTORS' INFECTING ABILITY MODULATION FOR *XYLELLA FASTIDIOSA* INVASIONS MANAGEMENT IN ITALIAN OLIVE ORCHARDS

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Summary

Recent estimates have revealed that more than 6.5 million olive trees in southern Italy have subdued to the *Xylella fastidiosa* infection, leading to the devasting Olive Quick Decline Syndrome (OQDS). This epidemic continues to expand, posing a significant threat to global olive oil production. OQDS has already resulted 30-34% reduction in ecosystem services provided by olive orchards and a 28% decline in associated biodiversity. Additionally, OQDS has annihilated productivity and the entire olive oil supply chain, causing considerable economic losses. To counteract the relentless spread of *Xylella*, Integrated Transmission Management (ITM) strategies are crucial. Reducing one vector per olive tree present in an olive orchard can confine *X. fastidiosa* within acceptable economic and environmental limits. Thus, monitoring and managing vector populations are crucial to curbing disease transmission. The complex interactions between insects and microorganisms are pivotal in the OQDS scenario. Understanding these interactions can provide insights into novel control strategies, such as disrupting bacterial symbiosis with Aphrophoridae foams, affecting the fitness of vector insects, and potentially reducing *X. fastidiosa* transmission. To counteract *Xylella* transmission effectively, biocontrol measures must be incorporated into IPM strategies for olive orchards. However, more than the current arsenal of vector antagonists is required. The entrance into the Europe of *Zelus renardii* shows promise in biocontrolling *Xylella* vectors. Furthermore, *Z. renardii*'s ability to manage other olive pests adds to its utility. *Zelus renardii*'s bionomics and its ability to regulate alarm pheromones via Brindley glands is crucial for its effective use in IPM strategies. The formulation of artificial diets for mass-rearing *Z. renardii* under controlled conditions can pave the way for its inundative release to enhance ITM. These biological and biotechnological control measures have the potential to significantly reduce *Philaenus spumarius* populations and the infective capacity of *Xylella* vectors within IPM strategies. This approach can also act preventively and protectively, reducing the risk of future infections and limiting repeated transmissions. Progress has been made in modulating the transmission abilities of *Xylella* vectors, while the challenge of OQDS and *X. fastidiosa* remains tricky. The availability of *Z. renardii* and exploring its capabilities offer a more sustainable and effective approach to managing this disease in olive production.

Resumen

Algunas estimaciones recientes revelan que más de 6,5 millones de olivos del sur de Italia han sufrido infección por *Xylella fastidiosa*, lo que ha provocado el Síndrome del Declive Rápido del Olivo (SiDRO). Esta epidemia sigue expandiéndose, lo que supone una importante amenaza para la producción mundial de aceite de oliva y de aceitunas. El SiDRO ya ha provocado una reducción del 30-34% en los servicios

ecosistémicos que proporcionan los olivares y un descenso del 28% en la biodiversidad asociada al cultivo. Además, el SiDRO ha aniquilado la productividad y toda la cadena de suministro del aceite de oliva, causando considerables pérdidas económicas. Para contrarrestar la incesante propagación de la *Xylella*, son cruciales las estrategias de Gestión Integrada de la Transmisión (GIT). La reducción de la población de *Philaenus spumarius*, vector de *Xylella fastidiosa*, a un individuo por olivo puede mantener el patógeno a niveles aceptables desde el punto de vista económico y medioambiental. Así pues, el seguimiento y la gestión de las poblaciones de los vectores son cruciales para frenar la transmisión de la enfermedad. Las complejas interacciones entre insectos y microorganismos son fundamentales en el escenario del SiDRO. La comprensión de estas interacciones puede aportar ideas sobre nuevas estrategias de control, como la interrupción de la simbiosis bacteriana con las espumas de los Aphrophoridae. Esto podría afectar la fitness de los insectos vectores y al menos potencialmente reducir la transmisión de *X. fastidiosa*. Para contrarrestar eficazmente la transmisión de la *Xylella*, se deberían incorporar medidas de biocontrol a las estrategias de gestión integrada de plagas de los olivares. Sin embargo, se necesita algo más que el arsenal actual de antagonistas del vector. La entrada en Europa de *Zelus renardii* resulta prometedora para el biocontrol de los vectores de la *Xylella*. Además, la capacidad de *Z. renardii* para controlar otras plagas del olivo incrementaría su potencial práctico. Las características bio-etológicas de *Zelus renardii* y su capacidad para regular las feromonas de alarma a través de las glándulas de Brindley son cruciales para su uso eficaz en las estrategias de control integrado de plagas. La disponibilidad de dietas artificiales para la cría masiva de *Z. renardii* en condiciones controladas, facilitará su liberación inundativa para la GIT. Estas medidas de control biológico y biotecnológico tienen el potencial de reducir significativamente las poblaciones de *P. spumarius* y la capacidad infectiva de los vectores de *Xylella* dentro de las estrategias de GIT. Este enfoque también puede actuar de forma preventiva y protectora, reduciendo el riesgo de futuras infecciones y limitando las transmisiones repetidas. En esta Tesis se ha progresado en la modulación de la capacidad de transmisión de los vectores de *Xylella*. A pesar de ello, el SiDRO y *X. fastidiosa* siguen siendo de difícil manejo. La disponibilidad de *Z. renardii* y la exploración de sus capacidades antagonistas ofrecen un enfoque sostenible y eficaz para gestionar esta enfermedad de los olivares que se explotan comercialmente.

Riassunto

Recenti stime hanno rivelato che più di 6,5 milioni di ulivi nell'Italia meridionale hanno ceduto all'infezione da *Xylella fastidiosa*, causando il devastante Complesso del Disseccamento Rapido dell'Olivo (CoDiRO od Olive Quick Decline Syndrome - OQDS). Questa epidemia continua ad espandersi, rappresentando una minaccia significativa per la produzione globale di olio d'oliva. L'OQDS ha già

provocato una riduzione del 30-34% dei servizi ecosistemici forniti dagli oliveti e un calo del 28% della biodiversità associata. Inoltre, l'OQDS ha annientato la produttività e l'intera filiera dell'olio d'oliva, causando notevoli perdite economiche. Per contrastare l'inarrestabile diffusione della *Xylella*, le strategie di Gestione Integrata della Trasmissione (GIT) sono fondamentali. La riduzione a un vettore per ogni olivo presente in un oliveto può confinare *X. fastidiosa* entro limiti economici e ambientali accettabili. Pertanto, il monitoraggio e la gestione delle popolazioni di vettori sono fondamentali per limitare la trasmissione della malattia. Le complesse interazioni tra insetti e microrganismi sono fondamentali nello scenario dell'OQDS. La comprensione di queste interazioni può fornire spunti per nuove strategie di controllo, come l'interruzione della simbiosi batterica con le schiume Aphrophoridae, influenzando la fitness degli insetti vettori e riducendo potenzialmente la trasmissione di *X. fastidiosa*. Per contrastare efficacemente la trasmissione di *Xylella*, le misure di biocontrollo devono essere incorporate nelle strategie IPM per gli oliveti. Tuttavia, l'attuale arsenale di antagonisti dei vettori non è sufficiente. L'ingresso in Europa di *Zelus renardii* è promettente per il biocontrollo dei vettori di *Xylella*. Inoltre, la capacità di *Z. renardii* di gestire altri parassiti dell'olivo aumenta la sua utilità. L'etologia di *Z. renardii* e la sua capacità di regolare i feromoni di allarme attraverso le ghiandole di Brindley sono fondamentali per il suo uso efficace nelle strategie IPM. La formulazione di diete artificiali per l'allevamento in massa di *Z. renardii* in condizioni controllate può aprire la strada al suo rilascio inondativo per migliorare la GIT. Queste misure di controllo biologiche e biotecnologiche hanno il potenziale di ridurre significativamente le popolazioni di *Philaenus spumarius* e la capacità infettiva dei vettori di *Xylella* nell'ambito delle strategie IPM. Questo approccio può anche agire in modo preventivo e protettivo, riducendo il rischio di infezioni future e limitando le trasmissioni ripetute. Sono stati fatti dei progressi nella modulazione delle capacità di trasmissione dei vettori di *Xylella*, mentre la sfida alla cura dell'OQDS rimane complicata. La disponibilità di *Z. renardii* e l'esplorazione delle sue capacità offrono un approccio più sostenibile ed efficace alla gestione di questa malattia nella produzione olivicola.

Chapter 1 – General Introduction

Insects represent the dominant organisms on Earth in terms of species number and biomass. Insect biodiversity occupies many ecological niches thanks to insect adaptability and consequent co-evolution opportunities with macro- and microorganisms [1–4].

Co-evolution between insects (host) and microorganisms (guest) leads to reciprocal interactions or symbiosis. Symbiosis is considered '*the cohabitation of distinct organisms'* [5], and the interspecific interaction can be negative (unbalanced), neutral (indifferent), or beneficial (mutualistic) [6]. Furthermore, the interactions can be ectosymbiotic if the guest microorganism thrives outside the host insect's tissues or endosymbiotic if the microorganism lives inside the host insect's tissues, including interstitia or specialised cells [7-9]. This PhD dissertation will focus on the ectosymbiosis between microorganisms and insects, considering cuticular any surface outside the insect body.

We consider regular insect symbiotic organisms proliferating on the outer cuticular surface, often within specialised structures. Ectosymbiotic microorganisms are insect-borne and physical-chemically attached to the insect's cuticle without interacting with organs [10, 11].

Ectosymbiotic microorganisms of economic importance also include *Xylella fastidiosa* Wells *et al*., 1987. *Xylella fastidiosa* settles in the lumen of the hindgut of a few Auchenorrhyncha species that use the xylem sap food plants [12-14]. *Xylella fastidiosa* can secrete chitinases, which increase biofilm formation by the bacterium, increasing its adhesion to the vector's cuticle while obtaining nutrients from the vector [15]. The vector ingests xylem sap for food [16], thus acquiring and transmitting the pathogen to the host plant [17, 18].

The quarantine bacterium *Xylella fastidiosa* subsp. *pauca* ST53 entered Italy (Apulia) from Costa Rica [19], inducing the consequent Olive Quick Decline Syndrome (OQDS) epidemic [20] on olive trees. *Xylella* demonstrates how an insect-borne alien bacterial species can invade new biogeographical areas to inflict considerable economic damage, which continues. Indeed, plants infected by *Xylella* cease production and die within a few years [17]. The bacterium decimated and continues to ruin olive trees in Apulia, southern Italy, inducing the OQDS [20] because encountered indigenous Aphrophoridae (Hemiptera, Auchenorrhyncha: Spittlebugs) xylem sap feeders capable of spreading the pathogen to *Olea europaea* L., 1753 [21-23]. Few Aphrophoridae can acquire and transmit *X. fastidiosa*, namely *Philaenus spumarius* L., 1758, *Philaenus italosignus* Drosopoulos & Remane, 2000 and *Neophilaenus campestris* (Fallén, 1805) (Hemiptera, Aphrophoridae) [17, 24]. Still, *P. spumarius* is the key vector of *X. fastidiosa* since it is the only one of the three Aphrophoridae usually feeding on olive trees [17].

Aphrophoridae-borne plant pathogen transmission could be interspecific or intraspecific. Vector–host–pathogen interactions determine whether a pathogen outbreak will lead to settlement, persistence, and epidemic development [25].

Vector-borne pathogen and vector control management raise interest because the interaction among the actors involved (e.g., vector, pathogen, and crop) causes relevant damage. Interaction is more substantial than that due to the Aphrophoridae (vector) or the *X. fastidiosa* (pathogen) alone.

Aphrophoridae adults have a persistent relationship with *X. fastidiosa* [26, 27]. Vector pest damage is greater than the sum of the actions of single insects because each inoculation propagates the damage of the insect-borne pathogen in space and time. An infection on a plant organ can propagate to the entire plant [28], depending on the size of the infected plant. A pathogen can continue to inflict damage after its vector death for years [29].

The plant pathogen impact originates from the vector's attitude to multiply the damage, infecting all the plants it can get and feed on in its lifespan. Once *P. spumarius* has acquired the bacterial pathogen, this will multiply in the vector foregut, making it transmissible at each food plant probing or feeding. The vector inflicts damage more than proportional to its food requirements [17]. The impact and the damages of *X. fastidiosa* are much more related to TARDIS (Time And Relative Dimension In Space, BBC®) vector factors dispersion than the vector total feeding ability.

Pathogen acquisition by vectors and transmission to olive trees include four scenarios. (I) Pathogen-free *P. spumarius* feed on an uninfected olive tree; the actors' status does not change (both are free from the bacterium). (II) Infective *P. spumarius* (with the pathogen) feeds on infected olive trees; the status of the actors does not change (they both have the pathogen already). (III) *P. spumarius* pathogen-free feeds on infected olive trees; only the status of the vector is modified. The vector acquires the pathogen and can transmit it to olive trees. Finally, (IV) infective *P. spumarius* feeds on uninfected olive trees; in this case, the status of the tree (from uninfected to infected) changes. Case IV represents the first infectious event that acts as a multiplier factor for spreading *X. fastidiosa* across the territory.

The infectious efficiency of *P. spumarius*, the high percentage of infective adults [30], and their mobility [31] concerning the rapid induced decline of olive trees strongly suggest that the *Xylella* vector must be considered the new key pest of olive trees [32–34].

Damage to infected plants occurs over the long term, ending in the death of the olive trees [35]. Given the infection's outcome, vectors can wipe out past investments, annual cultivation costs and future income of affected olive groves. Assuming that the disease cannot be treated, *X. fastidiosa* will annihilate susceptible plants.

The action threshold for vector control is low since any vector can potentially infect, even by probing, a host plant. Infections also multiply because feeding is performed repeatedly in the adult dispersion window.

Management of the current *X. fastidiosa* epidemics must control the first infections (transmission management) and not directly the vector population (vector management) [33]. There is a clear distinction between mere vector management, which considers managing vector population size (on eggs, juveniles, and adults), and transmission management, which is based on minimising the first transmission events by adults [36]. We assume treatments targeting adults (i.e., adult vector management) will match with effective pathogen infection management, such as in non-proportional damaging pests. However, when inappropriate timing occurs, adult vector management does not necessarily result in effective pathogen transmission control [36]. Indeed, the first control action against adults must coincide with their eclosion. In all other cases, any action will be ineffective in controlling transmission, even if they may eventually drastically reduce the adult population because vectors have already been infecting all the available – considering the time and space of the adult lifespan – food plants. The ineffective vector control facilitates the invasion of *X. fastidiosa* due to the wrong timing and the low control efficacy, allowing surviving vectors to infect all available food plants. Conversely, transmission control prevents additional infections after the first transmission, thus making plant damage proportional to vector numbers.

The delayed onset of OQDS symptoms makes managing *X. fastidiosa* invasion particularly tricky because the demarcation of the infected area boundary is symptom-dependent and does not consider infected but still asymptomatic olive trees. The search for *X. fastidiosa* infection to declare a new infected area is symptom-dependent. Often, the alert must be on time and far from the actual area of infection, further complicating the scenario for implementing effective management strategies.

Therefore, the ideal target for *X. fastidiosa* management would consist of killing as many adult vectors as possible at the time of their first feeding on olive trees [17, 33, 36]. This strategy would lower any vector infectious action to a direct proportionality. Effective infection management involves killing the part of the vector population that causes *2, 3... n* infections by mechanical and chemical control actions. Killing the same number of vectors when they have carried out *2, 3... n* infections on different plants will result in trivial and ineffective vector control. Transmission control in infected areas can significantly limit the spread of *Xylella* into disease-free territories and halt the epidemics. Mass-movement of infectious vectors will decrease with the management of juveniles and adults feeding on olive trees [36]. A low vector population will then make transmission rare and lead to isolating the bacterial pathogen in infected plants. The progressive death of diseased plants will reduce the disease to a few active foci, bringing the damage to a proportional event and simplifying its management. The number of surviving vectors will equal the

number of olive trees in a field, reducing the density to a maximum of one adult vector per plant. Reducing this number will keep the *Xylella* invasion within an acceptable threshold. In IPM farming, this will gradually reduce the intensity of physical and chemical control.

Olive IPM strategies involve the proper use of various control actions. This implies the use of resistant olive cultivars [37-39], *P. spumarius* juvenile mechanical and chemical control [17, 40], and infection chemical control against recently eclosed *P. spumarius* adults [33]. Adult vector biocontrol holds promise to reinforce the IPM strategy.

Several research groups have worked on identifying autochthonous natural enemies for *P. spumarius*. The actions of some natural enemies, such as *Ooctonus vulgatus* Haliday, 1833 (Hymenoptera, Mymaridae) [41], *Verrallia aucta* (Fallén, 1817) (Diptera, Pipunculidae) [42] or some spiders [43], are not efficient and prompt in controlling *P. spumarius* infections and populations, which can be from three to six million individuals per hectare [17]. Native predators specialised in *Xylella* vectors are not available. Evidence shows that the generalist predators (e.g., spiders) found in olive groves cannot contain *X. fastidiosa* infections [43].

An alternative could be using stenophagous predators of adult vectors as "living insecticides". These can be applied inundatively to implement or replace the chemical action and target the narrow window of vector feeding on olive trees. This inundative biocontrol action may also target secondary olive pests, i.e., Olive Moth, Olive Fly, *Latilica* spp. (Hemiptera, Issidae), *Pelionella cycliger* (Leonardi, 1908) (Hemiptera, Pseudococcidae) enforcing olive IPM overall.

Biological control of *P. spumarius* could involve the predator *Zelus renardii* Kolenati, 1856 (ZR, Hemiptera, Reduviidae). *Zelus renardii* is native to North and Central America [44] and widely distributed throughout the Americas, Asia, and Oceania [45]. *Zelus renardii* was first reported in Europe in 2011 in mainland Greece [46], then in Crete [47] and subsequently entered Spain, Italy, Albania, France, and Portugal [48-52]. *Zelus renardii* also entered Germany, Denmark, and the UK via grape transport from Italy and Greece [53, 54]. Other Balkan countries, the Czech Republic, Bulgaria, Austria, Cyprus, Turkey, and Israel [55-60], recently reported the presence of *Z. renardii* acclimatised in many countries of the Mediterranean Basin and continental Europe [60]. *Zelus renardii* is a non-invasive alien species, acclimatised and adapted to agroecosystems with little presence in anthropised urban and peri-urban ecosystems [58].

According to common opinion, *Z. renardii* is a generalist predator capable of disturbing the wild predator guild. This assumption derives mainly from simplified experiments involving a few species and individuals caged (physical interference) in small volumes with the Reduviidae [61, 62]. Recent evidence suggests that the predator is primarily stenophagous of hemipterans [63]. *Zelus renardii* chooses its prey based on their abundance [64] and on prey habitat, including the host plant, size, and mobility [65, 66].

Furthermore, the reduviid is often associated with honeydew that can attract guilds of potential predators. The honeydew appears to lure the reduviid, resulting in egg-laying and subsequent presence of juvenile *Zelus*. *Zelus renardii* has Holling's Type II functional response, i.e., it efficiently searches, suppresses, and consumes prey [64]. Furthermore, *Z. renardii* has a short prey handling time and high fecundity, and its egg-laying increases with increasing prey density [64]. Harpactorinae, including *Z. renardii*, have been well studied for biological control and successfully explored in integrated pest management systems. Besides, *Z. renardii* fitness is not affected by the *Bacillus thuringiensis* Cry1F, Cry1Ac and Cry2Ab toxins that the predator takes up while preying on *Spodoptera frugiperda* Smith, 1797 and *Trichoplusia ni* (Hübner, 1803) (Lepidoptera, Noctuidae) [67]. Following EU Ecosystems recommendations, this implies that IPM in olive groves can be enhanced by applying *B. thuringiensis* biocontrol [68].

Amongst different targets, the reduviid promptly attacks adults of *P. spumarius* and *N. campestris* as well as other olive pests such as *Bactrocera oleae* (Rossi, 1790) (Diptera, Tephritidae) [61]. *Zelus renardii* can reinforce mitigation actions against the *B. oleae*, which is already discouraged from laying eggs and has compromised symbionts due to further control actions [69–70].

Modelling experiments have demonstrated the potential efficacy of an inundation strategy with *Z. renardii* for *Xylella* vector control and mitigating infections and OQDS in Italy [36]. These models show that a few *Z. renardii* inundations fit the proposed IPM strategy to minimise the first *Xylella* infections below an acceptable threshold in two years [36].

Z. renardii further bionomics studies are required for its development as inundative biological control of *X. fastidiosa* vectors. This will minimise risks on non-targets and will increase predator performance. For instance, the mass breeding of *Z. renardii* has yet to be made available. This is required for field inundative releases.

Therefore, the objectives of this PhD dissertation are: (I) understanding the interplay between *P. spumarius* and *X. fastidiosa* infected plants focusing on vector spreading capabilities, (II) studying *Z. renardii* traits, a predator of *Xylella* vector for pathogen infection control and olive secondary pest guild management, and (III) developing *Z. renardii* artificial diets for its multiplication and future biocontrol studies of *X. fastidiosa* vectors.

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Chapter 2 – "Ectomosphere": Insects and Microorganisms Interactions

Abstract

This chapter focuses on interacting with insects and their ectosymbiont (*lato sensu*) microorganisms for environmentally safe plant production and protection. Some cases help compare ectosymbiont microorganisms that are insect-borne, -driven, or -spread relevant to endosymbionts' behaviour. Ectosymbiotic bacteria can interact with insects by allowing them to improve the value of their *pabula.* In addition, some bacteria are essential for creating ecological niches that can host the development of pests. Insect-borne plant pathogens include bacteria, viruses, and fungi. These pathogens interact with their vectors to enhance reciprocal fitness. Knowing vector-phoront interaction could considerably increase chances for outbreak management, notably when sustained by quarantine vector ectosymbiont pathogens, such as the actual *Xylella fastidiosa* Mediterranean invasion episode. Insect pathogenic viruses have a close evolutionary relationship with their hosts, also being highly specific and obligate parasites. Sixteen virus families have been reported to infect insects and may be involved in the biological control of specific pests, including some economic weevils. Insects and fungi are among the most widespread organisms in nature and interact with each other, establishing symbiotic relationships ranging from mutualism to antagonism. The associations can influence the extent to which interacting organisms can exert their effects on plants and the proper management practices.

Sustainable pest management also relies on entomopathogenic fungi; research on these species starts from their isolation from insect carcasses, followed by identification using conventional light or electron microscopy techniques. Thanks to the development of omics sciences, it is possible to identify entomopathogenic fungi with evolutionary histories that are less shared with the target insect and can be proposed as pest antagonists. Many interesting omics can help detect the presence of entomopathogens in different natural matrices, such as soil or plants. The same techniques will help localize ectosymbionts, localization of recesses, or specialized morphological adaptation, greatly supporting a robust interpretation of the symbiont role. The manipulation and modulation of ectosymbionts could be a more promising way to counteract pests and borne pathogens, mitigating the impact of formulates and reducing crop yield losses due to the lesser impact of direct damage and diseases. The promise has a preventive intent for more manageable and broader implications for pests, comparing what we can obtain using simpler, less-specific techniques and a less comprehensive approach to Integrated Pest Management (IPM).

INTRODUCTION

Insects have inhabited the Earth for approximately 480 million years, representing the dominant life form as species and biomass [1–4]. Their presence expresses the high biodiversity of insects in a wide range of ecological niches and results from their genetic plasticity, adaptability, and co-evolutionary processes with other organisms.

The influence between microorganisms and insects has led to the establishment of different types of interaction that can be summarised in various forms of interactions. De Bary [5] first defined symbiosis as "*the cohabitation of distinct organisms*". Symbiosis is here intended as a significant biological *liaison* between or among species. Symbiosis is one of the leading evolutionary drivers promoting natural biological novelty. Symbiotic relationships between prokaryotes and eukaryotes are present in all kingdoms of life [6].

Symbioses between two organisms can be broadly classified as mutualism, commensalism, or antagonism, depending on the interaction between the species involved. The impact of the symbiont on the amphitryon can highlight an evolutionary continuum between antagonism (negative interaction), commensalism (neutral interaction), and mutualism (beneficial interaction) [7]. Symbiotic relationships drive many interesting biological processes within individuals and at the ecological system level.

For the sake of this contribution, we consider ectosymbiont as the guest living out of the host body and endosymbiont as a guest living in the host body. Inside the amphitryon body, the guest may live within specialised host cells [8–10].

Mutualism provides an advantage for both species involved. Mutualistic microorganisms can give the host essential nutrients, protection from enemies, increase fitness, and mediate the host's interaction with other species [11]. Mutualists can be divided into obligate and facultative. Obligate or primary symbionts are microorganisms necessary for the host's survival. They tend to improve the nutritional aspects of unbalanced diets on which the host feeds [12]. Facultative symbionts are not essential for the survival of their host and have broader effects ranging from modifying nutritional aspects to manipulating reproduction. Secondary mutualists can often only be found in a fraction of the host population [11,12].

Commensal symbiosis represents a symbiotic relationship in which one organism benefits without associated costs [12,13].

Finally, parasitism or antagonism represents an unbalanced interaction in favour of the guest microorganism that takes advantage of the insect, generating a loss of fitness or causing host death. Antagonists may be obligate (host-specific) or facultative generalists. Antagonism between insects and entomopathogenic organisms results from co-evolution in which the pathogen aims to host exploitation better and improve its transmission. In contrast, the insect seeks to exclude the pathogen more effectively

by improving its defence strategies [14]. Both actors involved in antagonism adopt physiological, ecological, and ethological adaptations to maximise their fitness [15].

Manipulating and modulating these interactions represents counteracting plant pests and pathogens, mitigating related damages/symptoms that generate considerable food and economic losses. Moreover, the approach reduces the food and feeds contaminating microorganisms and the toxicological risk [16]. Such interactions also provide a basis to develop future research work in a relatively new field due to the vast diversity of insects and microorganisms in a broad range of trophic and ecological niches worldwide. The chapters below represent distinctive interactions, from the pure endosymbiosis to the pure ectosymbiotic case. Properly available techniques make discoveries and rising topics possible, and we consider the methods the real drivers in knowledge evolution [17]. We do not report the endless number of studies treating ectosymbiotic interrelationships, i.e., the studies on microorganisms thriving in the insect gut lumen reviewed by Steinhaus [18]. Moreover, we suggest the 1912 book by Peglion [19] and the 1913 study by Tonelli [20] to be among the first notes on insects transmitting ectosymbiotic plant pathogens.

Endosymbiotic Bacteria in Pests

The extensive literature on endosymbionts, such as *Wolbachia* spp., *Buchnera* sp., *Rickettsia* spp., *Cardinium* spp., and other species, evidences a close evolutionary link with the insects' hosts. Known effects include, among others, changes in trophic behaviour and marked effects that manipulate and interfere with the host reproduction and speciation pathways [21,22]. However, few studies have focused on weevils or *Philaenus* spp. interactions with their endosymbionts. A search on PubMed (https://pubmed.ncbi.nlm.nih.gov/ (accessed on 31 August 2022) with the "*Wolbachia*" query yielded 3740 records, but only 33 by adding the term "weevil". Similarly, "*Buchnera*" gathered 494 papers, including "weevil" in the query adding only one. The summary in Table 1 shows the results with different terms and combinations.

Table 1. Results of PubMed interrogations with query terms combinations[1].

Query Terms	*Wolbachia*	*Buchnera*	*Rickettsia*	*Cardinium*	Endosymbiont
Weevil	33	1	34	2	56
Rhynchophorus	0	0	1	0	2
Cosmopolites	0	0	0	0	0
Philaenus	3	0	2	1	2

[1]Dated 31 August 2022; default parameters.

No studies are available in PubMed for "*Philaenus*" and "*Bacillus*", while one is available for "*Cosmopolites*". Ten were found for "*Rhynchophorus*" and "*Bacillus*", mainly concerning *Bacillus thuringiensis* Berliner, 1915 antimicrobial activity and host immune response. No studies were found in PubMed when using the term "*Serratia*" with "*Cosmopolites*" or "*Philaenus*". However, *Serratia marcescens* isolates from *R. ferrugineus* [23,24] exist in publications.

Pathogens Spread by Monophagous Vectors: *Candidatus* Phytoplasma vitis

Knowing and studying the biology of a plant pathogen and its vector is necessary for epidemic management, especially when it comes to quarantine pathogens. A quarantine organism is a pest/pathogen of economic importance and yet to be present in an area or present but not widely distributed and officially controlled [25]. For EU member countries, quarantine organisms fall into "EU relevant quarantine organisms" and "EU priority quarantine organisms". Once introduced into a territory, "priority quarantine organisms" could have a more severe impact than "relevant quarantine organisms". Therefore, knowledge of the biology of the pathogen and its vector(s) can provide decisive help in eradication and containment actions. Vector-borne viruses, bacteria, and phytoplasmas are numerous, and many insects can potentially carry these microorganisms. Some hemipterans feed on mesophyll and sap, while others feed exclusively on xylem or phloem sap. This specialisation makes them suitable for transmitting pathogens that live in the circulatory system of plants [26].

Vectors may be specific for a microorganism; if the vectors are monophagous, the epidemiological process is straightforward to study and hypothetically easier to manage in the field. In some cases, multiple insects may transmit a pathogen. If the vectors are polyphagous, the epidemiological process is more complex to study and manage effectively [27,28].

An example of a pathogen spread by a monophagous vector is *Candidatus* Phytoplasma vitis, a quarantine organism included in the A2 EPPO list [29]. This organism is the etiological factor of Flavescence Dorée (FD), the most threatening of the Grapevine Yellows (GY) diseases in Europe [29,30].

FD first appeared in the 1950s in southwest France and other areas of Europe, North America, Asia Minor, and Australia [31,32].

The main symptoms of this disease are yellowing, downward curling of leaves, fruit abortion, stunted growth, and lack of lignification of new shoots [33].

The only wild vector of this phytoplasma is *Scaphoideus titanus* Ball, 1932 (Hemiptera, Cicadellidae), which feeds and completes its life cycle exclusively on grapevines [27,34]. *Euscelidius variegatus* (Kirschbaum, 1858) (Hemiptera, Cicadellidae) can only transmit FD under laboratory conditions [35].

Scaphoideus titanus is native to North America and entered Europe, presumably by transporting nursery material containing the insect's eggs [36]. It was first reported in France in 1958 [37] and in Liguria (Italy) in 1964 [38]. In Italy, the species is currently reported from the northern and southern regions.

Scaphoideus titanus is monovoltine and completes its life cycle exclusively on the grapevines. Egg hatching is gradual, beginning in mid-May and continuing until July. Post-embryonic development lasts about 40 days [29,38]. It feeds on the phloem sap of the vine and, during its trophic action on infected plants, acquires the pathogen in addition to nutrients.

Phytoplasmas are obligatory parasites of plants and vectors [29,39], and *Candidatus* Phytoplasma vitis infects the grapevine phloem and various organs of the vector insect (circulatory) and actively multiplies in both hosts (propagative) [26]. *Scaphoideus titanus* can assume phytoplasma both at the nymph and adult instars. In this case, infectivity does not disappear with the metamorphosis, persisting until the insect's death.

Under laboratory conditions, the adult insect requires a 7-day acquisition access period, a minimum latency of at least 7 days, and a 7-day inoculation access period. When the insect is in the nymphal stage, a latency time of 3-5 weeks is necessary after ingestion of the pathogen [40].

After a 13-day capture access period, the capture rate is 91.4% [40]. Vectors are crucial to the outbreak, and studying their feeding behaviour seems pivotal to understanding why some cultivars are more tolerant, as it appears that *S. titanus* prefers to feed on some cultivars over others. This behaviour probably depends on the phloem's chemical composition, making some cultivars less palatable. Cultivars' tolerance exists because of the intrinsic ability to deter the vectors [41]. Bressan [42] demonstrated how phytoplasma negatively affects the fitness of *S. titanus*, causing shorter adult' lifespans, lower fecundity, and a prolonged egg-hatching time. Endosymbiotic organisms, such as phytoplasmas, can be pathogenic for both hosts, i.e., the plant and the insect vector. However, this does not happen for ectosymbiotic organisms, which can develop a disease in plants without compromising any vital aspect of the vector. Ectosymbiotic microorganisms are transported from one plant to another simply by binding externally to the insect's body without interacting with the internal organs.

Before multiplying in the insect's organism, phytoplasma must pass the midgut and the salivary glands epithelia. Various glycoconjugates exist on the surface of these tissues, to which many pathogen adhesins bind. *Candidatus* Phytoplasma vitis binds with the VmpA adhesin to N-acetylglucosamine and mannose on the surfaces of the midgut and salivary glands of the vectors. Furthermore, the glycoconjugate patterns are very similar between *S. titanus* and *E. variegatus*, which may partly explain the specificity that *S. titanus* has for *Candidatus* Phytoplasma vitis [43].

Given the simplicity of the epidemic process, the management of one or more outbreaks may be easier with this type of pathogen.

EU member countries practice obligatory phytosanitary controls to manage FD spreading with immediate destruction of symptomatic plants and compulsory insecticidal treatments for vector control [44]. Monitoring in northern Italy has shown that the vector is present in large numbers, especially in abandoned vineyards and where there is inadequate pest management [45]. Vector population density is lower in managed vineyards. In Reggio Emilia province (Italy), the estimated vector density was 0.3–0.2 insects per plant in 2008–2009 [46].

Preventive monitoring allows to eradicate the pathogen and its vector quickly. The control of *S. titanus* is necessary for the success of eradication or containment actions and, considering a reduction in the use of insecticides in agriculture, the in-depth study of pathogen-vector interactions is essential to find new ways of managing an epidemic.

GENOMIC CLUES IN INSECT PATHOGENIC VIRUSES

Viruses have a close evolutionary relationship with their hosts, being host-specific and obligate parasites. Applying genomic and metagenomic approaches has uncovered several new viruses that remained hidden or have not entered already-described genera or families [47,48]. The research has led thus far to sixteen families of viruses infecting insects. The most studied include *Baculovirus* (Baculoviridae) and *Cytoplasmic Polyhedrosis Virus* (CPV, Spinareoviridae). Other pathogenic viral lineages in insects belong mainly to Reovirinae, Densovirinae, and Entomopoxvirinae [49]. Some viruses are the main ingredients of bioformulations applied for managing and biocontrolling some economically relevant insect pests [49,50]. However, the information available on the biology of insect viral pathogens is only partially exhaustive, given the extent of the phylogenetic radiations of their hosts.

Insect pathogenic viruses are less persistent than chemical pesticides. However, increased awareness of environmentally safe procedures has re-evaluated their use as biopesticides. Compared to synthetic pesticides, viruses offer crucial advantages such as high host specificity, selectivity, and no risk of environmental contamination. Insect pathogenic viruses are large, ubiquitous, and manifest high genomic plasticity [51]. The latter property allows them to select for increasing efficacy, persistence, and other valuable characteristics in pest management, including lack of activity *vs* concomitant parasitoids and predators [52].

The analysis of genomes to identify new insect-pathogenic viruses is a relatively recent research endeavour, also driven by the search for novel information on evolutionary processes eventually recorded in sequenced genomes. Genomic data have progressively revealed the natural history of known and new

host-pathogen associations, showing increased viral biodiversity—as indicated by the discovery of new species—as well as the introgression, to varying degrees, of viral genetic material into the host genomes. These processes range from the presence of "fossil" genetic fragments of viral origin to the introgression of actively expressed genes, which in some cases confer a specific advantage to the host, up to the integration of entire genomes [53]. Genetic exchanges between eukaryotes and viruses have often been considered residuals of previous viral infections. In some cases, gene integration processes provide new functions to the host, enriching its specialisation or functional adaptation to new trophic niches or habitats [54]. The production of critical genomic data from pests and the parallel advances of bioinformatics tools makes it possible to assess the real impact of exchanged genetic material on host biology and evolution.

The members of the Nudivirus, previously included in Baculoviridae, represent a distinct monophyletic sister group of dsDNA viruses present in several insect hosts [55–57]. They have non-retroviral species, such as an endogenous nudivirus integrated into the genome of the brown planthopper *Nilaparvata lugens* (Stål, 1854) (Hemiptera, Delphacidae) [53]. Several nudivirus-like genes exist in different host lineages, including Hemiptera and Hymenoptera, but only one nudivirus pseudogene infects *Philaenus spumarius* L., 1758 (Hemiptera, Aphrophoridae) [58].

Currently, two genomes of *P. spumarius* have been made available on NCBI. A search for the amino acid identity of the nudivirus *per os* infective proteins (PIF) in the *P. spumarius* genome, performed with TBLASTX [59], showed several positive, albeit short and fragmented, matches, presumably representing possible acquisitions of small genome fragments (Table 2, Figure 1).

Table 2. Top matches[1] of translated nudivirus PIF proteins, from different arthropod hosts, on the *P. spumarius* genome[2].

Query Protein	Virus	Acc. n.	N. of Matches	Max id. (%)	Lowest E-Value
PIF-1	*Nilaparvata lugens* endogenous nudivirus, isolate Hangzhou	KJ566575.1	87	60.0	0.002
PIF-2	*Drosophila*-associated nudivirus, isolate UA_Kan_16_57	MT496843.1	100	60.0	0.001
PIF-2	*N. lugens* endogenous nudivirus, isolate Hangzhou	KJ566558.1	100	52.0	2×10^{-4}
PIF-2	*Hyposidra talaca* nucleopolyhedrosis virus, isolate Hyta NPV-ID1	MT642700.1	8	45.7	0.004
PIF-2 (putative)	*Macrobrachium* nudivirus CN-SL2011	JQ804993.1	100	65.0	1×10^{-4}
PIF-2 (mRNA)	*D. melanogaster* PFTAIRE	NM_169147.2	50	60.0	2×10^{-6}
PIF-3 (complete cds)	*N. lugens* endogenous nudivirus, isolate Hangzhou	KJ566581.1	67	81.8	0.001
PIF-3 (putative, mRNA)	*Cotesia congregata*	FM201563.4	100	71.4	6×10^{-5}
PIF-4	*N. lugens* endogenous nudivirus, isolate Hangzhou	KJ566551.1	26	60.0	0.001

[1]Based on TBLASTX analyses of open access data available at https://www.ncbi.nlm.nih.gov/genome/7381.
[2]GenBank assembly GCA_018207615.1 (PRJNA602656) produced by Biello [60].

Figure 1: Distribution of best matches produced by TBLASTX on the *P. spumarius* PSPU08 genome using query sequences of nudivirus PIF proteins. Top hits of *N. lugens* nudivirus PIF-1 (A) and PIF-2 from nudiviruses of *Drosophila* sp. (B), *Macrobrachium rosenbergii* (C), and *D. melanogaster* (D). NCBI accession numbers of query sequences are KJ566575.1 (A), MT496843.1 (B), JQ804993.1 (C), and NM_169147.2 (D).

Viruses of weevils include the invertebrate iridescent virus 6 (*Chilo* iridescent virus), a single copy, linear dsDNA member of Iridoviridae, which parasites hosts from Coleoptera and other orders. The virus has also experimentally infected *Diaprepes abbreviatus* (L., 1758) (Coleoptera, Curculionidae), a severe weevil pest of *Citrus* spp. in Florida [61]. Other curculionid viruses include two undescribed macula- and bunya-like RNA viruses reported from eucalyptus snout beetles (*Gonipterus* spp.; Coleoptera, Curculionidae) [62], and an *Entomopoxvirus* found in the European spruce bark beetles, *Ips typographus* (L., 1758) (Coleoptera, Curculionidae) and in *Ips amitinus* Wood and Bright, 1992 (Coleoptera, Curculionidae) [63–65]. Finally, a severe disease of the red palm weevil *Rhynchophorus ferrugineus* (Olivier, 1791) (Coleoptera, Dryophthoridae) relies on the *Cytoplasmic Polyhedrosis Virus* (CPV), which produces polyhedral inclusion bodies in all host stages, drastically affecting the pest population density levels [66]. Weevils are also vectors of some plant viruses, such as single-stranded RNA *Tymoviridae* [67].

Interactions between Entomopathogenic Fungi and Pests

Biological control of invasive pests also bases on certain entomopathogenic fungi (EFs) that can infect hosts in agroecosystems and appear suitable for plant protection exploitation. For many years, the search for such species used their isolation from insect carcasses, followed by identification using conventional light or electron microscopy techniques. Thanks to the development of molecular methods, especially DNA sequencing and omics technologies, it is now possible to identify the most crucial EFs species and detect their presence in different ecological niches, including the soil or plant environments.

EFs number around 1000 species [68], the best-known being *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., and *Acremonium* spp. [69]. Infection usually occurs through propagules that germinate and invade the host body after contact; the invasive mycelium then colonises the host until it dies. Conidiation from emerging hyphae and/or the production of resting propagules follow the host death [70].

Among the EFs primarily used for pest control, some *Beauveria* spp. (Hypocreales, Cordycipitaceae) are widely used against, for example, the coffee berry weevil *Hypothenemus hampei* (Ferrari, 1867) (Coleoptera, Curculionidae) [15], the Asian corn borer *Ostrinia furnacalis* Guenée, 1854 (Lepidoptera, Crambidae), and the sweet potato weevil *Cylas formicarius* (Fabricius, 1798) (Coleoptera, Brentidae) [71]. Many studies have deepened the knowledge about the role of *Beauveria bassiana* (Bals.) Vuill, 1912, as its insecticidal activity is due not only to the hyphae penetrating and spreading in the host body, but also to the effect caused by

various toxins [72]. This fungus demonstrated its relevance in banana crops protection from *Cosmopolites sordidus* (Germar, 1824) (Coleoptera, Dryophthoridae) [73–75] due to its ability to significantly reduce the weevil survival [76].

Beauveria bassiana products are widely applied on banana plantations to manage *C. sordidus*. Another *Beauveria* species, *Beauveria caledonica*, is responsible for the lethal infections of *C. sordidus* in banana plantations in South America. This fungus produces secondary metabolites and can modulate the pest immune response [76,77]. Studies with *Metarhizium anisopliae* (Metschn.) Sorok, 1883 reported the potential of this fungus in controlling adult weevils [78].

Several studies are underway to control *P. spumarius*, indicated as the main vector of the bacterium *X. fastidiosa* involved in the OQDS (Olive Quick Decline Syndrome) in the Salento Peninsula (southern Italy). The insect can acquire and inoculate the bacterium from/to different host plants [79]; therefore, limiting the transmission of *X. fastidiosa* by managing its vector is essential. Recent studies analyse the ability of some *Trichoderma* spp. isolates in decreasing the survival of *P. spumarius* [80]. An innovative IPM approach may include developing EF-based biocontrol actions. EF also represents an essential source of natural molecules capable of affecting *P. spumarius* metabolism and reproduction, thus limiting the pests' indirect damage to plants [81].

Species of the genus *Trichoderma* are among the most studied and -used biocontrol agents worldwide. They not only produce benefits as plant growth promoters but also act, with various mechanisms, against other microorganisms in plant defence. Volatile and non-volatile compounds produced by some species of *Trichoderma* can be perceived by the olfactory structures of *P. spumarius* [81], modifying and directing the insect's food preferences towards other areas of reduced agricultural interest [81–83].

Although supported by valid research data, the information available in the literature on the exploitation of EFs as biocontrol agents still needs to be comprehensive. Critical data on the exploitation of EFs as practical means of biological control and information on the mechanisms involved in fungal-insect interactions still need to be improved in many world regions. Therefore, efforts are still required to identify and characterise new fungal strains to investigate their entomopathogenic.

MULTITROPHIC INTERACTIONS OF ENTOMOPATHOGENIC FUNGI, CROPS, AND INSECTS

Insect pathogens were isolated from Mediterranean soils (Alicante, SE Spain) using *Galleria mellonella* L., 1758 (Lepidoptera, Pyralidae) larvae baits [84]. Samples from 61 sites were from agroecosystems and forests, while soils under *Nerium oleander* L., 1753, gave results from natural environments and gardens. Entomopathogenic fungi (EFs) are the most frequent insect pathogens (32.8% soils). *Beauveria bassiana* is the most abundant species (21% soil). *Metarhizium anisopliae* (6.4%) and *Akanthomyces lecanii* (Zimm.)

Spatafora, Kepler and Shrestha, 2017 {*Lecanicillium lecanii* (Zimm.) Gams [=*Verticillium lecanii* Zimm.]} (4.8%) are less frequent. *Beauveria bassiana* also scored the highest virulence in a single soil sample (ca. 90% infected insects) and is the most frequent EF (77.8%) in soils under *N. oleander*. Soils from commercial crop fields of food security importance, such as bananas, are also reservoirs of EFs [85]. Reports indicate that *B. bassiana* is a cosmopolitan entomopathogen, especially in warm areas [86]. Economically important pests, such as thrips [87], aphids [88], or pine processionary (*Thaumetopoea pityocampa* [Denis and Schiffermüller, 1775] [Lepidoptera, Notodontidae]) [89], were detected naturally infected with EFs. *Beauveria bassiana* (isolate Bb203) also infected adults of the Red Palm Weevil, *Rhynchophorus ferrugineus* Olivier, 1790 (RPW), in the field (palm groves) just at the first weevil introduction in SE Spain [90]. *Beauveria bassiana* 203 proved more pathogenic to *R. ferrugineus* than strains from other hosts and sources [91]. The strain applied three times at three-month intervals to field palms naturally infested with RPW caused 70-85% insect mortality [92]. Therefore, EFs are present in arid environments and have great potential for IPM of severe insect pests [93,94].

EFs can also colonise plants and plant waste. The latter is the most frequent component of soil organic matter. Evaluation of the growth and multiplication (conidiation) of common entomopathogens rises from inoculation on (almond peels) and gardening (palm waste) substrates obtained from Mediterranean ecosystems by-products of agriculture [95]. The development of entomopathogens depends on the type of substrate. *Akanthomyces lecanii* grows and sporulates well on almond mesocarp, but *Paecilomyces farinosus* (Holmsk.) A.H.S.Br. & G.Sm., 1957 does not. *Beauveria bassiana* uses palm seed nutrients for growth and sporulation, and leaves of the Mediterranean dwarf palm *Chamaerops humilis* L., 1753 promote the growth and sporulation of both *A. lecanii* and *B. bassiana*. The date palm (*Phoenix dactylifera* L., 1753) has a mycobiota that includes profusely sporulating fungi (*Penicillium* spp. and *Cladosporium* spp.). *Fusarium oxysporum* Schltdl., 1824 saprotroph and an undescribed *Lecanicillium* c.f. *psalliotae* (Treschew) Zare & W. Gams, 2001 entomopathogen colonise red-scale (*Phoenicococcus marlatti* Cockerell, 1899 – Hemiptera, Phoenicococcidae) infested leaves [96]. Palm pathogens, entomopathogenic and saprotrophic fungi strongly interact with each other; *B. bassiana* strongly inhibits *Penicillium vermoesenii* [= *Nalanthamala vermoesenii* (Biourge) Schroers, 2005] (Figure 2), a fungal necrotrophy of palms.

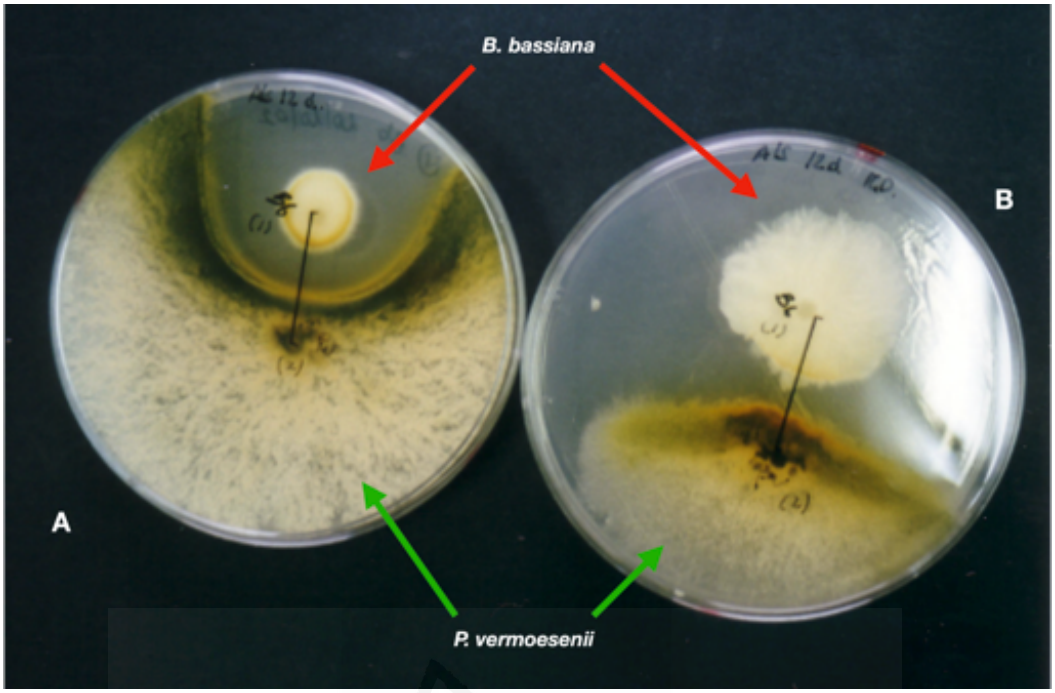

Figure 2: *Beauveria bassiana* (red arrows) inhibits the fungus palm pathogen *Penicillium vermoesenii* (green arrows). (A) Both fungi interact directly on the PDA medium. (B) The same two fungi on top of a dialysis membrane overlaid onto PDA (source: own photo).

EFs (*B. bassiana*, *Lecanicillium dimorphum* (J.D. Chen) Zare and W.Gams, 2001, and *Lecanicillium* c.f. *psalliotae*) artificially inoculated in living plants act as true endophytes [97]; fungi survive and spread in date palm (*P. dactylifera*) petiole tissues (parenchyma and vascular tissue) at least 30 days after inoculation. *Beauveria bassiana* is a natural endophyte from date palm roots [98]. This fungus was isolated from the roots of date palms in two coastal dune sites with high and low human impact in south-eastern Spain. Root colonisation by endophytic insect-pathogenic fungi has recently been reviewed [99]. Root and microbiota respiration [100] depletes oxygen in the rhizosphere. Fungal parasites of invertebrates, such as the nematophagous *Pochonia chlamydosporia* (Goddard) Zare and W. Gams, 2001 or the entomopathogens *B. bassiana* and *M. anisopliae*, breach chitin-rich barriers to infect the host. These biocontrol fungi can also ferment chitosan, a chitin derivative [101]. Apart from their application in biofuel production, this trait can be an adaptation for survival and insect infection by EFs in the rhizosphere. Entomopathogenic fungi are part of phylloplane and rhizosphere mycobiomes. Their endophytic behaviour allows them to colonise plant-derived substrates, affecting plant-volatile emissions during insect infestations [102]. Plant-derived substrates, such as rice grains, can be used for mass production and formulation of EFs [103,104].

Based on previous reports (see above) on the endophytic behaviour of EFs, several studies tested the response of palms to inoculation with these biocontrol fungi. *Beauveria bassiana*, *L. dimorphum*, and *L.* cf. *psalliotae* induced proteins in plant defence or stress response [105]. The plant immune system responds to

microbe-associated molecular patterns (MAMPs) derived from conserved structures (i.e., cell walls) of plant pathogens such as chitin [106]. Chitosan can permeabilise the membrane and kill plant pathogens such as bacteria and fungi in deacetylated form [107]. EFs and nematophagous fungi (NFs) are compatible with chitosan since they have evolved low-fluidity membranes [108,109] and branched cell walls rich in β-1,3-glucan [110]. Moreover, EFs and NFs contact chitin during host (insects and nematodes, respectively) infection. Chitosan modifies the transcriptome and biology of fungi and plants, causing cell stress [111]. Chitosan can enhance the pathogenicity of fungal parasites of nematode eggs [112–114]. These are close relatives of EFs, such as *Metarhizium* spp. [115].

Acoustics reveals that RPW larvae with *B. bassiana* infection have briefer movement and feeding activity [116]. We also have evidence that *B. bassiana* formulates used for RPW biocontrol in the field [92] repel adults of this insect pest [117]. Entomopathogenic fungi and close relative nematophagous fungi (*Pochonia* spp. egg parasites) emit volatile organic compounds (VOCs) capable of repelling *C. sordidus* [85] and RPW [117]. P201930831 and P202230103 insect repellents patented VOCs are on field trial for efficacy.

Finally, EFs are a component of plant and soil microbiomes. They are efficient insect pathogens with a multitrophic lifestyle, including plant endophytism, inducing plant defences and modifying insect pest behaviour with their VOCs, which work as low- environmental impact tools for pest management.

Native Entomopathogenic Fungi Used for Microbial Control of the *Rhynchophorus palmarum* (L., 1758)

In South America, economic palms such as the coconut (*Cocos nucifera* L., 1753) and the oil palm (*Elaeis guineensis* Jacq., 1897) are crops with social significance for the region. Industrial exploitation offers various raw materials for the cosmetics and food industry, in the settlement as construction materials, and in the traditional use of fresh coconut. Industrial processing induces employment and income opportunities for the community [118,119].

The incidence and damage of insect pests and plant diseases, which causes recurring losses on the farm and lower productivity [120,121], limit the overall production. The South American Palm Weevil (SAPW), *Rhynchophorus palmarum* (L., 1764) (Coleoptera, Dryophthoridae), causes crucial economic losses due to the cryptic larvae that burrow tunnels within the central cylinder of the palm stipes and apical meristem. The SAPW is black and sometimes reddish because of atypical colour polymorphism. It measures between 35 and 60 mm, presents sexual dimorphism between male and female, and the male snout is straight and robust. The male has stout brush-like setae on the front- clypeal head region, while the female rostrum is slender, lacking setae, and slightly arched dorso-ventrally [122,123]. A second species, *Dynamis borassi*

(Fabricius, 1802) (Coleoptera, Dryophthoridae), is similar enough to be misidentified. The presence and collection of adult specimens of *D. borassi* on Amazonian palm species, *Astrocaryum carnosum* F. Kahn and B. Millán, 1992, and *Astrocaryum chonta* Mart., 1844, provide information on weevil biology obtained from pupal cells collected in damaged inflorescences. The larvae were parasites by *Billaea rhynchophorae* (Blanchard, 1937) (Diptera, Tachinidae), which emerged from the pupal cells [124].

The geographic distribution of SAPW encompasses the Americas, from Argentina to California, and includes the Central American Antilles [125]. SAPW affects the primary area of commercial palm production on the continent and Brazilian regions of economic coconut and oil plantations [121]. SAPW spreads the nematode *Bursaphelencus cocophilus* (Cobb) Baujard (Rhabditia, Parasitaphelenchidae), which is responsible for inducing Red- Ring Disease (RRD) in palms [126,127]. The symptoms of RRD in palms are reddish lesions that gradually form in the stem [121].

The management of SAPW and RRD in coconut and oil palms is complex. However, chemical control has low efficiency in disrupting the SAPW-RRD association. An attempt at agronomical control uproots and burns the affected trees and reduces the infestation. However, this is a post-damage control action with a relevant significant environmental impact that also consists of greenhouse gas production. A more effective control action consists of the mass adult trapping by rhinchophorol coupled to traps with Synergic Blends of Attractive Sources (SBAS) and removing RRD-infected palms by keeping the RRD at low levels [122,128–131].

Concern over the mass trapping and felling of palms also suggests *in situ* biocoenosis studies identify new or neglected entomopathogenic microorganisms [132,133]. Highly virulent species and strains of native EFs can serve as effective bioinsecticides. Fungal strains native to the environment where they will be applied are fungi that have co-evolved with their host insects, such as certain strains of *B. bassiana* and *M. anisopliae*. These two represent the most widely-used entomopathogenic fungi in biological control [134–136]. Significant genetic diversity exists among the available collections, with a wide range of hosts and relevance to tropical and subtropical environments [137].

EFs play a central role in the Brazilian biopesticide market; these fungi mainly work in management of sugarcane spittlebugs or whiteflies in row crops via registered microbial formulation of *M. anisopliae* and *B. bassiana* [138]. That the number of registrations of biological formulations for pest control in Brazil is increasing (Table 3) [139] suggests the collection of relevant details among native biocontrol candidates.

Table 3. Several records of products for arthropod control in Brazil.

Product Records	2022	2021	2020	2019	2018
Insecticide	705	71	53	50	51
Microbiological insecticide	238	44	42	18	23
Biological Control Agent	69	6	3	6	5
Microbiological fungicide	66	19	15	6	8
Pheromone	46	2	1	0	3
Microbiological nematicide	46	6	12	6	1
Microbiological acaricide	42	12	10	6	2
Microbiological bactericide	5	0	0	0	0

Adapted source [138].

Recent research to control SAPW in Brazil has identified several native strains of highly virulent *B. bassiana* that can be differentiated to minimise resistance [140]. The criteria for selecting isolates for biocontrol originate in the insect mortality rates observed in bioassays and the efficiency of conidia production in the culture medium [141]. Several techniques allow fungi identification; the alpha taxonomy facilitates the clustering of collections, thus enabling performance estimation during pathogenicity tests [142] and subsequent molecular studies that are important to identifying and characterising a single native EF strain.

Advancements in molecular techniques, especially those based on DNA analysis by PCR, have enabled the development of rapid, accurate, and applicable methodologies for examining large samples to detect and identify different entities [143,144]. DNA profiles are powerful and sensitive tools to identify fungal isolates infecting a target population [143]. Sequencing ITS (Internal Transcribed Spacer) specific region is a routine technique to understand the phylogeny of EFs. Nuclear markers have highly conserved sequences and serve as barcode regions for identifying fungal species. Sometimes features have low resolving power, e.g., in some groups of ascomycetes [144,145]. The use of different loci, such as α-TEF (Translation Elongation Factor-1α), the nuclear intergenic region of the B locus (Bloc), and the larger subunits (RPB1 and RPB2) of RNA polymerase II, among others, helps [144–146].

The EF species with the most potential for development as bioinsecticides are those cosmopolitan ones in the environment where the microorganism will be applied [133]. Exotic species of EFs used in biocontrol may be ineffective in some pests due to adaptation to climatic diversity and differences in isolates from the host. Identifying native EFs is a promising alternative, especially concerning ecological suitability with native pest species and the more negligible effect on non-target organisms than exotic isolates [133,138,146].

Bactrocera oleae, *Colletotrichum* spp. Ectosymbionts and Olive Anthracnose in Mediterranean Areas

Olive (*Olea europaea* L., 1753) suffers from abiotic adversities, pest infestations, and bacterial and fungal or virus infections, hosting many non-pathogenic microorganisms [147–149]. The olive fly, *Bactrocera oleae* (Rossi, 1790) (BO; Diptera, Tephritidae; former *Dacus oleae*), is a key pest of olive groves in the Mediterranean basin [150]. This pest thrives where cultivated trees grow extensively and wild trees are indigenous [151]. We presume that agriculture was a significant driver for the expansion of cultivated and wild olive trees as sources of food, wood, and cattle fodder, despite the relationships between cultivated and wild olive trees in the Mediterranean still being determined.

The literature suggests that the interactions of the olive fly with fungal pathogens belonging to the genus *Colletotrichum* can have a significant economic impact on production [152]. *Colletotrichum* spp. are causal agents of Olive Anthracnose (OA). The species complexes *Colletotrichum boninense* Moriwaki, Toy.Sato and Tsukib., 2003 and *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc., 1884 can induce OA and impact orchard production in terms of quality and quantity [153]. *Colletotrichum* spp. are considered the most devastating fungal disease of olive trees, more aggressive in areas or conditions of high relative humidity [152]. Moreover, *Fusarium* spp. and *Alternaria* spp., together with some species of the Botryosphaeriaceae, can participate in drupe rots.

Fungal vectors [154], drivers [155], or spreaders could promote the fungal infection of drupes during ripening. Furthermore, oviposition wounds [156] may facilitate the fungal infection process, as in the case of the olive fly. However, injuries are not essential for infection of *Colletotrichum* spp. [157]. Koronéos [158] confirmed the indirect responsibility of the *B. oleae* in opening the way for the *Camarosporium dalmaticum* (Thüm.) Zachos and Tzav. Klon., 1979 (= *Sphaeropsis dalmatica*) in olives via the oviposition wounds. Koronéos also confirmed that the *C. dalmaticum* and the *Lasioptera berlesiana* (Paoli, 1907) (Diptera, Cecidomyiidae) are almost always present.

Interactions between insects and fungi participate in the ecological context, crop production, and human health [159].

Climate change and global warming expand most countries' olive fly [160] northern limit. However, the Lake Como area remains favourable because of mitigated winter temperatures. Northern Italy and the Apennines stay unfavourable due to winter cold weather dropping below 0 ◦C. Climate change erodes the olive flies' territories in southern regions due to lethally high summer temperatures [160].

Colletotrichum spp. may enter the drupes directly through the epicarp, but the severity of symptom expression and infection rate may increase if the BO injures the drupes [161]. In many European olive-

growing areas, a correlation between the incidence and severity of infections and *B. oleae* infestation is observed, probably due to the action of insect vectors or other spreaders of *Colletotrichum* spp. conidia.

The larval activity of *B. oleae* favours the infective process of *Colletotrichum* spp. and causes early fruit ripening, while the insect contributes to the spread of conidia [161].

Bactrocera oleae is also associated with bacteria [162,163]. Adults and larvae host a non-cultivable bacterium (*Candidatus* Erwinia dacicola), considered an obligate symbiont of the BO [163,164]. However, other bacteria are usually found in the digestive tract of wild olive flies and are probably transient residents ingested with the diet [165,166].

However, despite some direct evidence demonstrating the contribution of bacteria to larval development [167,168], the bacteria's roles in the BO's nutritional ecology still need to be resolved.

In general, interactions between organisms have an impact on their evolutionary history. In eukaryotes, insects and fungi predominate in abundance and species diversity [169]. Well-known cases of associations between insects and fungi occur in different ways, such as in the case of bark and ambrosia beetles [170], ants and termites cultivating fungi [171], or yeasts found in the gut of insects, wood wasps, and gall midges [172]. Spores or mycelia that insects ingest or mechanically carry can reach uninfected plants [173].

In plants, insects are involved in disease development through different types of action. Some of these are as follows. (I) Insects visit plant-infected organs exuding bacteria [174] of fungal conidia which dirty the arthropod bodies that spread them to other plants. (II) Insects can wound fruits, leaves, branches, shoots, stems, and roots, opening pathways for pathogens while feeding or laying eggs [175]. (III) Insects weaken plants by probing on them [176] and make plants more vulnerable to pathogens. (IV) Insects are also true vectors acquiring and transmitting propagules of fungi [177], bacteria [178], viruses [179], phytoplasmas [180], and protozoa [181]. Insect vectors transport pathogens from infected to uncontaminated plants by a well-defined and deterministic chain of events, initiating a new infective process and disease. The insects' active dispersion ability to find the appropriate host increases the effect of pathogens spreading [182]. However, the speed and mode of dispersal of pathogens, and their role in epidemics, depend on the type of contamination mechanism of the insect's body, either external (mechanical vectors) or internal (biological vectors) [183,184].

The role of the weevil *C. sordidus* in the epidemiology of the *Fusarium* sp. wilt of bananas in the field remains uncertain. Meldrum [185] considered the weevil an external spreader, but we need data on the possible presence inside the weevil body. Furthermore, the pathogen's dynamic of acquisition and permanence remains unknown, while it is a crucial factor in the role of *C. sordidus* in pathogen dispersion [186].

Moreover, introducing new pathogens is only necessarily followed by disease emergence if a second factor spreads the pathogen. Some pathogens may remain localised without specific vectors and cause no disease once introduced to new areas.

Many intra- or extracellular ecto- or endosymbionts thrive with insects [187]. The ectosymbiotic microbiome is being studied in the insect model *Bactrocera*/*Colletotrichum* to mitigate indirect damages [188]. Through molecular approaches, such as ITS and 18S rRNA sequencing, the fungal community of the insect is investigated, although their role still needs to be fully understood [188]. For instance, an analysis of the ITS base of the fungal gut microbiota of BO allowed the identification of a core formed by sooty fungi (*Cladosporium* spp.), plant pathogenic fungi (*Colletotrichum* spp.), and other less abundant taxa [189,190].

The Metagenomic data are scarce for the bacteriome, mycobiome, and virome of pests such as *Bactrocera* and beneficial predators [191]. Few data are available on host switching rates and multitrophic interactions involving ectosymbionts.

Moreover, the location in the insect body and the type of transmission of associated microorganisms during the vector life cycle or among individuals in the population requires a model study. We consider ectosymbionts those species regularly associated with the host insect occupying specialised insect structures or modifying the insect cell, tissue, organ, or system. Ectosymbionts could show morphological or behavioural changes needed to interact with the insect. Ectosymbionts of insects require recognising opportunistic, occasional, and symbiotic hosts to proliferate on the external cuticular surface.

Bubici [192] studied the microbiome profile of *Aleurocanthus spiniferus* (Quaintance, 1903) (Hemiptera, Aleyrodidae) on *Ailanthus altissima* (Mill.) Swingle, 1916. *Aleurocanthus spiniferus* has a wide host range, but the shift to a new host, *A. altissima*, could be associated with new endosymbionts. Specific methods based on morphology and molecular approaches will help to identify ectosymbionts, i.e., studying insect-associated microorganisms by light microscopy, SEM, Cryo-SEM, and combined with Next-Generation Sequencing (NGS).

MinION (Oxford Nanopore Sequencing Technology) characterises microbiome samples [193], identifies each microbe, and generates complete and closed genome assemblies, thus elucidating gene expression within microbial communities. Long Nanopore sequencing reads will provide improved genome assemblies, accurate identification of closely related species, and detailed analysis of full-length RNA transcripts from mixed microbial samples. Data will be provided in real-time, allowing immediate access to species identification, abundance, and antimicrobial resistance results.

Advances in mitigating damages and disease incidence would include the study of the pest itself and the characterisation of its ectosymbionts. Interfering with the microorganism's life cycle offers a new sustainable and effective management practices strategy.

Moreover, the study of the gut microbiota of insects is of great interest in medical research and economic exploitation in agricultural production [194]. Therefore, studies on the gut microbiota elucidate the identification of new ways to control crop pests, demonstrating that changes in phylogeny or diet can modulate insect populations and influence host fitness.

The gut microbiota plays a nutritional role in pests, as olive fly larvae depend on their gut microbiome to break down the phenolic compounds in unripe fruits [195].

Evaluating the effects of endo- or ectosymbionts on their hosts and consequently on plants and pathogens by studying insect-microbiota will contribute to a better understanding of insect ecology and explain their success in nature.

Grape Berry Moth (*Lobesia botrana*) Interaction with *Botrytis cinerea* and Ochratoxigenic *Aspergillus* Species

The European Grapevine Moth (EGVM) *Lobesia botrana* (Denis and Shiffermuller, 1775) (Lepidoptera, Tortricidae) is one of the main pests affecting vineyards worldwide, including all the European grape-growing areas [196]. This Lepidoptera is also present in America, where it is a quarantine pest subject to official control [197,198]. EGVM performs 2–4 generations per year on *Vitis vinifera*, depending on latitude, climate, and microclimate. The first generation of *L. botrana* is anthophagous, and developing on floral clusters is of lesser economic concern [199]. In contrast, the subsequent carpophagous generations thrive on berries during the early ripening process. The direct damage of the carpophagous larvae is caused by larval feeding on the unripe grape berries, resulting in a loss of grape weight and an unmarketable crop. However, the most significant damage originates from fungal and bacterial infections that drastically reduce the quality [196–200]. Penetration holes on ripe grape berries by third generation *Lobesia botrana* larvae promote the occurrence of several fungal and bacterial rots: we concentrate on fungal rots. Fungal rots can originate from infections by *Alternaria* spp., *Cladosporium* spp., *Penicillium* spp., *Rhizopus* spp., and grey mould caused by *Botrytis cinerea* Persoon, 1801 and *Aspergillus* black rot caused by black aspergilli species of Section *Nigri* [201–203]. The association of EGVM with grape grey mould led to one of the most important grape syndromes in the world and caused severe losses in table grape yields, provoking bad flavours and ruining bouquets in wine. The interaction of the *L. botrana* larvae with the *Aspergillus* black rot on clusters is also considered the primary source of ochratoxin A (OTA) in grapes [204,205]. OTA is the only mycotoxin for which a maximum regulatory level has been established in wine [206], making *Aspergillus* highly detrimental to viticulture.

On grape bunches, the larvae of *L. botrana* often associate with grey mould. Caterpillars can contribute to spore dispersal or act as spore drivers by trapping conidia in the cuticle ornamentation and faeces [201,202]. In addition, larvae-feeding wounds on grape berries promote the rapid colonisation of *B. cinerea*. For these reasons, larval activity spreads the grey mould under field conditions [207,208]. Research has shown that the presence of grey mould on grape berries increases the aggressivity and fitness of larvae and females [209–211]. According to Mondy et al. [209,211], *B. cinerea* is attractive to adults and first-instar larvae. The mould promotes the EGVM populations by increasing the survival and fecundity of larvae and reducing their development time. However, other authors have not observed any of these positive effects of grey mould on *L. botrana* populations [212–214].

Lobesia botrana is a significant risk factor for OTA under field conditions in a vineyard [215–217]. OTA is a secondary fungal metabolite, such as alternariol, alternariol monomethyl ether and tenuazonic acid [218,219]. OTA is nephrotoxic and hepatotoxic, in addition to other toxic properties. It is classified as potentially carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer [220]. *Aspergillus* bunch rot is a fungal disease that affects pre-harvest grapes. A complex of *Aspergillus* species in section *Nigri*, including *A. carbonarius*, *A. niger* and *A. tubingensis* [221,222], cause the bunch rot. The importance of these black aspergilli rose when OTA was found as a contaminant in grapes and grape-derived products [223,224]. These ochratoxigenic fungi are opportunists (saprophytes) that cause effects that are not always visible and commonly linked to limited yield losses [225]. Although they are always present in the field, they may develop massively on berries damaged by abiotic and/or biotic causes, from veraison to harvest, with a high incidence at ripening. This contamination is strongly related to climatic conditions, geographical regions (the southern Mediterranean climate is very favourable), vines cultivars and damaging pests [217]. Therefore, significant variations may occur from one year to the next. Where *L. botrana* completes three generations per year, and climatic conditions favour the infestation of EGVM larvae from the early veraison to the ripening stage, the control of the third generation is a crucial factor in reducing bunch damage. It also reduces rot at harvest [205–208].

Applying an effective, economical, and eco-friendly technique to control these agents simultaneously is impossible—most of the control strategies used so far rely on chemicals. However, the use of pesticides is increasingly discouraged due to the environmental pollution problems associated with high application rates. Pesticides could reduce biodiversity, the potential loss of key species such as bees and biological control agents, and even the generation of resistance in some invertebrate pest species [226]. Entomopathogenic fungi could represent an alternative solution for controlling these agents. These organisms are crucial natural control agents that limit insect populations in many natural and artificial ecosystems [227]. Many entomopathogenic fungi infect eggs, immature instars, and adults of many insect

species [228]. Studies have evaluated the efficacy of entomopathogenic fungi strains of genera *Beauveria* and *Metarhizium* on *L. botrana* [205,229,230] and the antagonistic effect of *Metarhizium anisopliae* on *B. cinerea* [230].

Further surveys will find new entomopathogenic fungi candidates for use in the biological control of *L. botrana*. New candidates should also consider their antagonistic activity towards *B. cinerea* and black aspergilli. Moreover, their compatibility with fungicides commonly used for grapevine diseases should be assessed.

PATHOGENS SPREAD BY POLYPHAGOUS VECTORS: *XYLELLA FASTIDIOSA*

Xylella fastidiosa (XF) has been known for many years in North America, where it was first isolated from grapevines affected by Pierce's disease [231]. It is the cause of numerous high-impact diseases and can infect over 550 plant species (80 families), although most remain asymptomatic [232]. *Xylella fastidiosa* is a quarantine pathogen registered in the EPPO A2 list. Its dangerousness is due to the enormous economic and landscape damage it can cause to an area. A strain of this bacterium, the one that causes the citrus disease defined as Citrus Variegated Chlorosis (CVC), is even included in the list of biological agents regulated by the US Agricultural Bioterrorism Protection Act of 2002 [233].

Xylella fastidiosa's first European issue was in 2013. Olive trees showing severe symptoms of desiccation [234] appeared in the province of Lecce (Italy). After the first European report, XF was also found in other EU and non-EU countries [235]. *Xylella fastidiosa* has three subspecies, each with a proper host range. The three main subspecies are subsp. *fastidiosa*, subsp. *multiplex* and subsp. *pauca* [236].

The transmission of the pathogen occurs with the help of xylem sap feeders. Insect vectors of XF belong to the suborder Cicadomorpha, and ca. 50 species have been identified worldwide [28]. The insect species transmitting XF are polyphagous on herbaceous and arboreal plants. They spend the juvenile instars feeding on herbaceous host plants. When they become adults, they also move to bushes or tree hosts [237,238].

Adult vectors that feed on xylem sap from an infected plant acquire the bacterium. Subsequently, the bacterial cells multiply, forming microfilm in the foregut vector lumina [182,239,240]. A non-circulative interaction exists [26] between the bacterium and the adult, where the XF behaves like a non-mutualistic ectosymbiont [182]. *Xylella* is peculiar among plant pathogens because there is no latency period after the acquisition. It has a propagative behaviour and a persistent presence in the adult foregut [241]. In the interaction between the bacterium and the vector, XF is the only one to benefit from. How vectors benefit from the association with *Xylella* is not clear, and the presence of bacterial cells in the precibarium changes feeding behaviours [242].

Xylella fastidiosa has many vectors, but some are more important because they are more widespread and efficient in their context. For the United States, *Graphocephala atropunctata* (Signoret, 1854) (Hemiptera, Cicadellidae) is the primary vector in the coastal areas of California, known mainly for spreading Pierce's disease [243]. *Draeculacephala minerva* Ball, 1927 (Hemiptera, Cicadellidae) is known for the spread of Almond Leaf Scorch (ALS) disease in central California [244]. In North America, the main *Xylella*-vector is *Homalodisca vitripennis* (Germar, 1821; Hemiptera, Cicadellidae) [245]. *Homalodisca vitripennis* is native to the southern USA and northern Mexico and has spread throughout the Americas through the displacement of plant material hosting its eggs [246]. Despite its low transmission efficiency, its extreme polyphagia and ability to travel great distances make it one of the most critical and dangerous vectors of *X. fastidiosa* [247]. *Homalodisca vitripennis* is also present in Oceania. *Homalodisca vitripennis* has yet to be detected in Europe and is included in the EPPO A1 list [248]. In Brazilian *Citrus* spp. groves, the main *Xylella*-vectors are *Aonidiella citrina* (Coquillett, 1891) (Hemiptera, Diaspididae), *Dilobopterus costalimai* Young Jr., 1977, and *Oncometopia facialis* (Signoret, 1854; Hemiptera, Cicadellidae) [249]. The primary vector in Europe is *P. spumarius*, a ubiquitous insect that effectively transmits *Xylella*, capable of rapidly spreading the bacterium [250]. It is considered the main cause of Salento's Olive Quick Decline Syndrome (OQDS) impact [233].

In the past, researchers thought that transmission of XF occurred without any specificity between vector and pathogen. However, recent studies demonstrate the implication of cell-to-cell signals in XF to colonise insect vectors' foregut [251,252]. Through the rpfF gene, XF regulates the production of small signal molecules called DSF (Diffusible Signal Factor), which depend on cell density [242]. When these molecules produced by individual bacteria accumulate in an environment, they cause a change in rpfF gene expression, stopping DSF production. When DSF production is blocked, the bacterium cannot effectively colonise the precibarium [242].

Bacterial adhesins and foregut surface carbohydrates play a role in vector-pathogen interactions because affinities depend on the polysaccharides. For example, N-acetylglucosamine inhibits cell adhesion to the chitin substrate [253]. Molecules influence the initial attachment of bacterial cells on the surface of the vector's foregut. The hemagglutinin-like proteins are decisive for XF adhesion to vector foregut polysaccharides [253]. In addition to haemagglutinins, XF produces other fimbrial and non-fimbrial adhesins. HXFA and HXFB appear essential for the first adhesion and colonisation of the foregut. FimA is involved in adhesion and aggregation [254].

Therefore, epidemics of XF are often very complex phenomena governed by many factors, such as the host plant species and vectors present and their context. Given the threatening nature of XF, in countries at risk of introduction, it is necessary to implement controls on imports of plant material, continuous monitoring, and in-depth studies on the presence of xylem-feeding leafhoppers, known as *Xylella*-vectors.

Studying the relationships between *Xylella* and its vectors and how to use this knowledge to develop plant protection products or epidemic management techniques is also necessary.

Furthermore, severe epidemics caused by pathogens such as XF have a considerable social impact. Therefore, XF requires constant education about quarantining plant pathogens and their impact on the territory. In the absence of proper political-scientific communication, binding control actions aimed at eradication or containment of the pathogen could be slowed down by the opposition of citizens and farmers, thus favouring the progress of the epidemic [233].

Detection of *Xylella fastidiosa* subsp. *pauca* from the Insect Vectors

Attempting to prevent the further spread of *X. fastidiosa* subsp. *pauca* ST53 in Apulia, the NPPO provides surveys in the "containment" and "buffer" areas, allowing olive tree sampling by appropriate spoiling techniques on the symptomatic olive foliage. Furthermore, Real-Time PCR with specific primers and following the EPPO procedures for detecting *X. fastidiosa* [255] allowed the detection of new infection foci. However, this method reveals the presence of the pathogen at a time that does not reflect the primary inoculation carried out by the insect vector through its feeding activity on the olive. Indeed, the inoculation of the pathogen could have occurred several months before the inspector collected the samples. Afterwards, one or more infected vectors may have reached other olive trees a few meters away, transmitting XF. This risk seems to increase if the sampling of olive leaves occurs in the year following the insect's feeding activity [256]. Therefore, this type of survey does not allow XF to be intercepted directly from the insect body, leading to an underestimation of the precise limit of the infection, allowing the bacterium to spread further in the territory. To reduce data uncertainty and better track the bacterium spreading in olive orchards, we suggest including *P. spumarius* adults sampling in olive groves from natural environments.

Scrutiny for the presence of the pathogen by the same EPPO procedures should run during spring (i.e., from the end of April) and autumn (i.e., September and October).

Symbionts of Red Palm Weevil

Studies on insect-microorganism iteration are steadily increasing, especially those focusing on the role of bacteria (as obligate or facultative symbionts) in the life cycle of their hosts [257–260]. Indeed, bacteria symbionts provide essential nutrients, degradation of food material, defence against natural enemies, and increase insects' fitness [261–263]. Obligate ectosymbionts are stably associated with the insect host, typically localised in specialised host organs. The olive fly ectosymbiont *Candidatus* Erwinia dacicola is a good example [264]. Facultative symbionts do not require a host for survival, may be temporarily

associated with the host, are generally horizontally transferred, or acquired from the environment, and may inhabit different organs of the insect (e.g., salivary glands, reproductive organs, etc.) or insect surfaces and play different roles in the insect's cell cycle. Pseudo-vertical transmission (vertical and horizontal symbionts acquisition) exists for facultative symbionts [260]. Some facultative symbionts are ectosymbionts like *Burkholderia* in the beetle *Lagria villosa* (Fabricius, 1781) (Coleoptera, Tenebrionidae) [265]. The growing scientific interest in the symbiont-insect relationship wishes to extend knowledge on insect biology further and identify new candidates and/or strategies for biological control of pests.

In this respect, the Red Palm Weevil (*R. ferrugineus*—RPW) has attracted increasing scientific interest in recent decades due to its devastating worldwide invasion of palms, resulting in severe economic issues [266]. Studies on the interaction between RPW and microorganisms mainly follow two research targets. The first searches for natural enemies for RPW biological control, and the second identifies ectosymbionts to disrupt their role in the RPW life cycle and insect-plant interaction [267–270]. Biological control emerges as an alternative to conventional management based mainly on chemicals, which entails severe concerns for human health, environmental pollution, and selecting resistant insects.

The natural enemies of RPW, here restricted to microorganisms, are bacteria, fungi, and viruses. Only one virus, *Cytoplasmic Polyhedrosis Virus* (CPV), is infectious in all stages of RPW [271]. However, data on CPV for biocontrol of RPW are limited to laboratory tests. As for fungi, *B. bassiana* and *M. anisopliae* are the two entomopathogenic fungi mainly studied for biocontrol of RPW [272]. *B. bassiana* can infect RPW eggs, larvae, and adults and be transmissible among adults. We expect that *B. bassiana* significantly affects the RPW population in infested palms, reducing the number of adults and their reproductive efficiency. Still, Besse [273] suggests that commercial oil formulation of *B. bassiana* has moderate results in the field. *Metarhizium anisopliae* is also a promising biocontrol agent for RPW [272]. Laboratory data confirmed the efficacy as high mortality *M. anisopliae*-treated RPW larvae and adults. A recent oil-in-glycerol formulation of *M. anisopliae* proposes a possible field application. The formulation is stable over time and can prolong conidial shelf-life compared to unformulated conidia [274]. Potential pathogenic bacteria for RPW mainly belong to the Gram-positive *Bacillus* sp., Gram-negative *Serratia* sp., and *Pseudomonas aeruginosa* [261]. The available data only concern studies conducted under laboratory conditions; *Bacillus thuringiensis*, *Bacillus sphaericus*, *Serratia marcescens*, and *P. aeruginosa* are the primary bacteria employed in bioassays and proposed as candidates for the biological control of RPW. Although promising, the use of bacteria for biocontrol is still debated, particularly about deployment strategies and human health concerns, as some of the proposed bacteria (e.g., *S. marcescens* and *P. aeruginosa*) have been responsible for human infections [275,276].

RPW-associated bacteria belong to different Taxa depending on geographic areas, palm species, and collection from larvae, pupae, adults, gut, or reproductive apparatus [23,277]. The widely identified Phyla are Proteobacteria, Bacteroides, and Firmicutes. The role played by RPW-associated bacteria is a topic of interest, which could yield valuable data for implementing new control strategies for RPW management. Considering *S. marcescens*, a facultative ectosymbiont of RPW, leads to understanding the role of bacterial symbiosis with the weevil [23]. The *S. marcescens* strains associated with RPW produce or not the red pigment prodigiosin and were regularly released during oviposition by females, as demonstrated through in vivo experiments with apples provided as the substrate for oviposition. The same Red-Pigment-Producing *S. marcescens* (RPPS) strains also exist in the reproductive apparatus and gut of dissected adult and virgin RPW and on the internal surface of pupal cases collected from infested palms. Strains of RPPS widely spread along the tissues of infested palms. Extensive studies have reported the antimicrobial activity of prodigiosin [278,279]. This finding is consistent with the antibacterial activity shown by RPPS strains collected from RPW vs both Gram-positive (*Bacillus* sp., *Paenibacillus* sp., *Lysinibavillus* sp. and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae*). In addition to the presence of a red pigment, the study conducted by Scrascia and colleagues [144] showed production from *S. marcescens* symbionts of a possible additional molecule (yet to be identified and characterised) with antibacterial properties. It is noteworthy that *Serratia* from RPW exhibits its antibacterial activity vs. *Bacillus* sp., which is a potential agent for the RPW biocontrol. Some strains of *S. marcescens* are known to produce volatile organic compounds with cytotoxic inhibitory activity against pathogenic bacteria, fungi, and nematodes [280].

Moreover, genetic information about encoding enzymes for plant polymer degradation exists in the genome of *S. marcescens* [281]. Thus, more than a pathogen of RPW, *S. marcescens* could play a role in the RPW life cycle, ranging from protection against natural enemies (due to its antibacterial properties) to metabolic abilities that would influence the interaction between insects and plants. Indeed, both cellulolytic activity and fermentative metabolism (extensively reported for *S. marcescens*) would allow the spread of RPW larvae within the palm tissues and explain the temperature increase detected within infested palms, favouring the insect's development [282,283].

APHROPHORIDAE FROTH NICHE

The juvenile instars of the superfamily Cercopoidea, known as froghoppers or spittlebugs, live in a surrounding liquid frothy "pond". Juveniles inject air bubbles into the submerging self-produced fluid. The references [284–288] suggest that froth originates from abdominal gland ducts and liquid faeces.

Tonelli [289] analysed the microbiological composition of the bacteria inhabiting the foam collected from nymphs of *Mahanarva fimbriolata* (Stål, 1854) (Hemiptera, Cercopidae). The molecular analysis of microbial community structures generated OTUs (Operational Taxonomic Units) and found three of the most representative 257 OTUs (relative abundance > 2%) to belong to Alphaproteobacteria (29.9%), Actinobacteria (14.0%), and Chloroacidobacteria (6.3%). Tonelli [289] also replicated the extraction of nucleic acids from the nymph gut and the underlying soil (291 and 288 OTUs). The comparison of the community structures obtained from the three environments showed that the OTUs' composition of the froth has more in common with the insect's gut (48 OTUs) than with the underlying soil (24 OTUs). The results exclude that the microbes in the froth originate from the soil. However, they point to gut communities as the primary source of microorganisms.

In the last decade, another family of Cercopoidea has come under the spotlight of the scientific community. Few Aphrophoridae play a primary role in transmitting xylem-inhabiting pathogens. *Xylella fastidiosa* subsp. *pauca* ST53 is the causal agent of OQDS in Italy and entered through an infected host plant or its adult vector [290]. Aphrophoridae species, notably *P. spumarius*, have become the key pests of Mediterranean olive trees [182].

The microbial community of the Juvenile Aphrophoridae Froth (JAF) and its symbiotic benefits remain unknown. Our results rise from the systematic sampling and inoculation in the Petri dishes of field-collected *P. spumarius* froth in sterile test tubes in 2021–2022. We plated the foam in Petri dishes on nutrient agar medium (Thermo Fisher Scientific, Waltham, MA, USA) by soaking sterile stabs.

Observation of the plates enabled the morphological recognition of three repeated colour patterns: violet-yellow, ochre-yellow, and straw-yellow (Figure 3). Firstly, Next-Generation Sequencing (NGS) identified the isolates belonging to these patterns; the sequences obtained made it possible to determine by database comparison (NANOPORE–WIMP) the presence of the genera *Microbacterium* (Actinobacteria, Actinomycetia), *Pseudomonas* (Proteobacteria, Gammaproteobacteria) and *Agrobacterium* (Proteobacteria, Alphaproteobacteria). The other isolates' complete identification could enrich this experimental result.

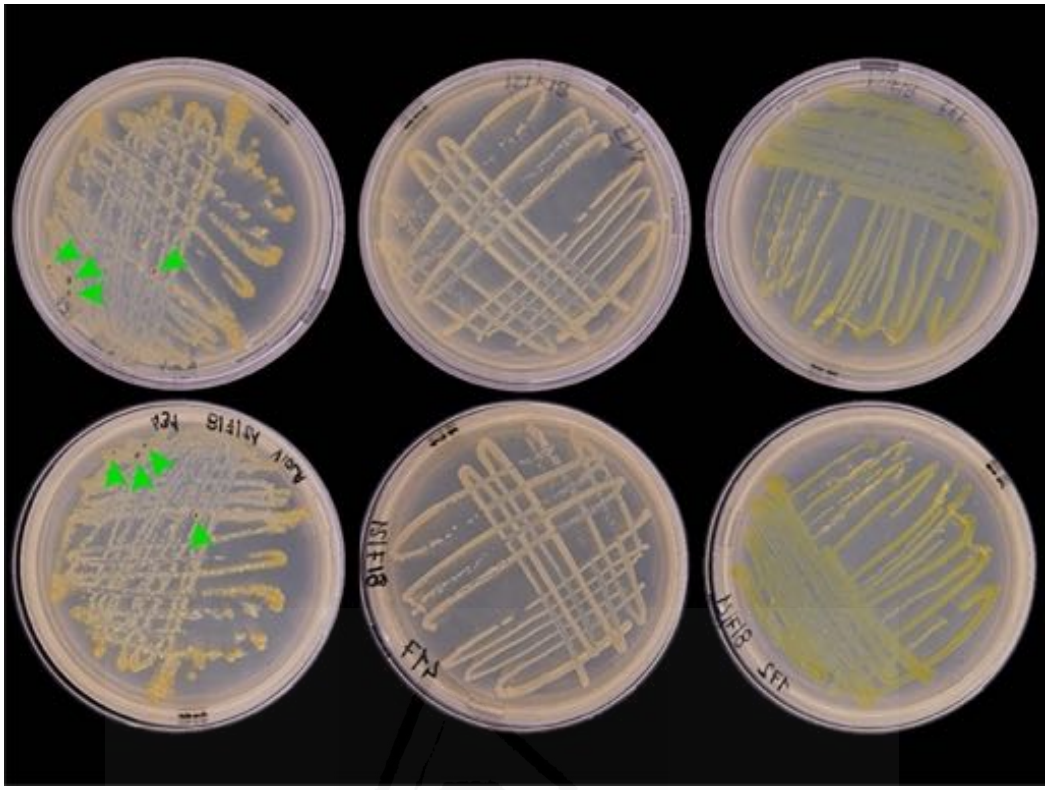

Figure 3: Bacterial isolates in Petri dishes from JAF, identified as genera (from left to right): *Pseudomonas*, *Agrobacterium*, *Microbacterium*; upper and lower Petri sides; green arrows indicate purple spots of the genus *Pseudomonas* (source: own photo).

The froth mass forms a barrier to the diffusion of atmospheric O_2 through the foam. The gaseous exchanges of juveniles occur by extending the abdomen outside the spittle mass. The insect then retracts the tip of its abdomen into the foam mass, producing new air bubbles in which the internal O_2 pressure is lower than atmospheric [291]. This aspect makes the froth environment even more restrictive to colonise.

It was necessary to recreate such growth conditions to validate the other microbial characteristics required to survive or grow in the froth environment. Oxoid™ AnaeroJar™ (Thermo Fisher Scientific, Waltham, USA) allowed the plate inoculation of JAF under controlled anaerobic and microaerobic conditions. The varying oxygen availability unveiled additional bacterial isolates currently being identified (Figure 4). We do not exclude the possibility that such organisms are the same as those isolable at regular oxygen rate but with the option of secondary micro-aerobiosis/anaerobiosis.

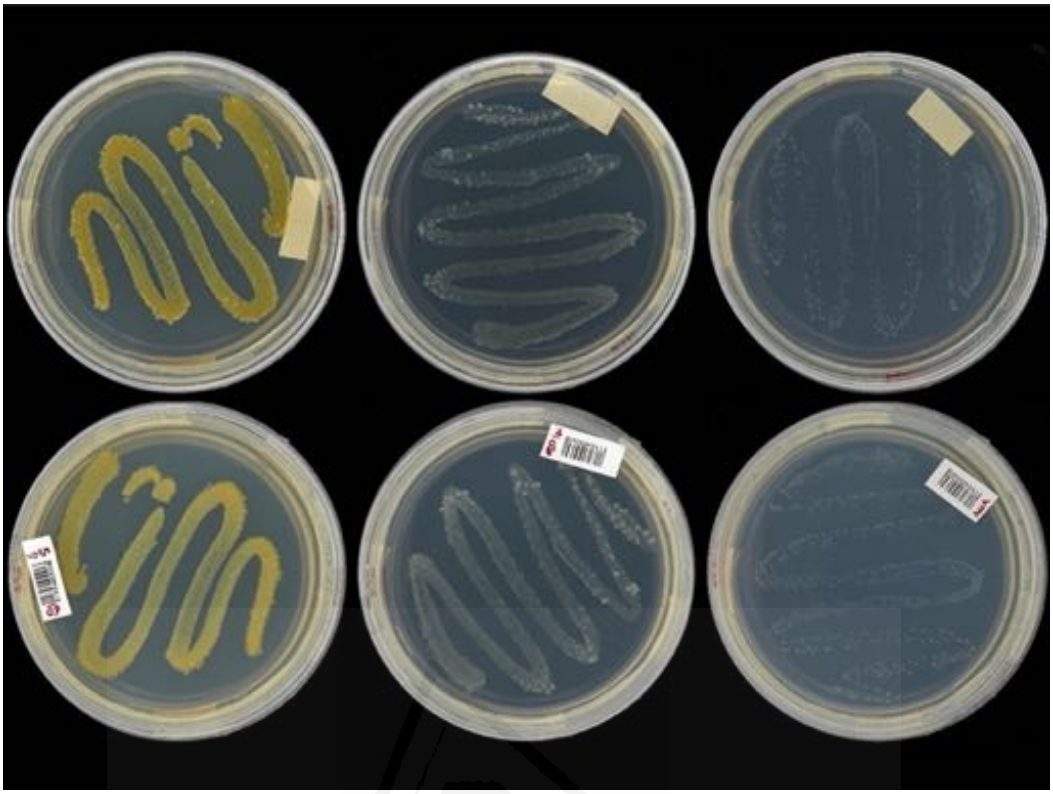

Figure 4: Bacterial isolates obtained by inoculation of unique JAF on Nutrient Agar incubated under uncontrolled oxygen (Left), microaerophilic (Middle), and anaerobic (right) conditions, respectively, using Oxoid™ AnaeroJar™; upper and lower face (source: own photo)

Spittlebugs nymphs inflate air bubbles in a self-secreted and egested liquid containing 99.30–99.75% water and Malpighian protein molecules [292]. The presence of these bacterial genera in the spittlebugs' froth implies the ability of microbes to utilise the substances in the foam.

Physiochemical functions have already justified the presence of proteins within the Cercopoidea froth matrix. Adequate water surface tension to maintain the froth's structure is allowed by the presence of the proteins in the foam of *Callitettix versicolor* (Fabricius, 1794) (Hemiptera, Cercopidae) [293–295]. However, microorganisms living in peculiar environments such as JAF could exploit such proteins.

Microbacteriaceae (*Microbacterium* spp.) are widespread bacteria. They have demonstrated a marked ability to utilise a wide range of substrates, and isolations have been reported from various matrices: air, water, soil, milk, phyllosphere, and insect gut [296].

Pseudomonas spp. and *Agrobacterium* spp. are two ubiquitous genera, often found in soil and phyllosphere. Contact contamination with these substrates can explain their presence in the JAF. However, a relationship with the insect is not excluded, as in the reported cases of a close host-crop relationship [297,298].

Defining a constancy in the insect class concerning symbiosis with gut microorganisms is difficult. In Hemiptera, it is possible to find cases in which some sap-feeders have little or no gut microbiota but depend on intracellular symbionts for specific nutrients [10]. The primary role of gut bacteria may supply functional components or participate in the digestion and detoxification of host harmful substances [299].

The excretory organs of Insects are the Malpighian tubules that extend into the hemocoel absorbing wastes, such as uric acid, pouring them into the hindgut for disposal. The hindgut manages a combination of nitrogenous and food waste, creating a proper environment for hindgut bacteria, leading to sorting differently from the foregut [300].

Therefore, the foam produced by Aphrophoridae still needs to be explored and defined on a microbial scale. Its chemical composition and the rarefied oxygen make it a suitable bacterial microhabitat. Such an ecological niche may host only well-adapted organisms that could have close relationships between microbes and the host insect. Finally, we cannot exclude that this ectosymbiosis may have evolved into a mutual benefit for both (e.g., protective antimicrobial synthesis) [301].

AUREOBASIDIUM SPP.: MULTITASKING BENEFICIAL MICROORGANISM

The yeast-like fungal genus *Aureobasidium* is naturally widespread in the carposphere and phyllosphere of fruits and vegetables. *Aureobasidium* species possess different enzymatic patterns closely related to biotechnological and agricultural uses [302]. These features favour their culture and employment as biocontrol agents against pathogenic fungi and pests [303].

Fungi, especially *Aspergillus* spp., *Cladosporium* spp., *Penicillium* spp., *Rhizopus* spp., and *Aureobasidium* spp., contribute to pollen composition. These fungi use extracellular enzymes to convert proteins, carbohydrates, and fats into antibiotics, organic acids, and other metabolites [304]. Within the *Aureobasidium* genus, several fast-growth, dimorphic fungi have poly-extremotolerant properties and produce mainly yeast-like cells involved in melanin wall deposits and are therefore called “black yeasts” [303]. The most widespread species is *A. melanogenum*, followed by *A. pullulans*. *Aureobasidium* spp. can synthesise different enzyme patterns, depending on species and strains [303]. *Aureobasidium melanogenum* produces pullulan, which is involved in resistance to environmental stresses such as UV radiation, high salt concentration, desiccation, strong oxidation, and heat. The strain TN3–1 is a large producer of pullulan and was isolated from natural glucose-rich honey. Furthermore, this strain disclosed high osmotic tolerance due to small vacuoles, trehalose, and glycerol in its cells [305]. From xylose, glucose, and sucrose, *A. melanogenum* biosynthesises liamocin, related to the release of Massoia lactone [303], the last compound being effective against *Fusarium* head-blight [303,306] and with larvicidal action against *Aedes aegypti* L., 1762 (Diptera, Culicidae) [307].

These metabolites can implement sustainable control means, counteracting the pollinator decline due to the depletion of plant biodiversity. Indeed, plant biodiversity depletion significantly reduces pollinators' nesting habitat and food resource availability [308]. Additionally, these means help prevent resistance to pesticides, such as insecticides, herbicides, and fungicides, that link closely to the target-specificity efficacy of these compounds [309,310].

Among *A. melanogenum*, strain CK-CsC is isolated from honeybee bread and produces cellulase, lipase, amylase, polygalacturonase, xylanase, proteinase, transferases, and mannanase, well-known as food additives [311]. Using this strain as a potential probiotic in the diet of honeybees (*Apis mellifera* L., 1758; Hymenoptera, Apidae) would improve the diet's nutritional properties and honeybees' health [311]. In addition, *A. pullulans* displays antifungal activity that controls some fungal pathogens (*Rhizoctonia solani*, *Monilinia* spp., *Neofusicoccum parvum*, and *B. cinerea*) and promotes plant growth.

Aureobasidium pullulans effectiveness is related to non-volatile and volatile organic compounds (VOCs), such as pullulan, degrading enzymes, siderophores, and aureobasidins. However, these VOCs are mainly responsible for the antifungal properties of *A. pullulans*. The formation of pullulan biofilm prevents fungal attachment and colonisation [312]. The antifungal activity of *A. pullulans* was also evaluated on strawberries to control root and crown rot and grey mould caused by *Phytophthora cactorum* and *Botrytis cinerea*, respectively. *Botrytis cinerea* infects strawberry flowers and remains latent until optimal environmental conditions and fruit ripening occur. Therefore, during the blooming stage, applications of chemical fungicide solutions [313] or biocontrol agent (BCA) suspensions are required, but an adequate covering of the flowers is not guaranteed [314]. Then, the dispersal of BCA by pollinating insects could help reach flower cavities without water use [315]. In this 2-year research, bumblebees (*Bombus pratorum* L., 1761; Hymenoptera, Apidae) used as a carrier were tested in the field, comparing two different dispersion devices, and demonstrating their similar dispersal efficacy. The cells of *A. pullulans* spread by the bumblebees during the blooming ranged between 103 and 105 cells per blossom. The efficiency in controlling grey mould ranged between 60–80% [315]. Similar results were obtained by Iqbal [310], evaluating the congeneric species *Bombus terrestris* (L., 1758) (Hymenoptera, Apidae) as a carrier of the same BCA. Entomovectoring methods for dispersing BCA may ensure high precision in reaching flowers at the right time, reduce treatment costs by saving water, and reduce the amount of product needed by 80 to 90%. In addition, dry application avoids moisture that could promote fungal infections [310]. Generally, bumblebees are better entomovectors than honeybees at low temperatures due to their higher metabolism in adverse thermal conditions. During the flight, the weight resistance of bumblebees with and without BCA loading was evaluated, which showed no significant differences and validated their loading capacity [310].

Finding new species of *Aureobasidium* spp. and improved culture conditions would make it possible to improve the extraction of natural metabolites with further biotechnological applications. This yeast-like fungus and/or related substances would be facilitated by its easy growth in a bioreactor with a liquid medium, avoiding the problems caused by low oxygen supply and concerns due to complex handling typical of filamentous fungi. *Aureobasidium* spp. derivatives are promising candidates to replace chemical pesticides to safeguard the environment and human and animal health. Furthermore, this eco-friendly One Health approach could encourage organic farming to improve the pollinating insect life cycles and promote biodiversity. Finally, bumblebees and entomovectoring methods could be the new frontier in the dispersion of biocontrol agents, with economic benefits.

CONCLUSIONS

Insects and microorganisms have a long history of interactions, and studying these phenomena provides a multifactorial view to deepen and complement the knowledge already acquired about the actors involved. These interactions act as an evolutionary engine that also makes it possible to overcome environmental stresses such as climate change. Indeed, developing strategies to safeguard the most vulnerable insect species could include the use of symbionts that can enhance their fitness towards a resilient or antifragile ecological response. The insects' reply consists of hosting microorganisms in their body as endosymbionts (ENS), living in the host's tissues and often infecting both the insect and the plant, or as ectosymbionts (ECS) living out of the body wall and often out of the cuticle. For example, selected strains of *Aureobasidium* produce probiotics that can improve the health of some pollinators by increasing their fitness to cope with biotic and abiotic stresses in agroecosystems.

Modifications induced by certain microorganisms can improve the environmental conditions favourable to the post-embryonic development of insects, as could be the case with the bacteria inhabiting the Aphrophoridae froth. Furthermore, they may establish antagonistic symbioses between the host plant and the pests, improving their fitness, as *S. marcescens* does with *R. ferrugineus*.

The microbial composition of juvenile aphrophorid foams could play an active role in the survival of naiades to abiotic or biotic agents. Their metabolisms could actively modify surface tension or add antibiotic or repellent effects, the latter case already documented by the topical irritability of palmitic and stearic acids found in the foam.

The JAF represents a hypoxic environment; rarefied oxygen selects microorganisms characterized by microaerophilia or anaerophilia. The scarcity of oxygen also joins dense glandular secretions from Batelli glands and nutrient scarcity because of the xylem sap origin of faeces and insect first food exploitation.

The set of microorganisms specialised to live in such an environment, and the interactions between them and the host constitute a micro-niche with its inputs and outputs of energy and matter. Such conditions form annually and persist for a limited time (equal to the post-embryonic development of its host). The origins and ways such microorganisms survive in the absence of JAF and reoccur in the following year remain unknown.

Some plant pathogens can exploit insects to spread in the environment to generate new infectious events. Phytopathogenic endosymbionts such as *X. fastidiosa* only carry out their contagious process in the presence of efficient insect vectors capable of acquiring and bearing them from plant to plant. Other plant pathogens can exploit the dissemination by insects such as ectosymbionts, e.g., *B. oleae* and *Colletotrichum* spp. or *L. botrana* and fungal rots agents.

The microbial communities that compose the microbiome of some insects can improve the fitness and adaptability of some pests, allowing them to do the host shift, e.g., *A. spiniferus* with *A. altissima*.

In addition, some microorganisms can interact negatively with the fitness of the host insect by being used as biocontrol agents. Entomopathogenic microorganisms (fungi, bacteria, or viruses) can reduce the incidence of certain pests (*R. ferrugineus*, *C. sordidus*, *R. palmarum*, *etc.*), reducing agricultural losses and allowing an eco-friendly approach.

The relevant detail consists in the places the microorganisms use to thrive with the insect. Despite the attention to ENS, living in the host's tissues and often infecting both the insect and the plant, a considerable amount of information suggests that ECS may be a more exploitable guild of guests for the sake of damage management. On the opposite side of the story, advantages may exist in exploring new associations among chosen ectosymbiotic bacteria and insects reared for food or feed, exalting the fitness of the final insect consumer.

Two models of control action depend on the insect-microorganism interaction. ENS are well-specialized multi-host restricted actors. Still, ECS shows many interactions, ranging from the simple microorganism driven by contamination to many morphologically complicated stories, i.e., *Candidatus* Erwinia dacicola or phoronts cascades as in *Rhynchophorus* spp. The RPW reminds the unexpected positive effects of its damage prevention offered by thiophanate-methyl were explained by observing that females contaminate the eggs at laying with a blend of yeast living in the lumina of female genitalia. Remarkably, females also release *Serratia* spp. and Nematoda at the same time.

The case suggests exploring other pest bionomics for similar associations because ECS management and replacement can easily disrupt the proper pest association, lowering the target insect fitness or uncoupling the pest from sensitive habitats.

Entomovectoring for disrupting pest-favourable natural ectosymbiotic interrelationships appears much more feasible, effective, and less impacting on wide-area context than formulates. Furthermore, disruption will act more easily *ex-ante* the pest damage supporting the antifragile intent for a gentle, non-invasive influx on artificial habitats.

In addition, certain VOCs produced by pathogenic invertebrate microorganisms (entomopathogenic or nematophagous fungi) can be used as repellents to manage high economic interest pests (*R. ferrugineus* and *C. sordidus*), generating a 'green' biotechnological means of pest control, and reducing food losses.

Therefore, the ectosymbiotic interactions between microorganisms and insects impact the evolutionary history of the actors involved [316]. Interactions can be a tool for the service of humankind to improve its impact on the environment. We must remember that generalist insect-driven microorganisms, much more than specialised insect-transmitted ones, cause the bulk of the damage to humankind. A better understanding of these interaction mechanisms is now possible by basic techniques of microorganism extraction from insect compartments and specialised structures coupled with rapid NGS sequencing of the whole available genome from the collecting site on the insect. Thus, combining techniques and re-discovering effective approaches to observations would enable scientific and technological progress to benefit our common home, the Earth.

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Chapter 3 – Brindley's Glands Volatilome of the Predator *Zelus renardii* Interacting with *Xylella* Vectors

Abstract

Alien species must adapt to new biogeographical regions to acclimatise and survive. We consider a species to have become invasive if it establishes negative interactions after acclimatisation. *Xylella fastidiosa* Wells, Raju et al., 1986 (XF) represents Italy's and Europe's most recent biological invasion. In Apulia (southern Italy), the XF-encountered *Philaenus spumarius* L. 1758 (Spittlebugs, Hemiptera: Auchenorrhyncha) can acquire and transmit the bacterium to *Olea europaea* L., 1753. The management of XF invasion involves various transmission control means, including inundative biological control using *Zelus renardii* (ZR) Kolenati, 1856 (Hemiptera: Reduviidae). ZR is an alien stenophagous predator of *Xylella* vectors, recently entered from the Nearctic and acclimated in Europe. *Zelus* spp. can secrete semiochemicals during interactions with conspecifics and prey, including volatile organic compounds (VOCs) that elicit conspecific defence behavioural responses. Our study describes ZR Brindley's glands, present in males and females of ZR, which can produce semiochemicals, eliciting conspecific behavioural responses. We scrutinised ZR secretion alone or interacting with *P. spumarius*. The ZR volatilome includes 2-methyl-propanoic acid, 2-methyl-butanoic acid, and 3-methyl-1-butanol, which are consistent for *Z. renardii* alone. Olfactometric tests show that these three VOCs, individually tested, generate an avoidance (alarm) response in *Z. renardii*. 3-Methyl-1-butanol elicited the highest significant repellence, followed by 2-methyl-butanoic and 2-methyl-propanoic acids. The concentrations of the VOCs of ZR decrease during the interaction with *P. spumarius*. We discuss the potential effects of VOC secretions on the interaction of *Z. renardii* with *P. spumarius*.

Introduction

Current global trade and the movement of plant material increase the introduction of alien species [1]. These species must overcome biotic and abiotic stresses to survive, colonise, and reproduce [2]. In this scenario, native species meet allochthonous ones, generating new interactions. These interactions can lead to mutualistic interplays (positive interactions) or biological invasions (negative interactions) [3]. Negative interactions can turn alien species into invasives, leading to infestations of plant pests or pathogen epidemics.

The quarantine bacterium *Xylella fastidiosa* Wells et al., 1987 (XF) subsp. *pauca* ST53 [4,5] entered from Costa Rica to Italy (Apulia), developing the consequent epidemic on olive trees, showing how an alien species can invade new hitherto-unexplored territories. Indeed, XF-infected plants ultimately cease

production and die within a few years [6]. In Apulia, XF encountered Aphrophoridae xylem-feeders (Spittlebugs: Hemiptera, Auchenorrhyncha), which acquire, transmit, and spread the bacterium in *Olea europaea* L., 1753 populations, a new host. Several Aphrophoridae can acquire and transmit XF. However, *Philaenus spumarius* L., 1758 (PS) is the main vector for population size and infective abilities [7]. The ability to transmit XF has changed PS's status from a marginal insect to a key pest for olive trees in the Mediterranean Basin areas where XF also occurs, leading to increased attention in managing PS infections [8]. The currently recommended strategy first suggests minimising PS populations during juvenile stages. Management then focuses on surviving adults to prevent acquisition and transmission or limiting infection events to one adult per plant to slow or stop XF invasion. The aim is to kill vectors during their first feeding on olive trees, particularly in areas still free from the bacterium [7].

The basis of the infection management strategy is the integration of several control actions. This strategy involves resistant olive tree cultivars, mechanical and chemical control of PS juvenile instars, and chemical and biological control of PS adults [7,9–14]. The biological control of PS can involve the predator *Zelus renardii* Kolenati, 1856 (ZR, Hemiptera: Reduviidae) [12,14], recently acclimated in the Mediterranean Basin [14,15]. The reduviid will attack adult PS and other olive pests such as *Bactrocera oleae* (Rossi, 1790) (Diptera, Tephritidae) [14]. Furthermore, ZR could be a potential biological control agent for other invasive alien pests recently entering Europe, such as *Macrohomotoma gladiata* Kuwayama, 1908 (Hemiptera, Psyllidae) and *Drosophila suzukii* Matsumura, 1931 (Diptera, Drosophilidae) [16,17]. As a biocontrol agent, ZR provides new interactions among alien and native species. These novel interactions offer the opportunity to unveil the mechanisms of integrating alien species into pre-existing trophic networks in the biogeographical area of entrance. Therefore, analysing how they interact with their biotic environment is relevant.

Olfaction is the primary sense by which insects perceive single or semiochemical blends [18,19], which are carbon-based volatile organic compounds (VOCs) vaporising at 20 °C and 0.01 kPa [20]. VOCs can play the role of interspecific (pheromones) and intraspecific (allelochemicals) communication [19,21–23]. Semiochemicals serve as olfactory cues [18,24,25], eliciting behavioural or physiological replies. Cimicomorpha and Pentatomomorpha (Hemiptera, Heteroptera) possess peculiar metathoracic glands [26], whose secretions dictate several adult insect behaviours [27]. Many Reduviidae species have pairs of Brindley's and metathoracic exocrine glands [27–31]. Secreted VOCs are involved in defence, alarm, and mating [29,31–33]. Brindley's glands have orifices located dorsally in the metathorax [34,35] and secrete alarm pheromones in the event of disturbance of the reduviids [29]. Metathoracic glands have lateral/ventrolateral outlets and dedicated dispersive apparatuses dictating aggregation, copulation, and defence [35,36]. The Harpactorinae subfamily, including *Zelus* spp., lacks metathoracic glands [37].

Zelus renardii uses semiochemicals for partner or prey searching [29,30,38–41]. ZR also supplements olfactory information with visual cues when encountering mobile prey [14]. In contrast, vibrational communication predominates in PS. Nevertheless, PS also utilises intraspecific chemical communication systems [42]. However, little knowledge exists on the chemical communication of PS underlying intraspecific interactions. PS is assumed to rely on vibrational rather than chemical communication to manage mating and other intraspecific interactions [43,44].

Therefore, this work focuses on ZR and PS VOCs, alone and in reciprocal interactions. We aim to establish background information to understand recognition among *Z. renardii* individuals and between ZR and its *P. spumarius* prey, the XF vector.

Materials and Methods

Insect Collection

ZR specimens for the study came from a September 2021–October 2022 collection in a *Citrus* CV orchard near Elche (38°14′54 N 0°41′43 W). ZR adults were collected using 60 mL single-use probes (Deltalab, Rubí, Spain) purposely perforated for ventilation. We obtained 18 ZR adults (males and females) for the study.

Adult PS thrived in a dicot-dominated field near Jijona (38°32′25 N 0°30′38 W) and were swept using a net from June to September 2022. PSs were transferred from the sweeping net into vented 5 mL microtubes (Deltalab, Rubí, Spain). We avoided mouth aspirator collection because abrupt draw-up would have inflicted low-pressure stresses on PS, eventually modifying the VOC Aphrophoridae profile.

Brindley's Glands

To demonstrate that Brindley's glands secrete VOCs capable of eliciting behavioural responses, we scrutinised ten ZR adults (five males and five females). The insects originated from 2018–2021 collections given during a study on reduviid mass-breeding performance. Adults were allowed to rest in the dark until December 2022 and then fixed in 75% *v*/*v* EtOH/distilled water, prepared using pure bioethanol (PVG Liquids N.V., Gent, Belgium). We took ten ZR adults collected over the years and available in the collection of the Forensic Entomology Laboratory of DiSSPA (University of Bari Aldo Moro, Italy).

Studying the insects, we first obtained a general view from the right side of the mesothorax and metathorax plus part of the abdomen. Then, we attempted to observe Brindley's glands on each half of every individual with light microscopy. Then, we focused on SEM evidence.

Each ZR was passed from EtOH to distilled water for 12–48 h to extract the alcohol. Later, we cut away and preserved the legs at the trochanter, prothorax, and abdomen at the third urite. After one/two days of

water replacement, gentle shaking, and rest in a vial on a warm plate (40–50 °C), we replaced the water with a fluid made by mixing 1/1 SDS (Sodium Dodecyl Sulphate in distilled water 50% *w*/*v*) and branded pure hand dishwashing soap. Porcelli [45] suggested a similar procedure, but we simplified the protocol by eliminating KOH, apart from the first two studied ZR specimens, which were also double stained [46]. The parts of each ZR were cleared first in soap and surfactant and then in Essig's Aphid Fluid (EAF) on a warm plate until they showed the cuticular details of Brindley's glands under a stereoscope. We cut each ZR part following the sagittal plane, obtaining two exposed Brindley glands. A Zeiss Phomi II and a Zeiss Tessovar, purposely modified for thick slide imaging and equipped with Olympus PEN cameras, were used to study, and perform bright field macro and microscopy imaging.

We used the same ZR parts scrutinised by light microscopy for the SEM study. The parts underwent steps from EAF to 75% EtOH, 99% EtOH, and propyl-acetate [7]; the last step was run in glassware with a minute outlet for slow solvent evaporation. After propyl-acetate evaporation, we obtained well-dehydrated cuticle parts for observations. Each ZR half was inserted into a Ø 12.5 × 8 mm height alloy SEM stub (Agar Scientific Ltd., Stansted, UK) using double-sided conductive tape (Ted Pella, Inc., Redding, CA, USA) [47], exposing the insect cuticle's internal surface. Insect parts were first imaged using a TM3000 Hitachi SEM in charge-up reduction mode. They were then lightly coated with gold/palladium sputtered by an Edwards S150 Ion Sputter Coater for 30″ and 8 mA current to observe more detail. The electron beam was accelerated at KV 5, 15, and 15 for analysis. In Cryo-SEM mode, the TM3000 was used with sublimated ultrapure water (Milli-Q® Lab Water Solution, Sigma-Aldrich, St. Louis, MO, USA), embedding the ZR parts at –25/–50 °C to expose the cuticle [48]. Meshwork and other imaging details were performed at –45 °C in charge-up reduction mode. Adobe Photoshop® (Adobe Incorporated, San Jose, CA, USA) was used to edit the images in grayscale and false colours.

Experimental Setup for VOC Detection

A Gas Chromatography–Mass Spectrometer was used to gather VOC data from one ZR, one PS, or one ZR + one PS per vial (HS, crimb, FB, 20 mL, clr, cert, 100 PK: Agilent Technologies, Santa Clara, CA, USA). We starved ZR and PS 24 h before their placement in vials at 23 °C under laboratory conditions.

The treatments targeted VOCs produced by a ZR or a PS alone in a vial or the VOCs from a net-limited interaction in the vial (Figure 1). A top circle (0.5 cm Ø) and a longitudinal net wall (2 × 0.5 cm) of polyurethane square mesh (1 × 1 mm) (Figure 1C) were used to limit the contact between the insects. An empty vial served as a control versus a vial with nets to discriminate an eventual "net effect". We performed seven biological replicates per treatment (ZR, PS, and ZR + PS).

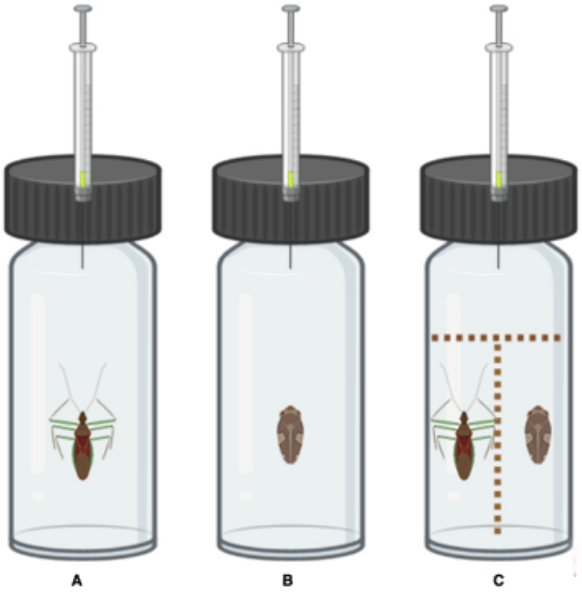

Figure 1. Experimental setup for volatile collection from insects. Vials: (**A**) *Zelus renardii* alone; (**B**) *Philaenus spumarius* alone; (**C**) *Z. renardii* and *P. spumarius* separated by nets (created with BioRender.com, accessed on 4 April 2023). N.B.: for pictorial fold, PS is portrayed with 3× magnification.

We also searched for VOCs produced upon PS aggregation. This test was comprised of four treatments (increasing the number of PS per vial), testing 18 PS in total (males and females) in trials with either 1, 2, 5, or 10 individuals placed in separate vials (Figure 2), performing three replicates.

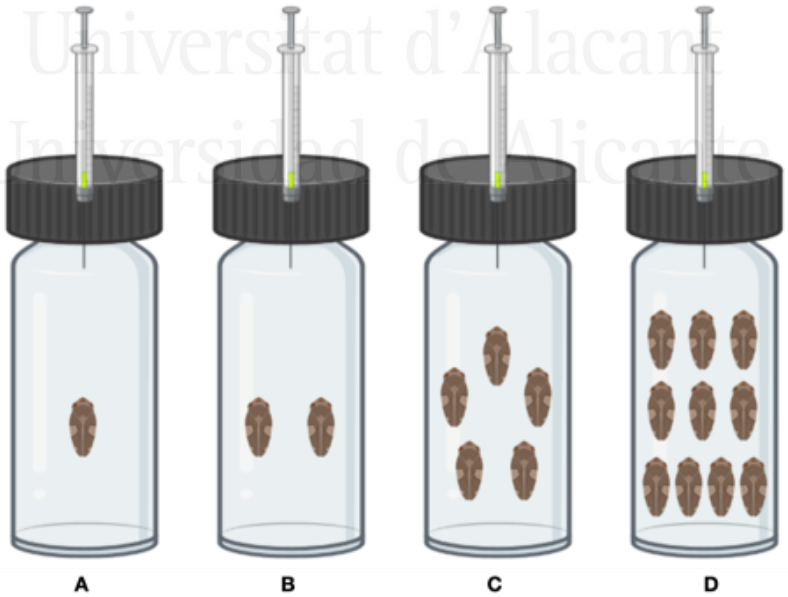

Figure 2. Experimental setup of *Xylella* vector VOC production. Vials: (**A**) 1 *P. spumarius*; (**B**) 2 *P. spumarius;* (**C**) 5 *P. spumarius*; (**D**) 10 *P. spumarius* (created with BioRender.com, accessed on 4 April 2023).

The solid phase microextraction (SPME) holder with a fused silica fibre (10 mm; Ø 80 μm; DVB/CWR/PDMS, Agilent Technologies, Santa Clara, CA, USA) absorbed VOCs by exposing the carrier's fibre to the vials' headspace for one hour at room temperature (approx. 23 °C).

GAS CHROMATOGRAPHY–MASS SPECTROMETRY (GC/MS) ANALYSES

The fibre placed into the GC injector (Agilent 5977B network mass spectrometer; Agilent model 7890B gas chromatograph; column: DB-624, length 30 m, 0.25 mm ID, 1.4 μm, Agilent) underwent a 4 min desorption at 250 °C in split/splitless mode. The fibre injector was a robot MPS (Multipurpose Sampler; Gerstel GmbH & Co. KG, Mlüheim an der Ruhr, Germany). The fibre was conditioned at 250 °C for 10 min before being injected into each sample. The chromatographic program used was started at 40 °C for 5 min and then later was increased by 5 °C/min up to 230 °C to maintain the temperature for 10 min. In total, the analysis time was 53 min. The ionisation source value for the electronic impact was 70 eV at 230 °C, with a mass range between 25 and 450 amu. The detector was a simple quadrupole at 150 °C. The NIST11 library allowed for the tentative identification of VOCs. After each chromatographic run, the software generated a chromatogram and VOC list.

The VOCs obtained were clustered into major VOCs with a match ≥50% and a peak height ≥100.000 ppm or minor VOCs with a match ≥50% and a peak height <100.000 ppm.

OLFACTOMETER ANALYSIS

Four-arm PET (polyethene terephthalate) olfactometers were used to study the behavioural response to selected VOCs in the volatilomes. Each olfactometer consisted of a central chamber, 12 cm in Ø and 6 cm tall, connected to four arms equally angled at 90°. Each arm was 3 cm in Ø and 6 cm long.

The lists of VOCs per treatment (ZR, PS, and ZR + PS) were compared using Venn diagrams to determine which VOCs were found most frequently in the GC/MS analysis. We selected the VOCs found in at least four out of seven replicates for the behavioural analysis.

Selected VOCs (2-methyl-propanoic acid, 2-methyl-butanoic acid, and 3-methyl-1- butanol) were tested separately in pouch dispensers (3.5 cm × 2.5 cm) of miracloth (Merck KgaA, Darmstadt, Germany). Each dispenser contained 2 g of 60 A silica gel (70–200 μ, Carlo Erba Reagents s.r.l., Cornaredo, Italy). We added 2 μL of the given pure compounds to the silica gel in each dispenser for the trials. One hundred and twenty tests (forty per compound) were performed to understand the effect of the selected VOCs on ZR behaviour.

We tested each VOC against four feral ZR males per replicate. We placed each VOC randomly into two of the four peripheral chambers, while the other two were left empty as negative controls. The tests were set up by rotating the ZRs between four different olfactometers and letting them rest for at least five minutes

between each test. Each ZR underwent olfactory stimulation for 30 minutes. We rinsed the olfactometer after each trial with n-hexane, ethanol, and distilled water and dried it with paper towels to remove any residual VOCs. Olfactometry was conducted under laboratory conditions (23 °C, approx. 60% HR) with a seasonal photoperiodic condition (approx. 10:14 L:D).

We assessed the number of times the predator approached the VOC and the negative control, recording the final ZR choice after half an hour. Forty olfactometer trials were run for each VOC.

CHEMICALS

Pure 2-methyl-propanoic acid and 2-methyl-butanoic acid were synthesised at the Institute of Organic Synthesis of the Department of Organic Chemistry at the University of Alicante (Spain), and the pure 3-methyl-1-butanol was from TCI (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan).

DATA ANALYSIS

A multivariate generalised linear model (GLM) with a Poisson distribution of the error was conducted to examine changes in the set of volatilomes of ZR alone that of ZR+PS. Furthermore, a 999-permutation multivariate analysis of variance (PERMANOVA) was used to analyse the Bray–Curtis' dissimilarity matrix. The treatment (ZR and ZR + PS) was a fixed factor. A betadisper was used to test the multivariate homogeneity of group dispersion. However, we used the F-value modification due to variances' lack of multivariate homogeneity [49]. A SIMPER analysis was used to determine which VOC contributed most to the differences. In addition, we conducted a univariate study of the variation produced in each VOC using a generalised linear mixed model (GLM) with a Gaussian family error distribution.

A GLMM with a binomial error distribution was used to determine the final choices of ZR to move towards or away from the VOC in the olfactometer tests.

A GLMM with Poisson distribution error was used to analyse the number of approaches to VOCs. The treatment (control and compound) and the experiment were the fixed factors.

Both models treated the individual identity of the insect as a random factor to control for the non-independence of repeated measures on the same individual.

All statistical analyses were performed using R version 4.1.2 (R Core Team, 2022) using "manyglm" from the "mvabund" package [50] for multivariate and univariate GLM models. PERMANOVA was used with the "adonis2" function of the "vegan" package [51]. Furthermore, the GLMM models [52] were used with the function "glmer" from the package "lme4", while the function "simulateResiduals" from the package "DHARma" [53] was used to perform the model diagnosis.

RESULTS

BRINDLEY'S GLANDS MORPHOLOGY

The right-side view focused on the adult ZR thorax and abdomen, showing relevant cuticular details in reflected light (Figure 3a). The marks indicated the pronotum (Figure 3a: 1), mesothoracic coxa (Figure 3a: 2), metathoracic coxa (Figure 3a: 3), second urite (Figure 3a: 4), third urite (Figure 3a: 5), first urite (Figure 3a: 8), hemelytron (Figure 3a: 9), mesopleuron (Figure 3a: 11), patchy cuticle areas (Figure 3a: 12), meshwork evaporatorium (Figure 3a: 13), and the Brindley's gland reservoir outlet (Figure 3a: 15), serving to put into context the observations of Brindley's glands and associated structures. Moreover, Figures 3 and 4 show details from the outside, while Figures 5 and 6 demonstrate the same areas and details from the inside of the insect. The same marks refer to the same details in all images. We scrutinised the same details, namely, the same parts from the same insect, using light and SEM microscopy, making the resulting description somewhat repetitive in numbering; a table of numbers/descriptions is provided to help follow the results (Table 1).

Table 1. List of *Zelus renardii* marks in pictures.

Number	Description
1	pronotum
2	mesothoracic coxa
3	metathoracic coxa
4	second urite
5	third urite
6	right spiracle of the second urite
7	right spiracle of the first urite
8	first urite
9	hemelytron
10	mesothoracic flap
11	mesopleuron
12	patchy cuticle areas
13	meshwork evaporatorium
14	Brindley's gland meshwork evaporatorium
15	Brindley's gland reservoir outlet place
16	gutter
17 (a–c)	trachea (and tracheal branches over Brindley's gland)
18	Brindley's gland reservoir
19	Brindley's gland units' cuticle
20	secretions (?)
21	abdominal finger

Macrography and SEM (Figure 3a,b) were conducted to identify the major cuticular elements: first (Figure 3: 7) and second (Figure 3: 6) abdominal spiracle; meshwork (Figures 3a,b and 4a,b: 14); gutter (Figures 3a,b and 4a,b: 16); abdominal finger (Figures 3a,b and 4a,b: 21); and mesothoracic flap (Figures 3a

and 4b,d: 10). The external side of the cuticle did not show any other relevant details. Transmitted bright-light microscopy showed the interior with one Brindley's gland reservoir per side. Each reservoir (Figure 5a–c: 18) is immediately below the first abdominal spiracle and slightly above the second abdominal spiracle (Figure 3a,b and 5a,c: 6, 7). The second (Figure 5a,c: 6) abdominal spiracle and meshwork (Figure 5a,c: 14) remained consistent with the external observations. In the last figures, minute drop-like sacculi (Figure 5a,c,e: 19) appeared to be associated and possibly connected with the reservoir. SEM showed the second abdominal spiracle (Figure 6: 6), the tracheal trunks and subdivision (Figure 6: 17a,b,c), and Brindley's reservoir (Figure 6: 18) sheltered by sacculi (Figures 5d and 6: 19) and partially hidden by tracheal subdivisions 17b and 17c (Figure 5d; 6).

The main tracheal trunk of the second spiracle was found to run to the metathorax, and its bifurcation stayed over the sagittal side of the gland (Figure 6). Reservoirs were membranous, flask-like, and slightly dorso–ventrally flattened (Figure 5a,b). The inflated reservoir measured approximately 0.12 × 0.10 mm, and no appreciable size differences were found between ZR males and females. Two bi-convex outlets corresponded to each meshwork-like metathoracic area (Figures 3a,b and 5c).

A mantle of drop-like cuticular sacculi covered each reservoir (Figures 5c,d and 6). We suggest that sacculi in ZR correspond to the B-type glandular units described by Barrett [54] in *Rodnius prolixus* Stål, 1859 (Reduviidae, Triatominae).

ZR glandular units were 5–6 μm in diameter (Figure 5d,e). In *R. prolixus,* the B-type sacculi joined Brindley's reservoirs by proper ducts randomly distributed and oriented toward the reservoir's internal surface. ZR detached sacculi show no duct and a single glandular unit type only. A-type secretory units with elongated, U-shaped sacculi [34,54] were not found in ZR.

Brindley's gland reservoirs in *Z. renardii* converge in a short cuticular duct opening just above the supra-coxal lobe of the metathoracic pleurae, as described in *R. prolixus* [28,34,54]. Brindley's gland outlets were funnel-like regressions, sheltered by a meshwork of cuticular microsculpture that decorated the surface (Figure 4a,b). The meshwork areas can act as evaporatoria, increasing the dispersing surface of the glandular secretion. Meshwork elements consisted of central plates (called "*chapeaux*" by Carayon [55]) with 3–5 holes connected to the adjacent ones by cuticular bridges forming the meshwork pattern (Figure 4a). Cuticular bridges delimited depressed areas called trabeculae [55]. A minutely decorated cuticular process extended along each metanotum side. Carayon [55] suggested a role for such a process called "*gouttière*" (Figure 4b). In ZR, the gutter started from the abdominal finger (Figure 4b: 21), ending in the mesothoracic flap (Figure 4b: 10). Layers and particles of solid residues of secretion (Figure 4c,d: 20) suggested that the mesothoracic flap and the metanotum gutter are evaporatoria for Brindley's glands or other thoracic glands of ZR. Indeed, we found another outlet with a meshwork-like area below the

mesothoracic flap, between the meta- and mesothorax membrane, like Brindley's gland outlets (Figure 4c: 13). In addition, the meta–mesothoracic outlets presented organic material, suggesting secretory activity. However, the structure, anatomy, and physiology of such a putative ZR meso–metathoracic gland were not further analysed here.

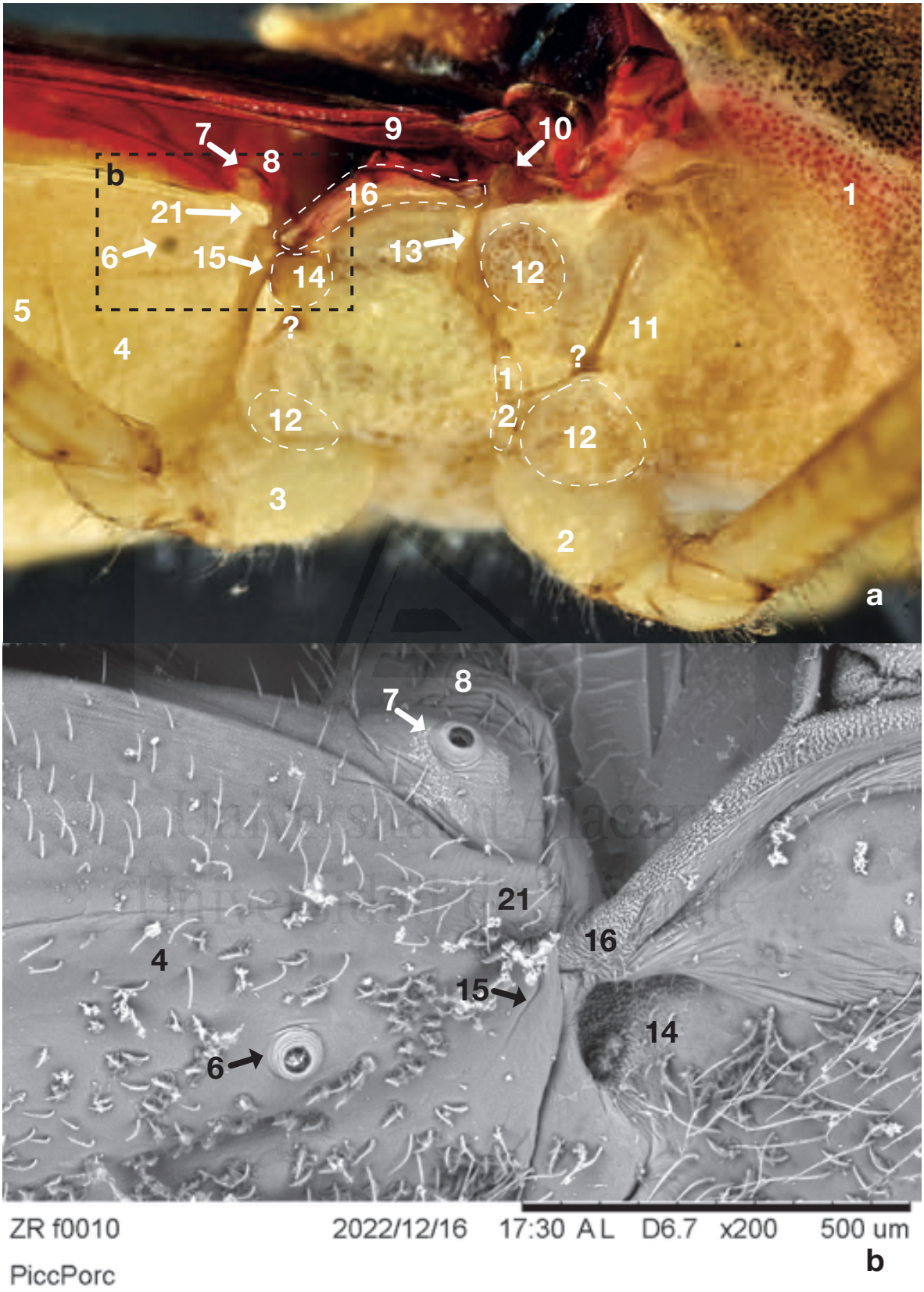

Figure 3. Right partial view of ZR's thorax and abdomen: 1 = pronotum, 2 = mesothoracic coxa, 3 = metathoracic coxa, 4 = second urite, 5 = third urite, 6 = right spiracle of the second urite, 7 = right spiracle of the first urite, 8 = first urite, 9 = hemelytron, 10 = mesothoracic flap, 11 = mesopleuron, 12 = patchy cuticle areas, 13 = meshwork evaporatorium, 14 =

Brindley's gland meshwork evaporatorium, 15 = Brindley's gland reservoir outlet place, 16 = gutter (*gouttière*), 21 = abdominal finger; (**a**) Tessovar light macroscopy; (**b**) Cryo-SEM.

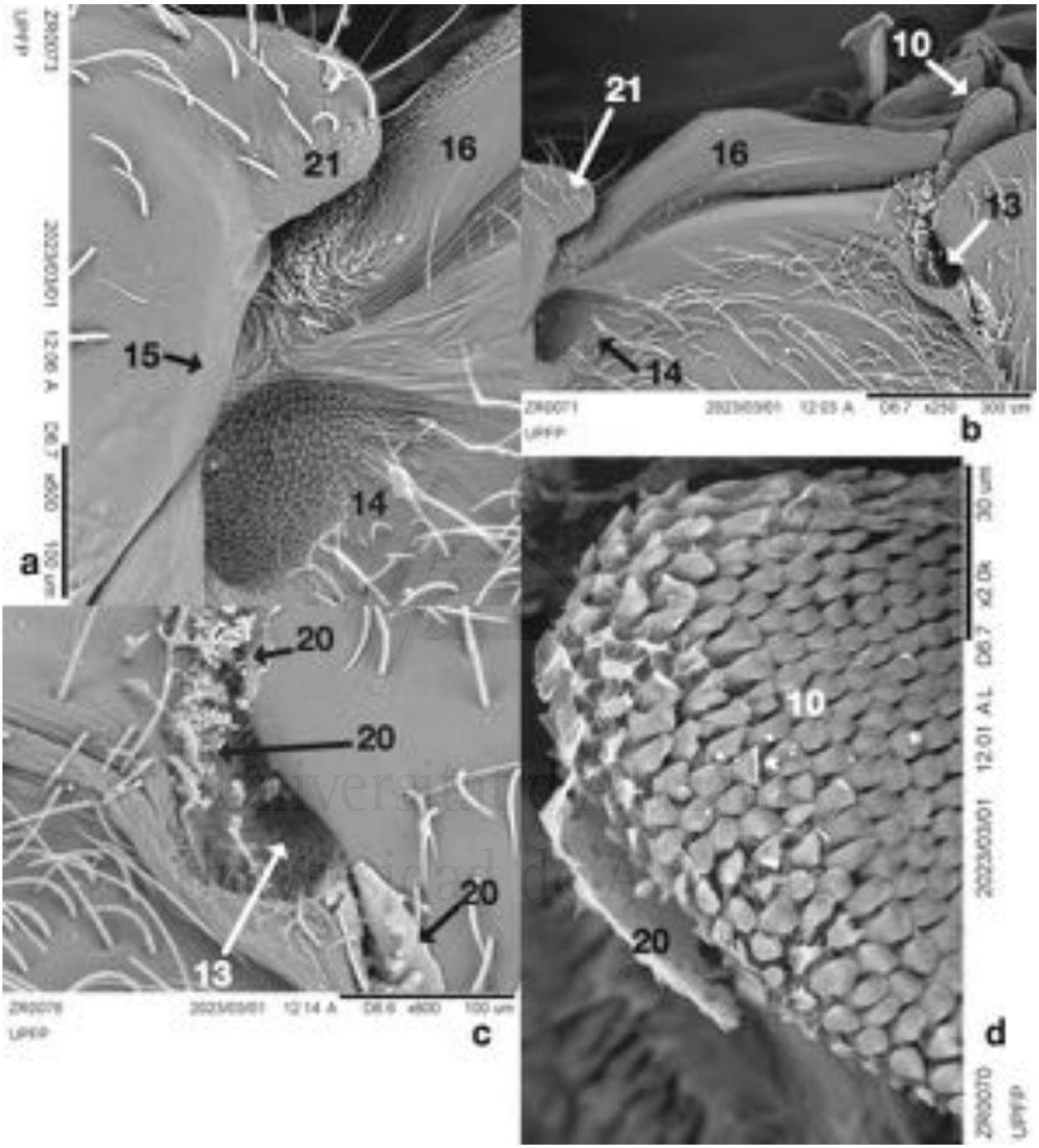

Figure 4. Details of right thorax and abdomen view: 10 = mesothoracic flap, 13 = meshwork evaporatorium, 14 = Brindley's gland meshwork evaporatorium, 15 = Brindley's gland reservoir outlet place, 16 = gutter (*gouttière*), 20 = secretions (?), 21 = abdominal finger; SEM.

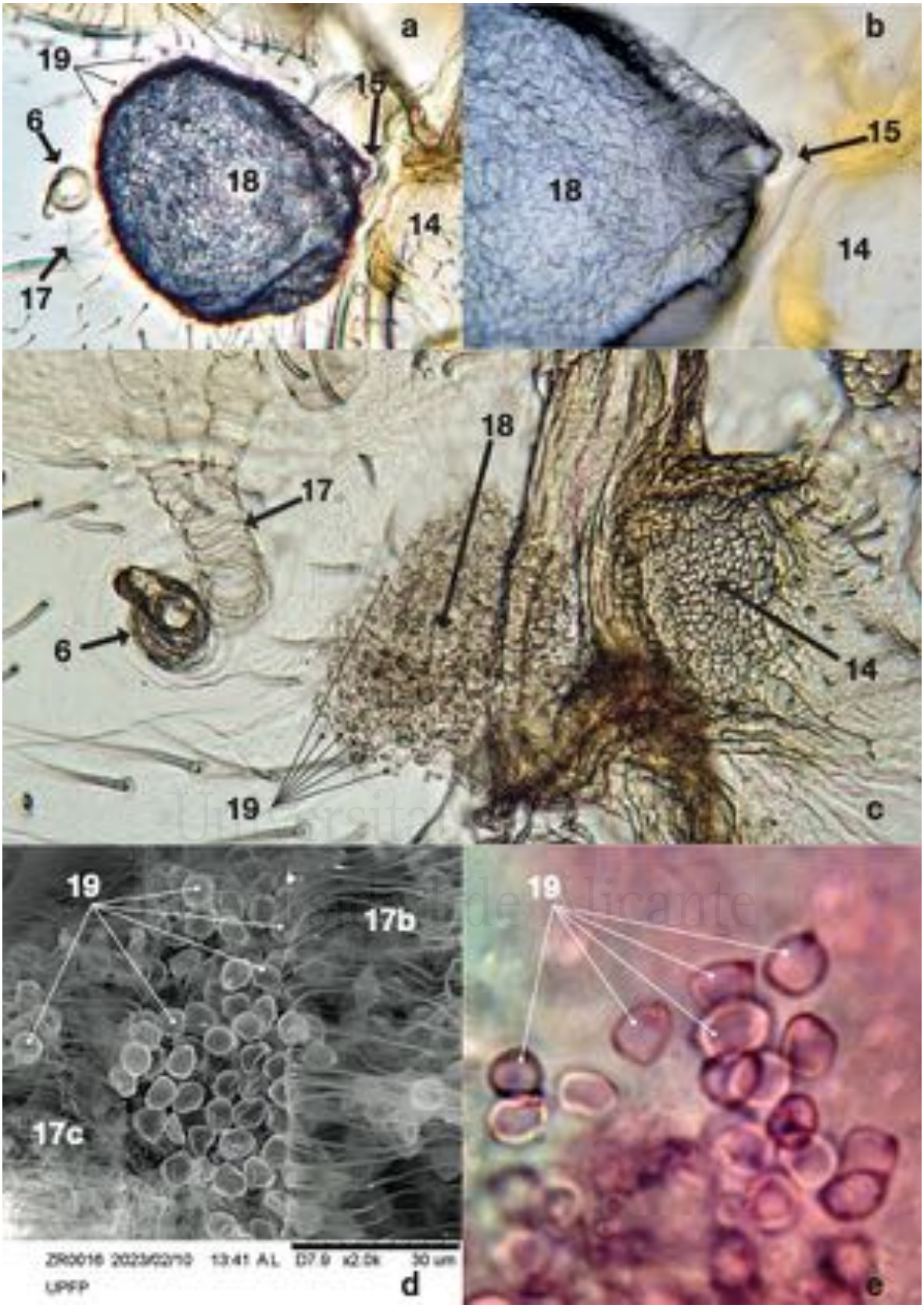

Figure 5. Details of Brindley's gland: 6 = right spiracle of the second urite, 14 = Brindley's gland meshwork evaporatorium, 15 = Brindley's gland reservoir outlet, 17 and 17b,c = trachea and tracheal branches over Brindley's gland, 18 = Brindley's gland reservoir, 19 = Brindley's gland units' cuticles; Phomi II light microscopy, SEM.

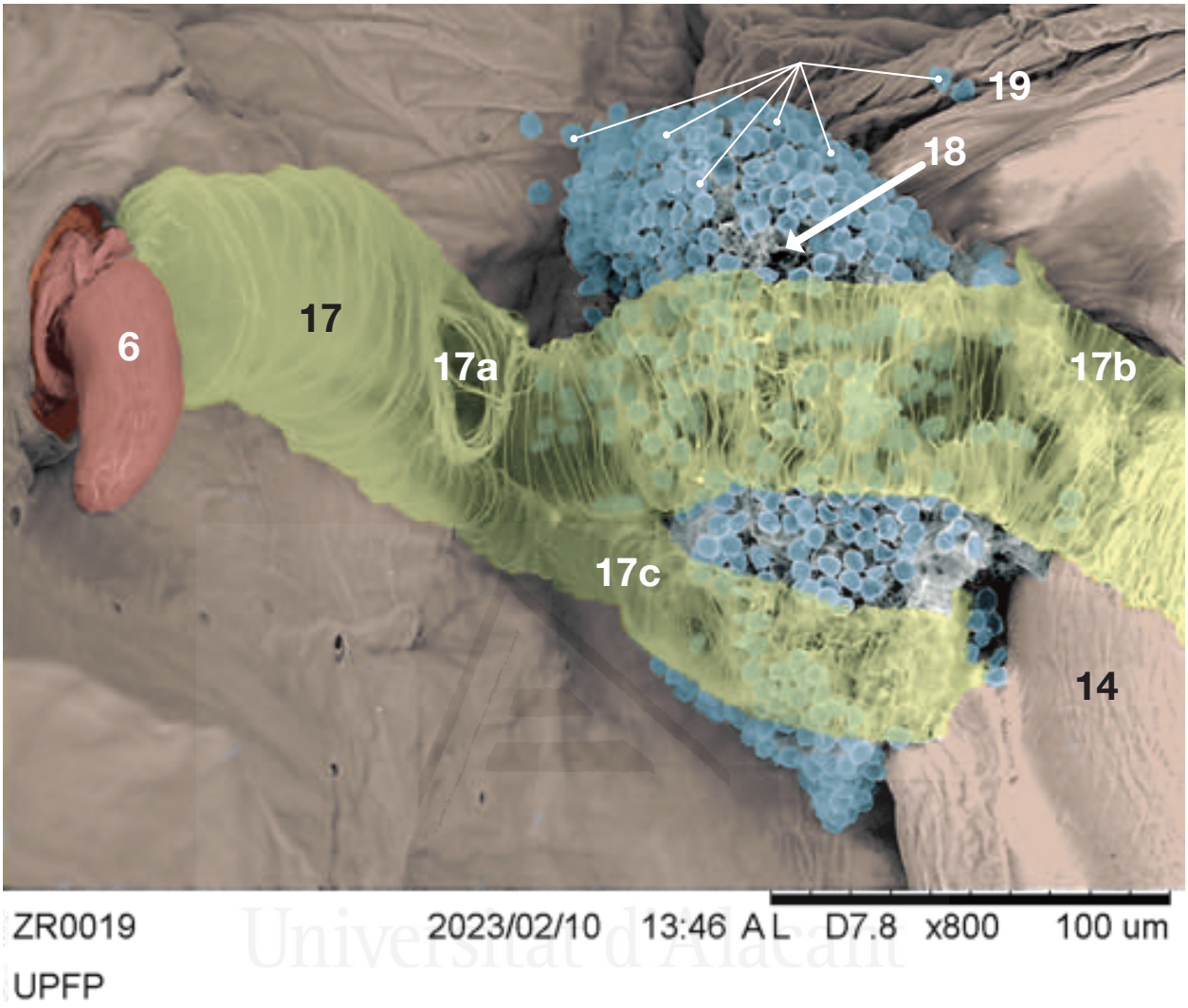

Figure 6. Brindley's glands false-colour SEM: 6 = right spiracle of the second urite, 17 = trachea, 17a = sign of a broken tracheal branch, 17b, c = tracheal branches lying over Brindley's gland, 18 = Brindley's gland reservoir, 19 = Brindley's gland units' cuticles.

VOCS PRODUCED BY ZR AND PS

We detected 86 VOCs across all experiments (Tables S1–S3). ZR alone produced 35 VOCs (Table S1), while PS alone only produced 13 VOCs (Table S2). Fifty VOCs existed in the interaction between ZR and PS (Table S3). ZR alone made only three major VOCs (8.6%). The remaining 32 VOCs (91.4%) were present in concentrations below 100,000 ppm.

PS alone did not produce major VOCs. 2,3-Butanedione (ca. 91,000 ppm) was the most abundant PS VOC. The ZR + PS interaction included 24% of major VOCs. Therefore, the largest part of the volatilome comprised minor VOCs (76%). 3-Methyl-butanal, 2-methyl-1-methylene-3-(1-methyl-ethenyl)-cyclopentane and propyl-cyclohexane were present in the individual volatilomes of both ZR and PS (Figure

7). However, two VOCs could be in the individual PS and the predator–prey interaction volatilome (Figure 7), namely, 2,3-butenedione and 2,4,6-trimethyl-benzaldehyde. Furthermore, 2,4,6-trimethyl-benzaldehyde was the only compound constantly found in all replicates of PS alone and the PS interacting with ZR.

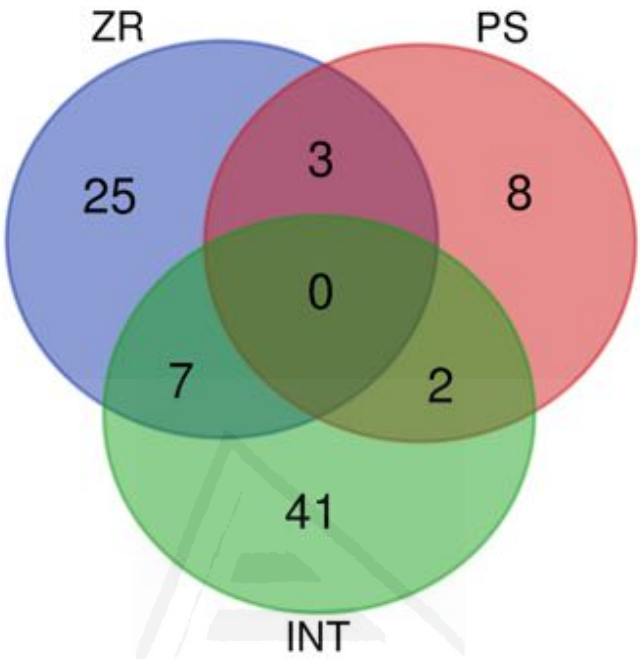

Figure 7. Venn diagram showing the volatiles shared between treatments. *Zelus renardii* (ZR), *Philaenus spumarius* (PS) and interaction between *Z. renardii* and *P. spumarius* (INT).

VOLATILOME OF ZR AND PS INTERACTION

Seven VOCs were found in the ZR + PS interaction and ZR alone (Figure 7 and Table 2). No VOC was present in all three treatments. Statistical analysis and olfactometry tests were performed on VOCs present in at least four out of seven replicates. Therefore, we only considered 2-methyl-butanoic acid, 2-methyl-propanoic acid, and 3-methyl-1-butanol because we detected all three in ZR and ZR + PS interactions (Table 2).

Table 2. VOCs detected in *Z. renardii* alone and *Z. renardii–P. spumarius* interaction. Abbreviations: M-VOCs = major VOCs; m-VOCs = minor VOCs; R.T. = Retention Time; P.H. = peak height; No. Rep. = number of replicates for the chemical. In bold are the VOCs found in at least half of the replicates.

Compound	R.T. (min)	P.H. (ppm)	Match (%)	No. Rep.
M-VOCs				
3-methyl-1-butanol	12.656	256,956	87	5
2-methyl-propanoic acid	15.262	802,433	95	7
2-methyl-butanoic acid	18.643	507,820	68	5
2-methyl-pentanoic acid	18.741	2,106,728	72	1
m-VOCs				
2-pentanol	10.884	34,370	83	1
2,4,4,6-tetramethyl-hept-2-ene	24.076	50,924	50	1
1-ethyl-2-methyl-cyclohexane	24.079	54,621	50	2

The multivariate GLM revealed significant differences in the volatilome between ZR and ZR + PS (*p-value* = 0.01). Furthermore, PERMANOVA also showed a significant difference (*p-value* < 0.05). SIMPER analysis indicated that the variable contributing most to the differences between ZR and ZR + PS was 2-methyl-propanoic acid, with 61.7%, followed by 2-methyl-butanoic acid, with 35%. However, only the contribution of 2-methyl- propanoic acid was significant (*p-value* = 0.03). When ZR interacted with PS, the amounts of 2-methyl-butanoic acid and 2-methyl-propanoic acid decreased (Figure 8). In contrast, 3-methyl-1-butanol increased (Figure 8). However, there was no significant change between the three VOCs produced by ZR alone and ZR + PS (GLM, *p-value* > 0.05) (Figure 8).

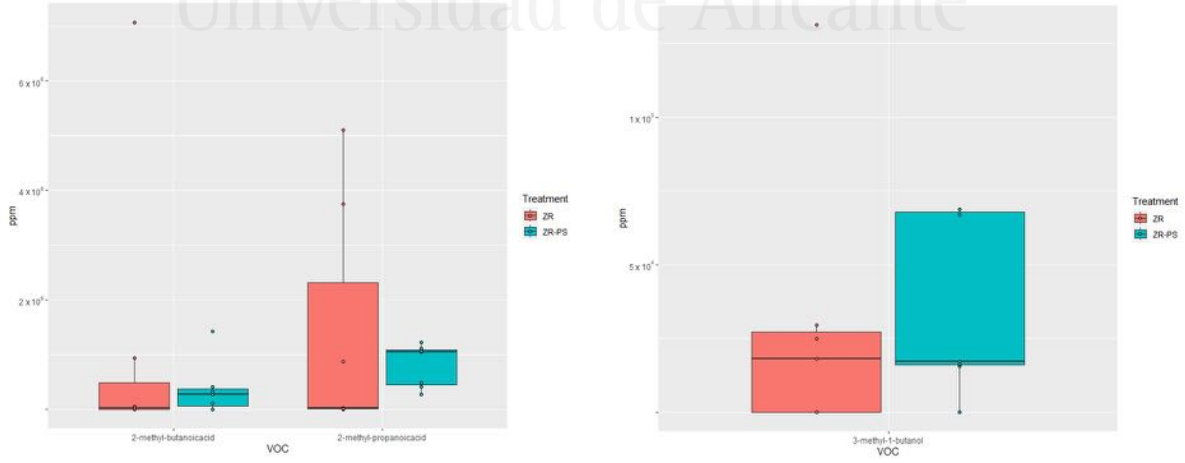

Figure 8. Amount of 2-methyl-butanoic acid, 2-methyl-propanoic acid, and 3-methyl-1-butanol in ZR alone (red box plots) and during ZR–PS interaction (blue box plots).

VOLATILOME OF *PHILAENUS* AGGREGATION

The volatilome linked to the progressive aggregation of PS revealed ten VOCs (Table S4). All were minor VOCs (<100,000 ppm). VOC production was higher when PS was alone (six VOCs) than during intraspecific interaction with two or more (up to ten) con- specifics (3–4 VOCs) (Table S4). 2,4,6-Trimethyl-benzaldehyde was the only VOC always present regardless of the number of PS interactions, with an average concentration below 20,000 ppm.

ZR RESPONSE TO VOCS SELECTED FROM PREY INTERACTION

2-Methyl-propanoic acid was the final choice for *Z. renardii* in 30% of olfactometer tests. The movement was 27.5% for 2-methyl-butanoic acid and 17.5% for 3-methyl-1-butanol. All differences between the choice of control vs. a VOC were highly significant (*p- value* < 0.05; *p-value* < 0.001) (Figure 9).

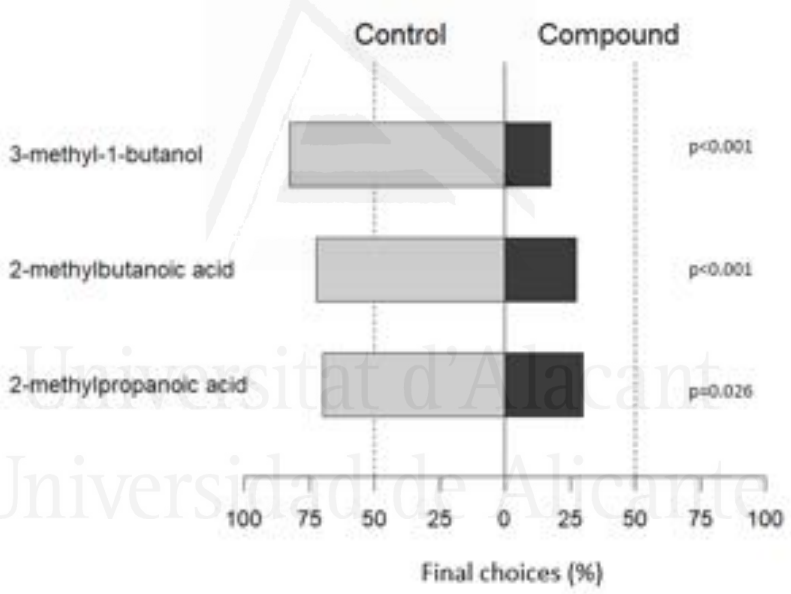

Figure 9. The final choice of *Z. renardii* when given a VOC (3-methyl-butanol, 2-methyl-butanoic acid, or 2-methyl-propanoic acid) or the control (empty—no VOC).

The average number of approaches (movements towards a given stimulus) per test to 2-methyl-propanoic acid was 0.4 ± 0.59 vs. 1.35 ± 1.23 to the control (Figure 10A). The GLMM showed significant differences between treatments (*p-value* < 0.001), whereas there were no significant differences between experiments (*p-value* = 0.06). Similarly, the average number of approaches per test was significantly lower in the case of 2-methyl-butanoic acid (0.4 ± 0.49) than in the case of departures (1.2 ± 1.01) (*p-value* < 0.001) (Figure 10B). The average number of approaches per test to 3-methyl-1-butanol was also significantly lower

(0.32 ± 0.52) compared to the departures (1.47 ± 0.84) (*p-value* < 0.001) (Figure 10C). No significant differences existed between experiments (*p-value* = 0.95).

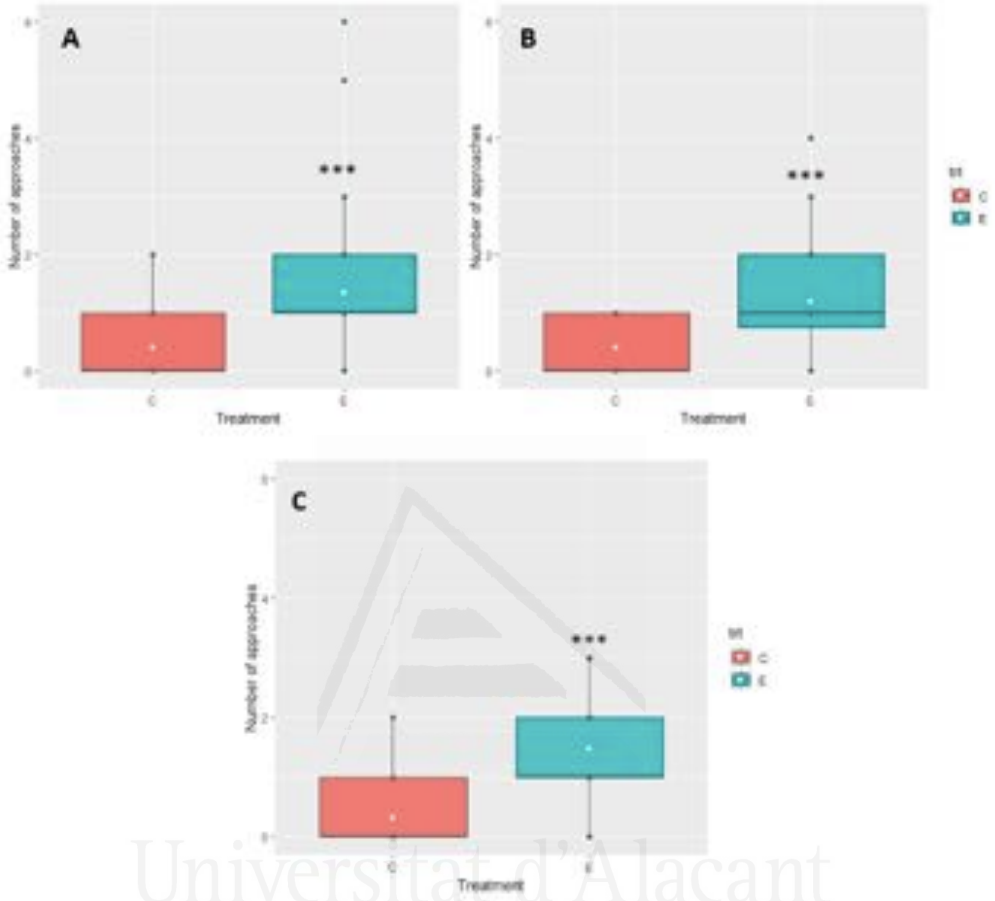

Figure 10. Average approaches of *Z. renardii* to VOCs: (**A**) 2-methyl-propanoic acid; (**B**) 2-methyl- butanoic acid; (**C**) 3-methyl-1-butanol. *** *p-value* < 0.001. Abbreviations: C = compound, E = empty.

DISCUSSION

A pair of Brindley's glands are found in each ZR, male or female. These glands are in the abdomen, before the second spiracle, opening toward the metathorax, and slightly below the first spiracle. The placement and general morphology of Brindley's gland assemblages in ZR may refer to the "type *diastomien périadénien* (F)" of Carayon ([55] page 741). Each reservoir collects the secretion from ca. 600 exocrine units. Single unicellular glands resemble the "B-type" glandular unit described in *R. prolixus* [54].

Further scrutiny will confirm the presence of one or more kinds of secretory units that should correspond to several VOCs. Each unit possesses a duct, presumably, but details of the units are beyond the scope of this study. Our study, with a few processed specimens for light microscopy and uncoated or sputtered

parts for SEM observations, minimises artifacts and provides evidence of the presence of Brindley's glands in ZR.

Zelus renardii and *P. spumarius* revealed differences in VOC production. The predator ZR uses olfactory communication, having a greater quantity and variety of VOCs than PS. PS aggregation caused a reduction in the number of VOCs detected by GC/MS.

Philaenus spumarius has fewer antennal sensory structures than other Auchenorrhyncha species, yet these structures can perform an olfactory function [56,57]. However, reducing VOC diversity with aggregation suggests PS prefers vibrational rather than olfactory communication between conspecifics [43,44]. Despite this, male PSs respond positively to female VOCs, indicating the species' ability to perceive likely sexual olfactory stimuli, confirming the functionality of olfactory receptors [42]. VOCs in our PS volatilome were not frequently detected, so no tests were conducted to study their role in the behavioural responses to these XF vectors. Identifying the sex pheromones of PS could lead, in future studies, to developing traps for their monitoring and management in olive orchards.

The widest variety of VOCs was found in the ZR + PS interaction, suggesting that the predator rather than the prey contributes mostly to this increase. Cimicomorpha, to which ZR belongs, possess well-developed odour glands capable of producing different semiochemicals for intra- and interspecific communication [26]. Cimicomorpha VOCs include short-chain organic acids, alcohols, short-chain aldehydes and esters, alkanes, monoterpenes, aromatic alcohols, and aldehydes [27,58]. We found many of these compounds in this ZR study.

In the ZR + PS interaction and when ZR was alone, the most abundant VOC was 2- methyl-propanoic acid. In the adults of Reduviidae Triatominae, this acid is released by Brindley's glands when subjected to stress or dangerous situations, independently of the individual's sex [27,29,59–65]. The gland system of adults of *Zelus* ssp. (Reduviidae, Harpactorinae) includes only Brindley's glands [30]. At high doses, 2-methyl-propanoic acid elicits alarm responses in adults and juveniles of *Triatoma infestans* (Klug, 1834) [62,63] and *R. prolixus* [64]. In contrast, this acid in low doses and mixtures with other organic acids in the blends produced by Brindley's glands has attracted the juvenile stages of *T. infestans* [66]. 2-Methyl-butanoic acid makes up the volatilome of ZR and has been found to be secreted in Brindley's glands and, together with its derivatives, is part of the alarm pheromone blend of *T. infestans* [27]. Therefore, the production of these organic acids suggests that they may perform similar functions in ZR bionomics.

3-Methyl-1-butanol has never been reported among the compounds released by Reduviidae and can be considered one of the precursors of 2-methyl-butanoic and 2-methyl propanoic acids. 3-Methyl-1-butanol can be a derivative of isopentenyl pyrophosphate or its isomer dimethylallyl pyrophosphate. These isomers are derivatives of mevalonic acid, formed by coupling 3-unit acetyl-coenzyme A [67]. The oxidation of 3-

methyl-1-butanol, commonly known as isoamyl alcohol, gives 3-methyl-butanoic acid [68]. 2-Methyl-butanoic acid can originate from 3-methyl-1-butanol or putative precursors (isopentenyl and dimethylallyl pyrophosphate) by hydrolysis, hydrogenation, and oxidation. However, 2- methyl-butanoic acid can also come from the poly-acetate pathway. Furthermore, 2-methyl-propanoic acid (or isobutyric acid) can also be formed from 3-methyl-1-butanol by successive transformation into 3-methyl-butanoic acid (primary oxidation) and 3-methyl- 2-oxobutanoic acid (secondary oxidation), before finally undergoing decarboxylation. Thus, 3-methyl-1-butanol may precede the two more abundant organic acids detected in the ZR volatilome in storage in the gland reservoir. Possible transformation into 2-methyl- butanoic and 2-methyl propanoic acids occurs by spontaneous oxidation, hydrolysis, hydrogenation, and decarboxylation occurring in the environment.

3-Methyl-1-butanol, 2-methyl-propanoic, and 2-methyl-butanoic acids individually stimulated ZR to move away to areas of the olfactometer devoid of olfactory stimuli (controls), suggesting their role as alarm pheromones, as is already known for other reduviid species [27,64]. These three substances could compose the alarm pheromone blend of *Z. renardii*, with a predominance of 2-methyl-propanoic acid. Further studies will include the estimation of Kovats' retention indices for each VOC using authentic standards and could investigate how combinations of these compounds could influence the behaviour of the ZR.

The VOCs detected are substances with low molecular weights and high volatility, typical characteristics of compounds that stimulate defensive behaviour [69]. The characteristics of VOCs allow them to quickly reach the olfactory receptors of conspecifics and to be rapidly eliminated after a disturbance. In this way, risk communication and the defensive response of conspecifics are immediate [69].

The secretion of alarm pheromones induces behavioural changes in the conspecific that detects them [70]. These consist of a series of defensive behaviour strategies, which may include the detection of danger (defence), avoidance of the threat (escape), or deterrence through attack (fight) [69]. ZRs run away and show defensive strategies, reacting to all three VOCs tested separately. This evidence confirms that 2-methyl-propanoic acid, 2-methyl-butanoic acid, and 3-methyl-1-butanol act individually as alarm pheromones in ZR. 3-Methyl-1-butanol elicited the highest escaping behaviour, followed by 2-methyl-butanoic and 2-methyl-propanoic acids.

The concentration of volatile organic acids shows a significant standard deviation when the predator is alone, whereas it is more stable during the interaction. The minor fluctuations could be due to our experimental systems' separation networks between prey and predator. The predation behaviour of *Zelus* includes ambushing or prey stalking [71]. The presence of stable support during the interaction with PS, which provides the predator with the opportunity to hide by ambushing the prey, could cause the ZR to perceive a reduction in stress, thereby stabilising the production of alarm pheromones.

When the predator is alone, it releases significantly higher amounts of 2-methyl-propanoic acid and 2-methyl-butanoic acid, reducing their secretion in the presence of prey. Predators can modulate the secretion of alarm pheromones, and ZR can reduce the release of alarm pheromones to optimise disguise during the ambush and increase the predation rates. Only 3-methyl-1-butanol increases during the interaction, eliciting an alarm response in ZR. The presence of this compound is connected to the reduction of 2-methyl-propionic acid and 2-methyl-butanoic acid, which could be precursors.

Predators use kairomones to locate their prey in natural habitats. Kairomones emitted by prey or synomones released by host plants can attract predators or parasitoids [72,73]. Prey can, likewise, develop mechanisms to detect the predator's presence that elicit defensive responses to escape the threat of predation [69]. In addition, the alarm pheromones of some species can induce defensive behaviour in other species sharing the same habitat [70]. The same VOCs may mediate different intra- and interspecific responses [74]. Perception of predators is a crucial adaptation for reducing predation risk and maintaining prey fitness [75].

Modulating alarm pheromone production may also mark the predation area of ZR, keeping conspecific prey competitors at a distance. Some plant pests and predators use territory marking to reduce intraspecific competition for food sources. For example, females of more than 20 species of Tephritidae (Diptera) of the genera *Ceratitis*, *Anastrepha*, *Rhagoletis*, and some *Bactrocera* [76] mark the host with pheromones at oviposition sites by dragging the surrounding area with the ovipositor [77]. Host-marking pheromones inhibit further and subsequent egg-laying, reducing competition from various larvae for the same food source and cannibalism events in favour of species fitness. Moreover, pheromone marking is also known for several predator species belonging to different insect orders [78]. During the search for prey, the larvae of some Coccinellidae and Chrysopidae deposit oviposition-deterring pheromones (ODPs) to induce conspecific females to not lay eggs near insect colonies or areas already occupied [79–82]. ODPs avoid intraspecific competition for the same food resources, favouring the suitability of predators [81].

CONCLUSIONS

Zelus renardii has a pair of Brindley's glands on the second urite that release their secretion through metathoracic outlets. Brindley's glands are present in males and females of ZR and produce alarm pheromones.

Zelus renardii uses its capacity to produce VOCs. *Philaenus spumarius*, the main *Xylella* vector, uses vibrational communication much more than chemical communication, having a lesser ability to secrete VOCs.

Zelus renardii, in situations of stress or danger, can produce a mixture of substances that act as alarm pheromones towards conspecifics. This bouquet consists of 2-methyl-propanoic acid but also 2-methyl-butanoic acid and 3-methyl-1-butanol as significant components.

The predator modulates the secretion and release of this blend depending on the presence of stress or prey. When ZR interacts with PS, it reduces the production of alarm pheromones and the possibility of being detected by its prey. Alternatively, the modulation of alarm pheromone production may help the predator mark its predation territory, displacing conspecific competitors, thus reducing competition.

Future evidence of the ability of *Z. renardii* to mark its predatory area may lead to plans for massive releases of the predator to contain the *Xylella* vector population without incurring cannibalism or predatory competition.

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SUPPLEMENTARY MATERIALS

Table S1: VOCs produced by *Z. renardii* only. Abbreviations: M-VOCs = major VOCs; m-VOCs = minor VOCs; R.T. = Retention Time; P.H. = Peak Height; N° Rep. = Number of replicates in which the chemical was detected. In bold are the chemicals found in at least half of the replicates.

Zelus renardii				
Compound	**R.T. (min)**	**P.H. (ppm)**	**Match (%)**	**N° Rep.**
M-VOCs				
2-methyl-propanoic acid	15.838	1631335	82	5
2-methyl-pentanoic acid	19.333	8615617	72	1
2-methyl-butanoic acid	20.346	2016627	76	4
m-VOCs				
2-pentanone	6.123	22245	74	1
2-butanol	6.800	29187	78	1
3-methyl-butanal	8.476	28567	91	2
2-pentanol	10.095	28326	83	1
3-methyl-1-butanol	12.035	50936	82	4
1-ethyl-4-methyl-cyclohexane	17.722	44109	81	1
3,5-dimethyl-octane	18.126	13186	50	1
3,6-dimethyl-octane	18.484	33890	90	1
propyl-cyclohexane	18.600	41630	72	2
methoxyacetic acid, 2-ethyl-cyclohexyl ester	19.739	44727	50	1
1,1,4-trimethyl-cyclohexane	19.744	40179	58	1
cis-1-methyl-4-(1-methylethyl)-cyclohexane	20.224	25588	72	1
1-nonyl-cycloheptane	20.227	19839	59	1
cyclooctanemethanol	20.387	25796	61	2
1-methyl-3-propyl-cyclohexane	21.101	32210	80	1
cis-2-oxabicyclo[4.4.0]decane	21.105	20596	72	1

3,5-dimethyl-3-heptene	21.232	20442	53	1
4-propyl-3-heptene	21.233	22782	57	2
octacosyl trifluoroacetate	21.402	20228	72	1
2,6-dimethyl-nonane	21.700	70108	81	2
(1-methylpropyl)-cyclohexane	22.142	20244	50	1
(2-methylpropyl)-cyclopentane	22.144	29617	55	1
(2-methylpropyl)-cyclohexane	22.327	62063	83	1
methoxyacetic acid, 2-ethylhexyl ester	22.994	37049	72	1
8-methyl-heptadecane	23.356	45083	59	1
3,4-dimethyl-undecane	23.358	22567	78	1
1-ethyl-2-methyl-cyclohexane	24.073	17192	59	1
2,4,4,6-tetramethyl-hept-2-ene	24.081	17826	53	1
trans-1,3-dimethyl-cyclohexane	24.082	14294	50	1
2-methyl-1-methylene-3-(1-methylethenyl)-cyclopentane	34.108	18235	64	1
2,5-bis(1,1-dimethylethyl)phenol	38.214	14754	64	1
2-methyl-propanoic acid, 1,3-propanediyl ester	39.667	23619	50	1

Table S2: VOCs produced by *P. spumarius* only. Abbreviations: m-VOCs = minor VOCs; R.T. = Retention Time; P.H. = Peak Height; N° Rep. = Number of replicates in which the chemical was detected. In bold is the chemical found in at least half of the replicates.

Philaenus spumarius				
Compound	**R.T. (min)**	**P.H. (ppm)**	**Match (%)**	**N° Rep.**
m-VOCs				
cyclohexane	6.004	20063	90	1
2,3-butanedione	6.114	91014	90	1
3-methyl-butanal	8.482	32148	93	2
dimethyl-disulfide	11.958	20061	95	3
propyl-cyclohexane	18.600	13245	50	1
benzeneacetaldehyde	24.729	21050	82	2

2-phenoxy-ethanol	31.247	9980	89	3
2,4,6-trimethyl-benzaldehyde	32.535	27425	95	7
isobornyl acetate	34.102	19715	64	1
7,7-dimethyl-2-methylene-bicyclo[2.2.1]heptane	34.103	18591	60	1
2-methyl-1-methylene-3-(1-methylethenyl)-cyclopentane	34.105	17908	58	1
isobornyl formate	34.106	16291	69	2
hexanedioic acid, bis(2-ethylhexyl) ester	49.789	38055	64	1

Table S3: VOCs produced by the interaction between *Z. renardii* and *P. spumarius*. Abbreviations: m-VOCs = minor VOCs; M-VOCs = major VOCs; R.T. = Retention Time; P.H. = Peak Height; N° Rep. = Number of replicates in which the chemical was detected. In bold are the chemicals found in at least half of the replicates.

***Zelus renardii – Philaenus spumarius* interaction**				
Compound	**R.T. (min)**	**P.H. (ppm)**	**Match (%)**	**N° Rep.**
M-VOCs				
3-methyl-1-butanol	12.656	256956	87	5
2-methyl-propanoic acid	15.262	802433	95	7
5-(1-methylethylidene)-1,3-cyclopentadiene	16.905	138665	72	1
2-methyl-butanoic acid	18.643	507820	68	5
2-methyl-pentanoic acid	18.741	2106728	72	1
1-ethyl-2-methyl-benzene	20.806	115927	93	2
4-cyclohexyl-decane	22.338	147675	90	1
1,2,4-trimethyl-benzene	22.832	140645	94	1
4-ethyl-decane	22.951	217784	76	2
1-butyl-2-propyl-cyclopentane	23.852	103884	58	1
5-ethyl-1-nonene	23.854	128398	56	2
1-(ethenyloxy)-octadecane	29.014	122934	80	1
m-VOCs				
2,3-butanedione	6.116	25207	85	2
2-pentanol	10.884	34370	83	1
2,4-dimethyl-hexane	13.082	66112	80	1
2-(pentyloxy)-ethanol, acetate	17.365	21772	50	1
1,3,5,7-cyclooctatetraene	18.012	47404	90	3

hexanoic acid, cyclohexyl ester	18.265	23572	50	1
4,5-dipropyl-octane	18.841	65768	50	1
2,5,6-trimethyl-decane	19.304	57766	59	1
octadecyl-trifluoroacetate	20.777	63657	53	1
2-ethyl-1,3-dimethyl-cyclohexane	21.031	50495	72	1
cis-1-ethyl-2-methyl-cyclohexane	21.108	55471	72	1
2-amino-6-methyl-benzoic acid	21.692	21331	52	2
γ-terpinene	23.457	87288	96	2
2-ethyl-1-hexanol	23.608	22146	80	2
1,2,4,5-tetramethyl-benzene	23.777	79533	94	1
7-methyl-2-decene	23.988	59328	52	1
2,4,4,6-tetramethyl-hept-2-ene	24.076	50924	50	1
1-ethyl-2-methyl-cyclohexane	24.079	54621	50	2
4-ethyl-1,2-dimethyl-benzene	24.559	84189	76	2
4,5-dimethyl-nonane	24.883	24371	53	1
1-ethenyl-3-ethyl-benzene	25.029	33284	91	1
(2-methyl-2-propenyl)-benzene	25.031	34302	56	1
3-methyl-eicosane	26.586	32955	59	1
cis-1,4-dimethyl-cyclooctane	27.351	34427	90	1
cyclododecane	27.351	34416	93	2
3-ethyl-benzaldehyde	28.502	19743	55	1
4-dodecene	29.016	88184	55	1
decanal	29.018	26787	82	2
4-methyl-tetradecane	30.197	78737	72	2
benzothiazole	30.466	19724	93	2
2,4,6-trimethyl-benzaldehyde	32.543	42232	92	6
thymol	32.835	98201	95	1
6-methyl-1-octene	32.936	21872	70	1
2,4,6-trimethyl-benzoic acid, methyl ester	33.470	29379	93	3
butanoic acid, heptyl ester	34.705	13714	59	1
cyclotetradecane	34.758	37934	95	1
2-(methylamino)-benzoic acid, methyl ester	35.364	20864	97	1
3-ethyl-5-(2-ethylbutyl)-octadecane	36.358	52995	80	2

Table S4: List of VOCs detected by *P. spumarius* progressive aggregation. Abbreviations: m-VOCs = minor VOCs; R.T. = Retention Time; P.H. = Peak Height, PS= *P. spumarius*.

***Philaenus spumarius* intraspecific interaction**							
Compound	**R.T. (min)**	**P.H. (ppm)**	**Match (%)**	**1PS**	**2PS**	**5PS**	**10PS**
m-VOCs							
dimethyl-disulfide	11.970	34445	97			X	X
3,6,6-trimethyl-bicyclo[3.1.1]hept-2-ene	18.874	20553	90			X	
1-ethyl-2-propyl-cyclohexane	19.747	19633	50	X			
(S)-1-methyl-4-(1-methylethenyl)-cyclohexene	22.464	14935	90		X		
1,5-dimethyl-1,5-cyclooctadiene	22.472	22190	96	X			
1,2,3,5-tetramethyl-benzene	22.639	15393	90	X			
(+)-2-bornanone	27.948	24886	95	X	X		
2,4,6-trimethyl-benzaldehyde	32.557	16781	86	X	X	X	X
methyl-eugenol	34.810	48146	98	X			
2,5-bis(1,1-dimethylethyl)-phenol	38.235	17342	78			X	X

Table S5: PERMANOVA analysis of the volatilome between *Zelus renardii* alone and *Zelus renardii* interacting with *Philaenus spumarius*.

Factor	Df	Sum Sqs	R2	F	P.value
Treatment	1	0.6274	0.1669	2.4043	**0.03**
Residual	12	3.1311	0.833		

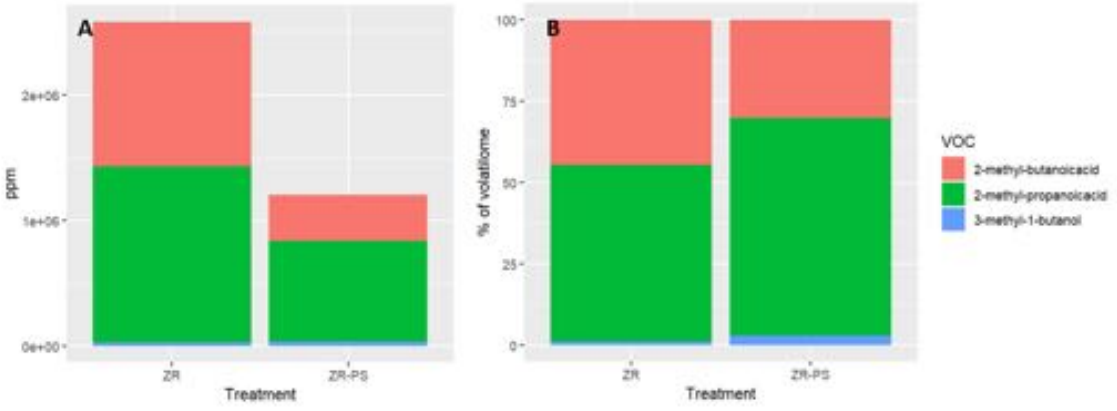

Figure S1: A) parts per million of each VOC of volatilome in each treatment. B) % of each VOC of volatilome in each treatment. Abbreviations: ZR = *Zelus renardii* alone; ZR-PS = *Zelus renardii* interacting with *Philaenus spumarius*.

Table S6: SIMPER analysis showing the volatile that most contributed to the observed differences between groups.

Compound	Contribution	Cumulative contribution	p.value
2-methyl-propanoic acid	0.617	0.617	0.034
2-methyl-butanoic acid	0.359	0.976	0.520
3-methyl-1-butanol	0.024	1.000	0.591

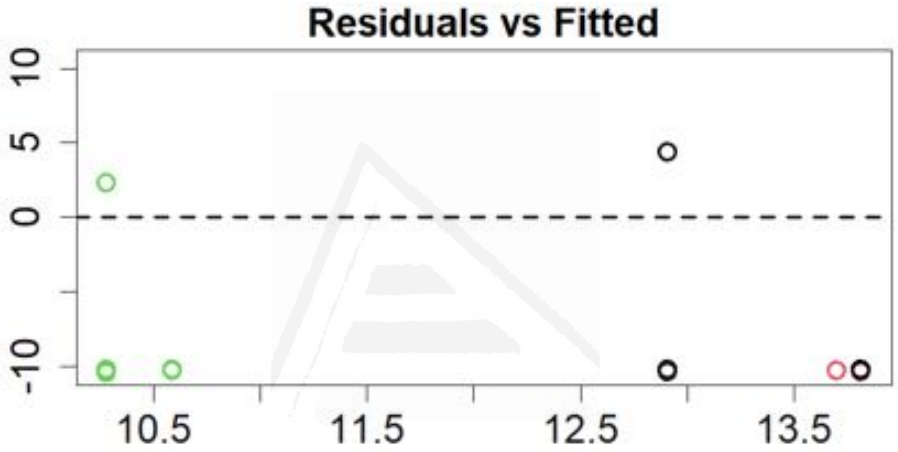

Figure S2: Residual versus fits plot of multivariant glmm with Poisson error distribution.

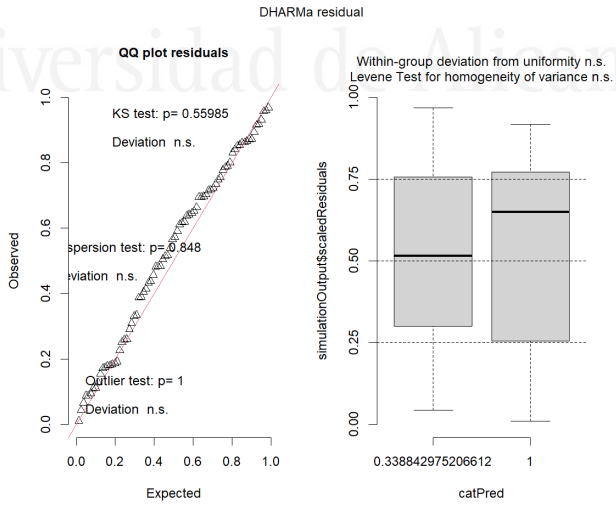

Figure S3: Diagnostic plots of GLMM model (final choice) of 3-methyl-1-butanol acid.

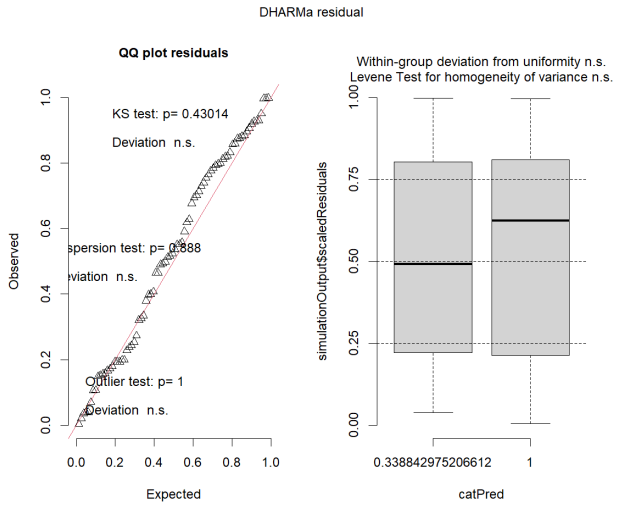

Figure S4: Diagnostic plots of GLMM model (final choice) of 3-methyl-1-butanol acid.

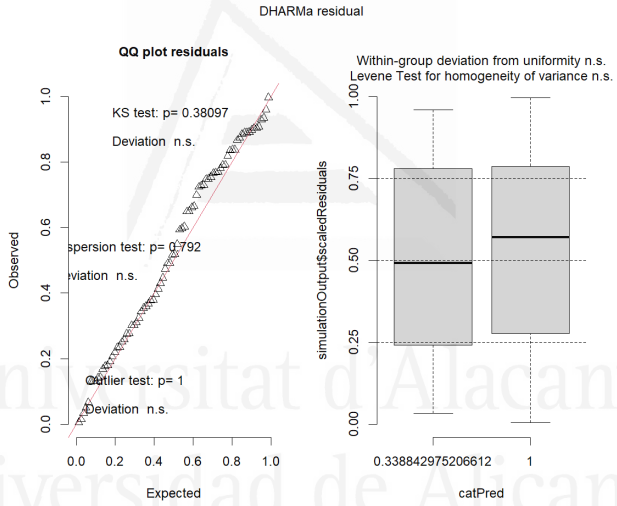

Figure S5: Diagnostic plots of GLMM model (final choice) of 2-methyl-propanoic acid.

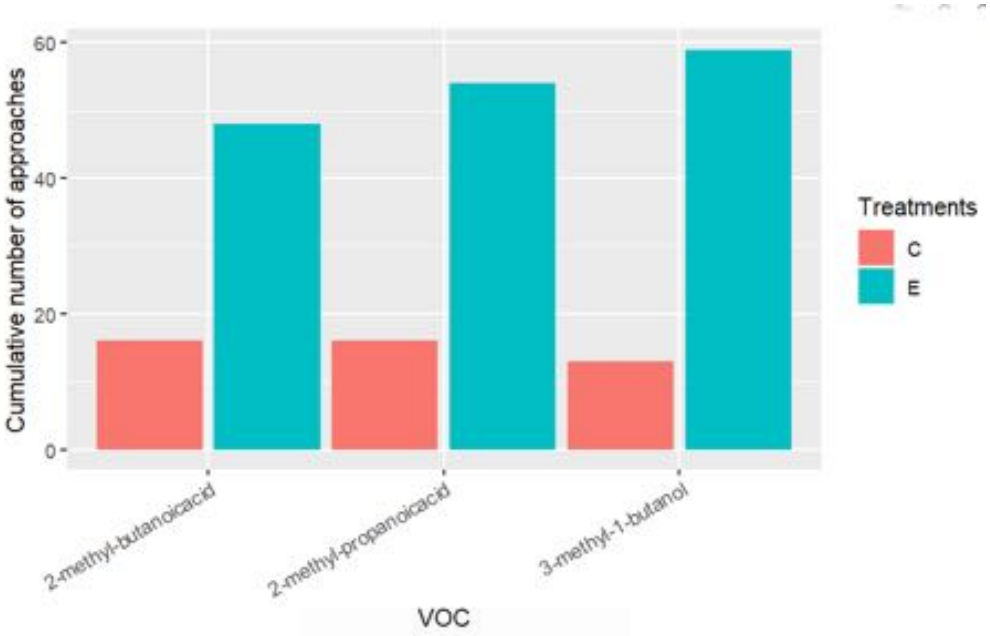

Figure S6: Cumulative number of approaches for each VOC in each treatment. Abbreviations: C = compound; E = empty (control without stimulus).

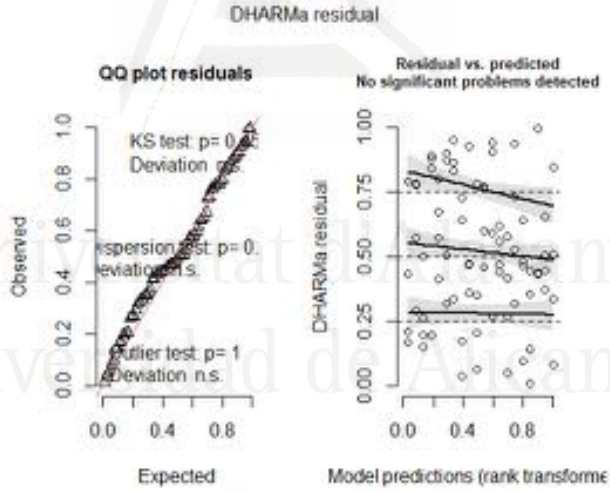

Figure S7: Diagnostic plots of GLMM model (number of approaches) of 2-methyl-propanoic acid.

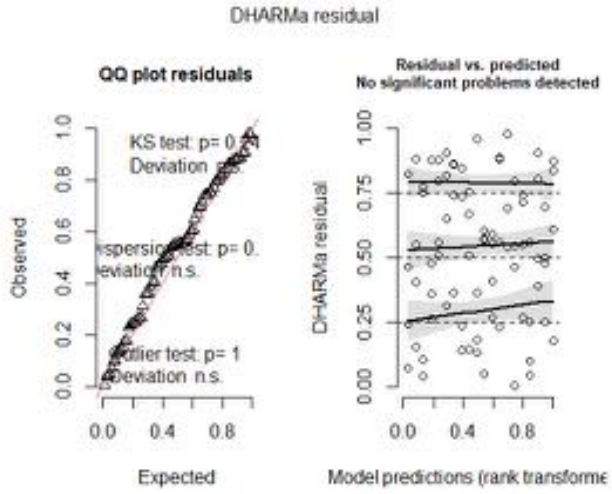

Figure S8: Diagnostic plots of GLMM model (number of approaches) of 2-methyl-butanoic acid.

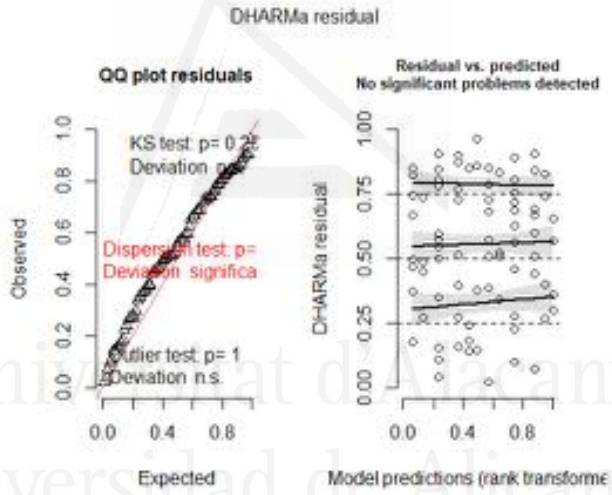

Figure S9: Diagnostic plots of GLMM model (number of approaches) of 3-methyl-1-butanol acid.

Chapter 4 – Performance of Artificial Diets for *Zelus renardii* Rearing

Abstract

Mass production is a prerequisite for using natural enemies in integrated pest management and organic farming. Natural enemies in agroecosystems include predators, which can subdue prey while maintaining adequate population densities. The Leafhopper Assassin Bug (LAB), *Zelus renardii* Kolenati, 1856, can act as a natural enemy in agroecosystems because it is stenophagous on hemipterans, selecting them based on size and mobility. Some of LAB's prey include *Philaenus spumarius* (L., 1758), *Bactrocera oleae* (Rossi, 1790), *Drosophila suzukii* (Matsumura, 1931), and *Macrohomotoma gladiata* (Kuwayama, 1908), suggesting this reduviid as a biocontrol agent in various contexts. The involvement of LABs in biocontrol strategies presupposes their mass-rearing. LABs can be effectively mass-reared using live prey, but there is no knowledge of their rearing using artificial diets. Our data show that mass-rearing of *Z. renardii* is feasible with oligidic, meridic, and holidic artificial formulations. Four artificial diet formulations allowed for the post-embryonic development of LAB to be completed entirely in captivity. The accumulated degree-days (ADD) method accurately predicts the growth of LABs based on heat accumulation, estimating that up to three generations could grow per year in captivity.

Introduction

Worldwide, more than 230 naturally antagonistic arthropod species [1] are reared, mostly parasitoid and predatory insects [2]. The mass-rearing of insects involves operations to increase the reproductive potential, or fecundity, of the reared species [3]. An organism's reproductive potential, or fecundity, is the number of eggs a female produces during her lifetime, as opposed to fertility, which represents the rate of eggs hatched relative to the total number of eggs a female lays [4]. Mass-rearing aims to produce many healthy and reproductive individuals [5]. Mass-rearing is a prerequisite for using natural enemies in IPM and organic farming [6] for inundative biocontrol actions. There is a growing interest in the mass rearing of insects in biofarms due to the use of these arthropods in food and feed production [7,8]. Rearing systems are simplified microcosms that create artificial niches, providing the optimal conditions for successful mass-rearing [9,10]. Insect biofactories produce fertile and voracious genetically stable individuals, i.e., capable of transmitting selected traits to their offspring [11]. Mass-rearing requires control of temperature, photoperiod, and feeding [11]. The diet can consist of live prey for predators, hosts for parasitoids, or complex artificial media. The most common practice is rearing natural enemies on live insects; however, this is demanding in cost and labour and is often unsustainable. Furthermore, using live insects poses

health problems by exposing the antagonists to epizootics (e.g., entomopathogens, such as fungi, viruses, or bacteria) [12].

An artificial diet usually reduces mass-rearing costs, making it sustainable and safer. Bogdanow [13] first started developing artificial diets for insects, leading to the development of modern formulations. The diet formulation must adapt to the needs of the antagonist, adjusting the fraction of acquirable nutrients depending on the species and instars. In nature, nutrient availability limits feed quantity and quality, shaping the antagonist's feeding habits [10]. The composition of artificial diets can be chemically defined (holidic), chemically semi-defined (meridic), or chemically undefined (oligidic) [14,15]. The components of a diet must meet the requirements of palatability, nutritional completeness (ratio of macro- to micronutrients), stability (chemical, physical, and biological), and insect bioavailability [10] for each antagonist entity.

Natural enemies in agroecosystems include predators [16] that prey on insects that they can subdue while maintaining adequate population densities [17]. Almost all insect orders [18] have predatory species, of which predatory hemipterans contribute most to the natural and applied biological control of pests [19,20]. Most predatory hemipterans belong to the Geocoridae, Nabidae, Pentatomidae, and Reduviidae genera [17]. Several reduviids thrive on natural or artificial diets [17,21–27]. Artificial populations of *Rhynocoris* spp., *Platymeris* spp., and *Zelus* spp. [27] serve as natural enemies in agroecosystems.

Zelus is one of the largest Reduviidae genera, with 71 species [28,29], and is native to Tropical America [30]. Globalization and trade in fresh crops have allowed *Zelus renardii* (Kolenati, 1856), otherwise known as the Leafhopper Assassin Bug (or LAB), to enter and spread in several European countries, where it has acclimated [31–53].

Zelus renardii is a stenophagous predator on Hemiptera and selects its prey based on size and mobility [54]. LAB uses visual stimuli to direct the movement of potential prey through ambushing, stalking, or entangling [55–58]. LAB preys on *Bactrocera oleae* (Rossi, 1790) (Diptera, Tephritidae) and *Philaenus spumarius* (L., 1758) (Hemiptera, Aphrophoridae), of which the latter is a vector of *Xylella fastidiosa* Wells et al., 1987 (Xf) subsp. *pauca* ST53 [59–62]. The Xf subsp. *pauca* ST53 in Italy is responsible for the Olive Quick Decline Syndrome (OQDS), which can lead to the death of olive trees in a few years [61]. Evidence suggests that LAB can be a biocontrol agent in olive grove IPM [54]. Numerical experiments have demonstrated the potential efficacy of the inundative control strategy with LAB against Xf vectors [63]. Inundative control with LAB could stop new infections and reduce Xf transmission to a manageable threshold [63,64]. Therefore, the use of LAB adults for biological inundative control of Xf infections depends on the mass rearing of the predator. Furthermore, LAB preys on invasive pests recently introduced in Europe, such as *Macrohomotoma gladiata* Kuwayama, 1908 (Hemiptera, Homotomidae) and *Drosophila suzukii* (Matsumura,

1931) (Diptera, Drosophilidae) [65–67]. We tested live adult *Drosophila melanogaster* Meigen, 1830 (Diptera, Drosophilidae) (Dm) preys vs. five artificial diet formulations to select the optimal diet for the mass rearing of *Z. renardii*.

Materials and Methods

Insect Sources

Feral adult LABs were collected from citrus trees harbouring a mixed infestation of *Aleurocanthus spiniferus* (Quaintance, 1903) and *Aleurothrixus floccosus* Maskell, 1896 (Hemiptera, Aleyrodidae) [68] on the "Ernesto Quagliariello" University of Bari Aldo Moro's campus (N 41°06'37''; E 16°52'58'') in May 2020. After capture, the LABs thrived with *D. melanogaster* in one Petri dish each to avoid possible cannibalism. Eight pairs were mated randomly daily for one week in ventilated 9 cm ⦰ Petri dishes under laboratory conditions. After mating, the females and males returned to their Petri dishes. One hundred and ninety-eight newborns populated six cohorts. Each cohort originated randomly from 33 eggs laid by all females, and the newborns were isolated in ventilated 3 cm ⦰ Petri dishes 24 hours later. Each cohort used a single diet throughout the entire life cycle (Figure 1C). The five immature instars in the post-embryonic development of the LABs [69] were numbered from N1 to N5, plus Ad for adults. Filter paper disks soaked in sterile distilled water maintained the RH at around 80± 5 %. Additionally, the paper roof of the disks provided a good surface for walking and moulting. The laboratory conditions varied seasonally, with a minimum temperature of 18°C and a maximum temperature of 25°C, using air conditioning in the summer and heating in the winter, as reported by Liccardo et al. [63]. Weekly replacing the Petri dishes reduced the contact of LABs with contaminants, mitigating and avoiding epizootics, and daily cleaning removed debris and faeces.

Diet Delivery

The artificial diets were initially liquids to test the feral LABs' acceptance. Aliquots of 0.25 ml of the liquid diets were poured into a microtube (Eppendorf) cap and delivered to the 9 cm ⦰ Petri dishes (Figure 1A). After a liquid diet acceptance, we gelled the liquid with technical agar (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). We obtained cylinders of jellified diet by punching the gel with plastic cocktail straws. N1 and N2 ate on cylinders that were 0.2 ⦰ x 1 cm (0.03 cm^3) in size, while N3, N4, and N5 and the adults accessed cylinders that were 0.5 ⦰ x 1 cm (0.2 cm^3) (Figure 1B). A small piece of alloy or plastic avoided jelly/filter paper contact, improving cleanliness and mitigating contamination. Feral adults immediately accepted the liquid formulation of the diets below. Based on this response, we chose the formulations to feed the newborns.

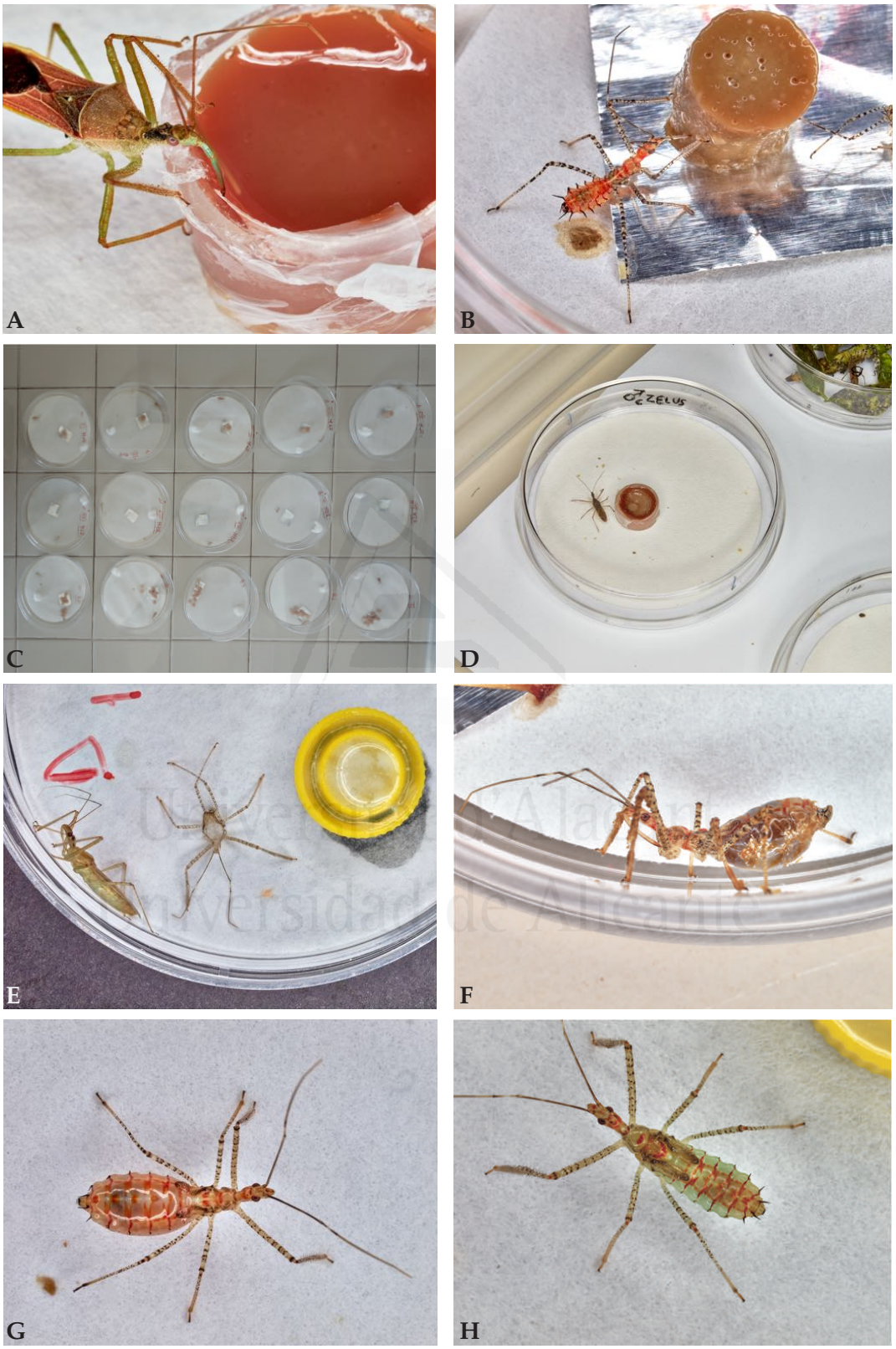

Figure 1: A) feral adult of *Z. renardii* accepting D0 liquid formulation; B) third juvenile instar (N3) feeding on gelled D0; C) mass-rearing of a cohort of *Z. renardii* in the laboratory; D) adult male of *Z. renardii* obtained with D0; E) teneral

adult of *Z. renardii* after metamorphosis, reared with D1; F) side view of a fourth juvenile instar (N4) engorged, after feeding on D0; G) top view of a fourth juvenile instar of *Z. renardii* (N4) engorged, after feeding on D3; H) top view of a fourth juvenile instar (N4) not engorged.

DM: LIVING PREY, *DROSOPHILA MELANOGASTER*

Mass-rearing the LABs with live prey required a continuous supply of adult *D. melanogaster* (vinegar fly—Dm). The vinegar flies thrived in four-liter Plexiglas flasks on an artificial meridic medium consisting of 58.9 g of sucrose (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 58.9 g of maize meal (Bonomelli s.r.l., Zola Predosa, Itay), 2.5 g of agar (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 50 g of fresh brewer's yeast (Lievital®, Lesaffre Italia S.p.A., Trecasali, Italy), 0.5 g of methyl 4-hydroxybenzoate (methylparaben) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and 1.7 ml of propionic acid (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) per liter of sterile distilled water.

The vinegar flies nourished a cohort of 33 LABs for their entire life span, with a sustained rate of five adult *D. melanogaster* every other day per LAB. Mouth aspirators transferred the adult vinegar flies from their flasks to each LAB Petri dish, daily removing the carcasses.

D0: BEEF-LIVER AND EGG-YOLK-BASED OLIGIDIC DIET

The D0 diet had an oligidic base (OB) obtained from 200 g of fresh, homogenized organic beef liver with 20 g of organic egg yolk and 30 ml of a 30% (w/v) sucrose solution. The D0 formulation consisted of 50 g of OB, 1 g of technical agar, 1 g of ascorbic acid (an antioxidant) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and 0.5 g of methyl 4-hydroxybenzoate (methylparaben) in 100 ml of sterile distilled water.

D1: HOLIDIC DIET BASED ON MERITENE MOBILIS®

Meritene MOBILIS® is a nutritionally complete food supplement with chemically defined carbohydrates, proteins, lipids, minerals, and vitamins. The D1 formulation comprised 20 g of Meritene MOBILIS® (Nestlé Health Science, Nestlé S.A., Vevey, Switzerland), 1 g of agar, 1 g of ascorbic acid, and 0.5 g of methyl 4-hydroxybenzoate (methylparaben) in 100 ml of sterile distilled water. The jar of Meritene MOBILIS® reports its composition (Table S1).

D2: HOLIDIC DIET BASED ON MERITENE MOBILIS® AND KCL

The D2 formulation corresponded to the D1 formulation by adding potassium chloride (KCl). KCl increased the K content of the artificial diet according to the physiological importance of the element. Moreover, K and Na are indispensable in the reactions of excitable tissues (e.g., nervous tissue), and potassium is also involved in pH regulation in cells and body fluids [10].

The D2 formulation comprised 20 g of Meritene MOBILIS®, 0.3 g of KCl (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 1.5 g of agar, 1 g of ascorbic acid, and 0.5 g of methyl 4-hydroxybenzoate (methylparaben) in 100 ml of sterile distilled water.

D3: HOLIDIC DIET BASED ON NIDINA® 2 OPTIPRO®

Diet D3 bases on Nidina® 2 OPTIPRO® (Nestlé Baby&Me, Nestlé S.A., Vevey, Switzerland), which is a powdered skimmed milk baby food with a balanced supply of protein, vitamins, and minerals. To obtain the D3 formulation, 20 g of Nidina® 2 OPTIPRO®, 1 g of agar, 1 g of ascorbic acid, and 0.5 g of methyl 4-hydroxybenzoate (methylparaben) were added to 100 ml of distilled water. The jar reports the composition of Nidina® 2 OPTIPRO® (Table S2).

D4: MERIDIC DIET BASED ON MERITENE MOBILIS®, KCL, AND OB

The D4 formulation included 20 g of Meritene MOBILIS® plus 1 g of agar, 10 g of O.B., 0.3 g of KCl, 1 g of ascorbic acid, 0.5 g of methyl 4-hydroxybenzoate (methylparaben), and 100 ml of distilled water. As with the other diets, D4 was gelled and distributed in cylinders of different sizes depending on the predator's instar.

MANAGEMENT OF REARING DATA AND PICTURES

A FileMaker® (FileMaker Inc., Santa Clara, CA, USA), purposely set up as the multimedia database [70], was running on an iPad (Apple Inc., Cupertino, USA) tablet, monitored all relevant biological rearing events, including the following: hatching, moulting, metamorphosis, diet type, sex of adult, date of death, the apparent cause of death, etc.

Several Olympus PEN cameras (Olympus Corporation, Tokyo, Japan) were mounted on a Zeiss Tessovar macro/microsystem (Carl Zeiss GA, Oberkochen, Germany) to record .ORF picture shots. All experiments occurred in the Forensic Entomology Laboratory of the DiSSPA - UNIBA Aldo Moro.

DATA ANALYSIS

The rearing procedure lasted thirteen months in mean laboratory conditions, as described by Liccardo et al. [63], with a reasonable temperature seasonal cycle [71]. The accumulated degree-days (ADD) method measured the life cycle length of the LABs based on the different diets.

Indeed, insects are ectothermic, poikilothermic organisms whose body temperature changes mainly depending on the environment that they develop in [10], plus their metabolic heat increments. The speed

of insect development primarily depends on temperature [72]. According to the formula reported by McMaster and Wilhelm [73], we used ADDs to measure accumulated heat units over time as follows:

$$ADD = \sum_{n}^{1} days \left\lfloor \frac{(Tmax - Tmin)}{2} \right\rfloor - Tbase$$

where n is the number of rearing days, *Tmax* and *Tmin* are the daily maximum and minimum temperatures, and *Tbase* is the development threshold in *Z. renardii*, which is 15 °C [74].

The sex ratio in each diet was analysed using a chi-squared test, followed by a post hoc analysis based on the residuals of Pearson's chi-squared test for count data [75]. Additionally, the mortality of each diet was analysed using the Fisher's test, followed by a post hoc test of pairwise comparisons between proportions. In both cases, the Bonferroni *p-value* correction was applied in the multiple comparisons to reduce the probability of type I errors.

A new variable (cumulative ADDs) was created by summing up the ADDs of each immature instar up to the adult per individual better to analyse the effect of diet up to the adults. An ANOVA test with a Welch's correction was performed due to the lack of homogeneity of the variances. A Games–Howell test was run for significant differences, as it does not assume equal variances [76].

For studying the effect of diet on ADDs at each insect instar, A Kruskal–Wallis test was performed for each instar because of a lack of homogeneity of the variances and the assumption of a missing normal distribution of the residuals. Diet was the fixed factor and ADD was the derivate value. In addition to the descriptive statistics, a predictive model produced the ADD forecast for each instar using a random regression forest (randomForest package) [77]. Insect instar and diet served as predictor variables. The plotmo package was used to plot the consequent five hundred trees. In addition, the rpart.plot package [78] was used to create a decision tree. All plots and statistical analyses were run in R software v. 4.2.2. [79].

RESULTS

SEX AND MORTALITY RATIOS

The chi-squared test showed a significant relationship between diets and sex composition (*p-value* < 0.05) (Figure 2A). In Dm and the meridic artificial diet D4, the proportion of males was higher, while in D1, the balance between both sexes was equal. Furthermore, D3 and D0 showed a higher proportion of females, although this composition was only significant in D0 (*p-value* < 0.05).

As for the effect of diets on mortality, diet D2 had the highest mortality, with 100% mortality (Figure 2B). On the other hand, the Dm had the lowest mortality. However, the mortality of diet D2 versus the rest of the diets only showed significant differences (*p-value* < 0.01).

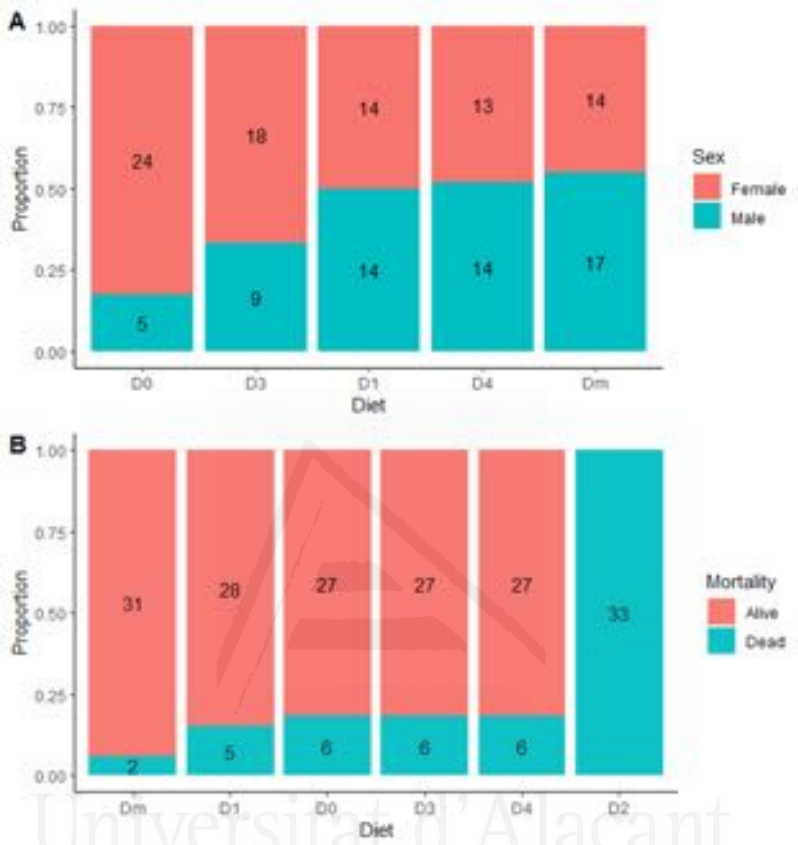

Figure 2: Proportion of (A) sex ratio and (B) mortality rate per diet of the reared *Zelus renardii*.

EFFECT OF DIET ON THE ADDS

Diet D4 showed the highest cumulative ADD value (2191.98 ± 448.72 ADDs), followed by diet D3 (2131.81 ± 321.27 ADDs) (Figure 3). On the other hand, the Dm showed the lowest value (1923.96 ± 466.98 ADDs), closely followed by diet D1 (1931.89 ± 255.40 ADDs). The Welch–ANOVA test found significant differences in the cumulative ADD values (*p-value* = 0.001) (Table S8), although the Games–Howell post hoc test only found significant differences between diets D1 and D4. Noteworthy is the greater dispersion of the data in the Dm and the artificial diet D0 compared to the rest of the artificial diets.

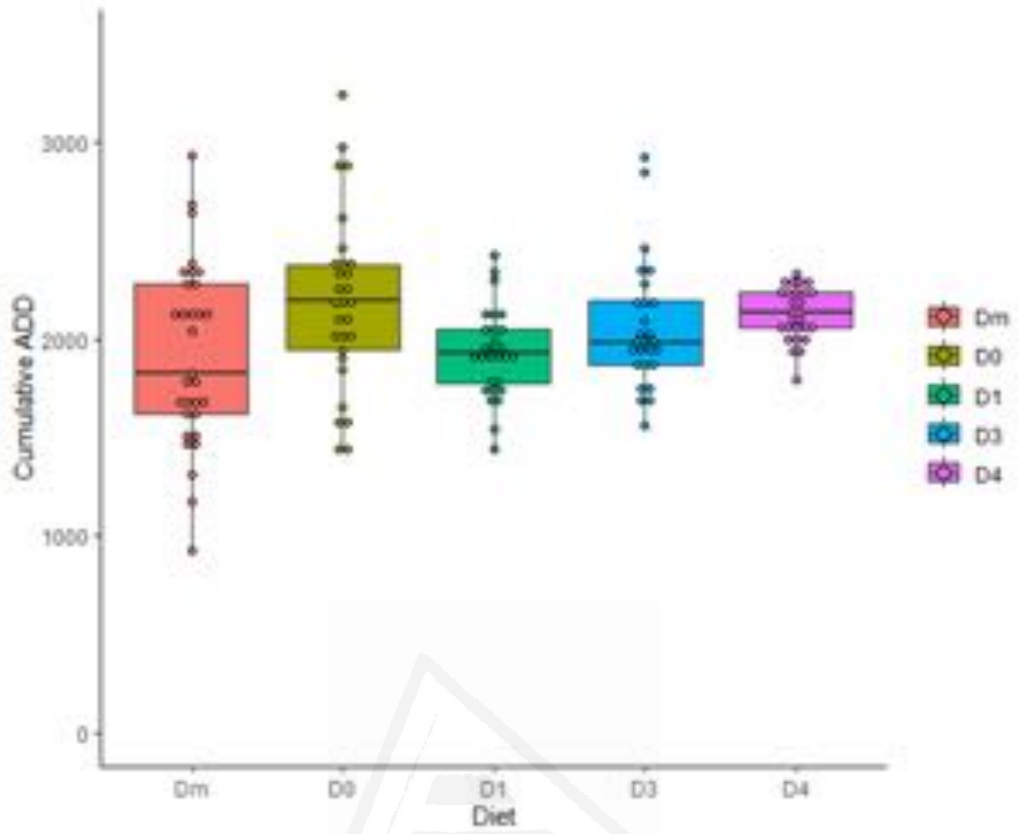

Figure 3: Effect of each diet on cumulative ADDs necessary to complete post-embryonic development.

EFFECT OF DIETS ON EACH INSTAR

The Kruskal–Wallis' analysis of cumulative ADDs per instar showed significant differences between diets at each instar (Figure 4). At the N1 instar, all artificial diets showed higher ADD values than the Dm. On the other hand, the artificial diets D3 and D4 led to significantly higher ADD values than the artificial diets D0 and D1.

At the N2 instar, the differences in ADD values between the Dm and D1 were insignificant; however, they remained between the Dm and the rest of the artificial diets. Furthermore, at the N3 instar, the D0 and Dm did not show significant differences. However, Dm and D0 had lower ADD values than other artificial diets.

At the N4 and N5 instars, the Dm and the D0 diet only showed significantly lower ADD values. However, the comparison of Dm to the rest of the artificial diets resulted in non-significant differences.

Finally, Dm and D4 had significantly lower ADD values for the adults than the rest of the artificial diets. The high dispersion of data in the N2, N5, and Ad instars of artificial diet D0 was noteworthy; furthermore, all diets showed a high dispersion of data at the N5 and Ad instars.

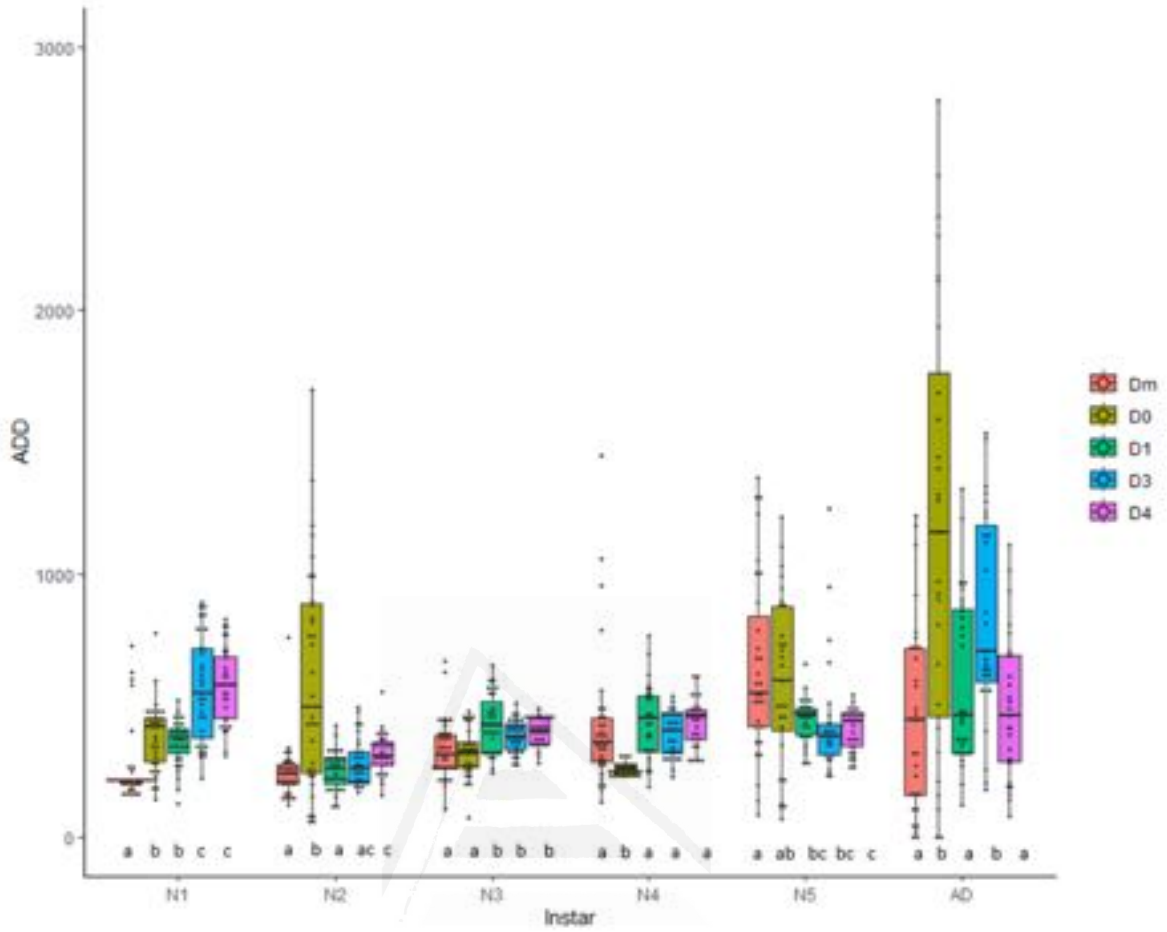

Figure 4: Effect of diet on ADDs for each post-embryonic instar. Abbreviations: N1 = first instar; N2 = second instar; N3 = third instar; N4 = fourth instar; N5 = fifth instar; Ad = adult.

The predictive model (Figures S1–S3) explained 32.46 % of the variance with the instar and diet variables. The most important variable for predicting ADD values was "instar". The model predicted a higher ADD value at instars N5 and Ad (Figure 5). On the other hand, the instar with the lowest predicted ADD value was N2, followed by N1 (Figure 5). Regarding the ADD values expected for each diet, Dm required the lowest number of ADD, closely followed by D1 and D0. In contrast, individuals fed the D3 and D4 diets accumulated more ADDs.

In the predictions of ADDs according to diet and instar, an interaction between both factors was observed. At instars N1, N2, N3, and N4, the D1 diet had the closest value to the Dm diet. Furthermore, D1, D3, and D4 would accumulate fewer ADDs at the N5 instar compared to D0 or Dm. Finally, in the case of Ad, Dm collected fewer ADDs. Nevertheless, the artificial diets allowed for a more significant accumulation of ADDs by the insects, especially D0.

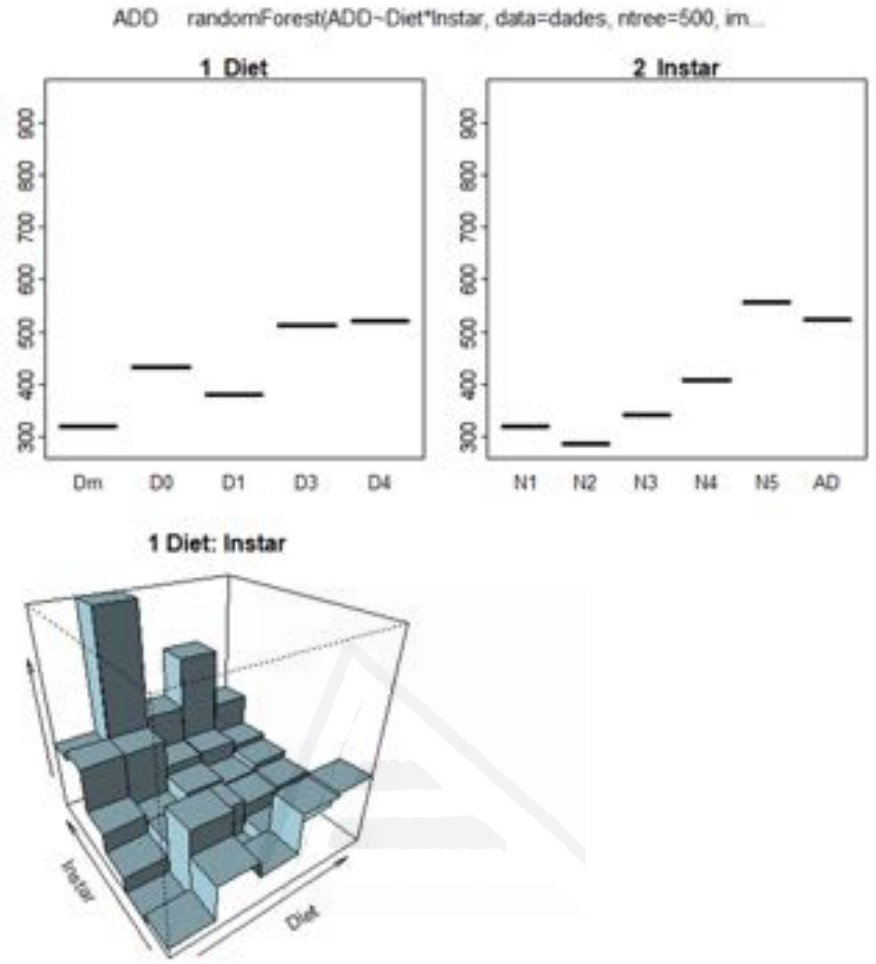

Figure 5: Random Forest plots. The upper plots (1 "diet" and 2 "instar") indicate expected ADDs based on diet or instar. The lower plot shows expected ADDs based on diet and depending on instar. Abbreviations: N1 = first instar; N2 = second instar; N3 = third instar; N4 = fourth instar; N5 = fifth instar; Ad = adult.

The decision tree separated the Ad instar from the rest at the first node, which indicated a different prediction of ADDs based on the diet compared to the rest of the instars (Figure 6). The next node separated D3 and D0 from the rest, from which a higher number of ADDs was expected, especially for D0.

As for the rest, regardless of the diet, the N2, N3, and N4 instars accumulated low ADDs as compared to the rest of the diets and instars. In the case of instar N1, the D0, D1, and Dm diets accumulated fewer ADDs than the rest of the artificial diets.

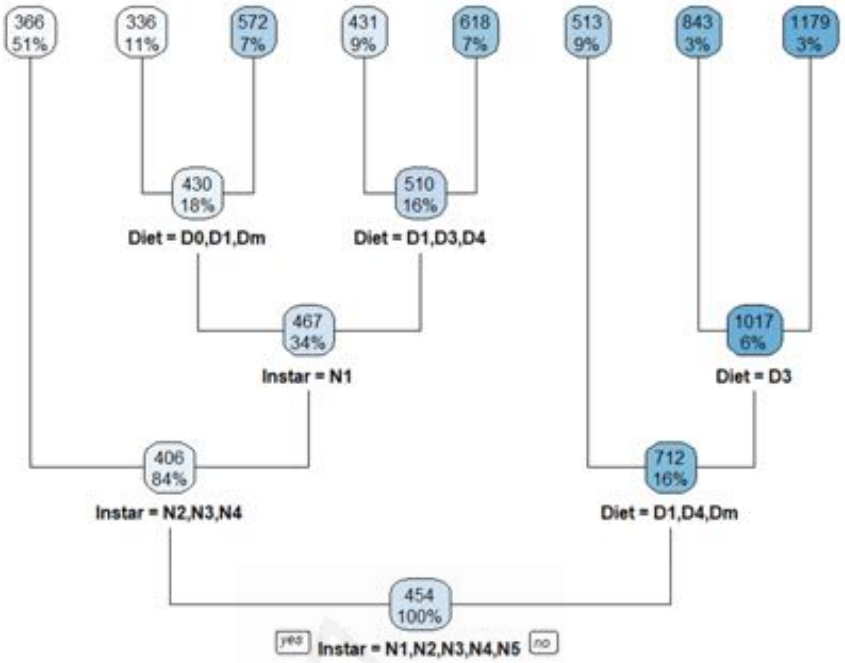

Figure 6: Decision tree to predict the ADDs required for *Z. renardii* to complete its post-embryonic development based on diet and/or instar.

DISCUSSION

The mass production of predators represents a crucial precondition for their successful use in biocontrol programs. Few predatory reduviids are commercially available for pest biocontrol [27]. Until now, there was no information regarding the mass rearing of *Z. renardii* using artificial diets.

The success of mass rearing depends on the quality and type of artificial diet [27], as well as the propensity of the species to live in captivity. LAB is well adapted to mass rearing with live prey [54] and with oligidic, meridic, and holidic artificial formulations. LAB completed its life cycle from egg to adult with all the proposed formulations except for D2. The diets were palatable, stable, nutritional, and bioavailable for LAB, such that they completed the life cycle as expected from an effective artificial diet formulation [5]. All diets that enabled the completion of post-embryonic development ensured a growing population trend according to the species' reproductive potential, as predicted by life tables, according to Carey [80] (Tables S3-S7).

The diets D0, D1, D3, and D4 allowed for the mass rearing of LAB in captivity. The D1 formulation showed the best performance, comparable to that of Dm. We will conduct subsequent studies on mass-rearing techniques and customize the diet to the needs of the predator. A successful combination of species-specific diet and rearing technique will allow testing different options for using *Z. renardii* as a biocontrol.

Furthermore, the performance of the diets for ADDs differed concerning the LAB instars, indicating that nutritional requirements could be instar-specific.

Generally, the mass rearing of insects in captivity intrinsically imposes a change in the genetic structure of the reared species [10], exerting selective pressure on the cohort. Mass rearing can influence sex ratios, but little knowledge exists about the factors influencing sex determination in Reduviidae [27]. In *Zelus* spp., sex determinism occurs chromosomally [81], with a feral assortment LAB percentage of 50:50 (♂: ♀) [69]. Adult LABs obtained with Dm, D1, D3, and D4 maintained a sex ratio of ca. 1:1, whereas the sex ratio of adults reared on the oligidic diet D0 had a percentage of 17:83 (♂: ♀). The D0 formulation may have resulted in differential mortality between the sexes, favouring females, even though female insects generally exhibit greater sensitivity to food stress than males [82]. The possibility of having a diet that brings more females to sexual maturity would allow more individuals to be used in biocontrol actions.

Having abundant individuals in one or more cohorts will allow us to test the efficacy of LAB against other prey-size classes, increasing the predator's effectiveness on release. Similarly, several mixed LAB cohorts of different instars can achieve prey size-dependent predation on other sympatric target pests. We know that young LABs prey on *Drosophila* spp. or *Megaselia* spp. larvae [54], and LAB adults mostly ignore these small insects but quickly subdue *B. oleae*. These observations suggest using different LAB age cohorts in red fruit or olive management. Prey size selection may also influence cannibalism in mass rearing; however, the topic requires further investigation due to contradictory experimental evidence [54]. A diet that maximizes LAB's fitness will allow maximum LAB efficacy for inundation against target prey, such as Xf vectors and similar hemipterans. However, an abundant population of Reduviidae will better tolerate intensive treatments during selection and sanitization from entomopathogens, achieving acceptable absolute individual survival after the process. The sanitization of LABs, in turn, will result in a reasonable basis for the subsequent reproduction of LAB lines.

Rearing LAB lines using "prey learning" can help obtain adults that are promptly aggressive towards a compliant target in terms of size and kairomones. The intention is to utilize LAB's learning evidence to catch live prey. The accumulation of experience during rearing has also been a technique attempted for parasitoid learning [83]. We suggest that LAB is sensitive to manipulation by VOCs or other semiochemicals [84].

ADDs or GDDs (growing degree-days) accurately predict insect development based on heat accumulation [85]. Each insect requires specific heat accumulation to carry out post-embryonic development; DDs (degree-days) interpret this accumulation. The application of ADDs is primarily used in forensic entomology [86–88] and in predictive models for the control of plant pests [85,89–91]. The use of ADDs in mass rearing allows for predicting the time required to complete post-embryonic development,

regardless of the season in which rearing occurs, opening the prospect of continuous annual rearing. With a constant temperature and by using eggs as a biofix (starting point for ADDs), it was possible to predict the duration of LAB's cycle in captivity (estimated ADDs per stage shown in Tables S1-S5). With an average daily temperature of 25 °C, LAB accumulated enough ADDs to produce between two and three generations/year in captivity, as it occurs in nature [32].

CONCLUSIONS

Today, LAB is well established in the European and Mediterranean regions and is moving toward central Asia. This opportunity is valuable to test an alien antagonist such as LAB against endemic Palearctic pests (e.g., *P. spumarius* and *B. oleae*) or invasive aliens (e.g., *D. suzukii* and *M. gladiata*). The first mass occurrence of LAB was in Italy while preying on *M. gladiata*, an invasive alien pest of ornamental figs. Planting ornamental figs and the subsequent construction of appropriate ecological corridors favoured the success of *M. gladiata* and its propagation in the EPPO region. The same corridors also favoured the Mediterranean spread of *Z. renardii*. This occasion was valuable to experience the interaction of an alien antagonist from the Far West struggling in Middle Earth against a pest from the Far East. The predation of LAB on *M. gladiata* prompted us to investigate the feasibility of rearing a predator for release in agricultural and urban areas.

The efficacy of LAB rearing with artificial diets shows the potential for producing and using this predator as a biocontrol agent for inundation programs. The diets tested, while effective in allowing LAB to complete its post-embryonic development, require further refinement concerning predator and stage-specific requirements.

ADDs in mass rearing allow predicting the time required to complete post-embryonic development, regardless of the rearing season. The predator can produce between two and three generations/year in captivity.

We have no evidence of a negative ecological impact of LAB, whose population may self-regulate through cannibalism. Furthermore, we have no evidence of the establishment of LAB in olive groves or other orchards. The opportunity to manipulate *Z. renardii* with semiochemicals and mass rearing with different diets provided a chance to understand the general and applied aspects of using the reduviid in pest management.

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SUPPLEMENTARY MATERIALS

Table S1: Ingredients and nutritional information of Meritene® MOBILIS® (Nestlé® Health Science, Nestlé S.A., Vevey, Switzerland).

Ingredients					
Skimmed milk, milk protein, maltodextrin, inulin, minerals (calcium carbonate, magnesium carbonate, magnesium citrate, zinc gluconate, iron phosphate, calcium phosphate, copper gluconate, manganese sulphate, sodium selenite), sunflower oil, vitamins (C, E, niacin, pantothenic acid, B1, B6, A, B2, folic acid, K, biotin, D, B12), emulsifier: soy lecithin.					
Nutrients	**Unit**	**100g of Meritene® MOBILIS®**	**Nutrients**	**Unit**	**100g of Meritene® MOBILIS®**
total fat	g	3.5	**Minerals**		
carbohydrates	g	36.5	potassium	mg	800
fiber	g	24	chloride	mg	500
proteins	g	44.8	calcium	mg	950
salt (=Na(g) x 2.5)	g	1	phophorous	mg	510
Vitamins			magnesium	mg	250
vit. A	µg	590	iron	mg	6
vit. D	µg	23	zinc	mg	6.7
vit. E	mg	10	copper	mg	0.62
vit. K	µg	70	manganese	mg	1.1
vit. C	mg	180	selenium	µg	41
vit. B1	mg	1.4			
vit. B2	mg	1			
vit. B6	mg	1.4			
folic acid	µg	170			
vit. B12	µg	3.9			
biotin	µg	39			
patothenic acid	µg	4.4			

Table S2: Ingredients and nutritional information of NIDINA® OPTIPRO® 2 (Nestlé® Baby&Me, Nestlé S.A., Vevey, Switzerland).

Ingredients					
Skimmed milk, partially demineralised and fractionated whey powder, vegetable oils (palm, rapeseed, coconut, sunflower), maltodextrin, lactose, soluble fibres (galacto-oligosaccharides from milk and fructo-oligosaccharides), minerals (calcium, potassium, sodium, magnesium, iron, zinc, copper, iodine, selenium), emulsifier: soy lecithin, vitamins (C, E, pantothenic acid, niacin, A, B1, B6, riboflavin, folic acid, biotin, D,K, B12), whey protein, *L. reuteri*					
Nutrients	**Unit**	**100g of Nidina® 2 OPTIPRO®**	**Nutrients**	**Unit**	**100g of Nidina® 2 OPTIPRO®**
total fat	g	23.6	**Minerals**		
carbohydrates	g	59	sodium	mg	190
fiber	g	2.9	potassium	mg	550
proteins	g	9.6	chloride	mg	360
salt (=Na(g) x 2.5)	g	0.48	calcium	mg	560
Vitamins			phophorous	mg	310
vit. A	µg	530	magnesium	mg	50
vit. D	µg	9	iron	mg	7.3
vit. E	mg	9.3	zinc	mg	5.2
vit. K	µg	46	copper	mg	0.35
vit. C	mg	85	manganese	µg	50
thiamine	mg	1	fluoride	µg	<60
riboflavin	mg	1.2	selenium	µg	8.6
niacin	mg	4.4	iodine	µg	13
vit. B6	mg	0.54	**Other nutrients**		
folic acid	µg	118	linoleic acid	mg	3800
vit. B12	µg	1.2	α-linolenic acid	mg	460
biotin	µg	16	lactose	g	35.5
pantotenic acid	mg	5.2			

LIFE TABLES

Life tables allow us to assess the actuarial properties of an insect species by analysing the causes of mortality to know the population trend (T) between one generation and the next. When $T > 1$, the population increases, while $T < 1$ decreases. If $T = 1$, the population is in equilibrium: usually, this situation is only found in populations in natural ecosystems. We used life tables to quantify the effectiveness of different diet formulations on the cohorts of *Z. renardii* tested. We used the classical life tables (Carey, 2001) as a model for understanding the dynamics of *Z. renardii* cohorts by organising age-specific mortality and survival data under laboratory conditions. We reported age-specific mortality rates for the life tables during the experimental period. Life expectancy for each stage is calculated in ADD, and all causes of death are

grouped by instar. The life tables consider cohort survival (*lx*), age-specific mortality (*qx*), age-specific survival (*px*), life expectancy per instar (*ex*) and distribution of deaths (*dx*) as main functions, as reported in Carey (2001). We estimated the population trend (*T*) since the average number of eggs a female *Z. renardii* lays during her lifetime. The calculation considered the number of females obtained per treatment. On average, a female of *Z. renardii* can lay 27 eggs per egg mass and 13 egg masses during her cycle, according to the observations of El-Tom (1965).

Table S3: Life table of the cohort of 33 *Zelus renardii* reared with Dm (*Drosophila melanogaster*). Abbreviations: EG = egg; N1 = first instar; N2 = second instar; N3 = third instar; N4 = fourth instar; N5 = fifth instar; AD = adult; *x* = number of living specimens; *lx* = cohort survival rate; *px* = age-specific mortality rate; *qx* = age-specific mortality rate; *dx* = distribution of deaths per *x*; *ex* = life expectancy per instar in ADD (Accumulate Degree-Days); *T* = population trend.

Dm						
Instar (x)	**Number living(N)**	**Fraction surviving (lx)**	**Age-specific survival (px)**	**Age-specific mortality (qx)**	**Deaths distibution (dx)**	**Expectation of life (ADD) (ex)**
EG	33	1.00	1.00	0.00	0.00	2527.5
N1	33	1.00	1.00	0.00	0.00	2399.5
N2	33	1.00	1.00	0.00	0.00	2137.1
N3	33	1.00	0.97	0.03	0.03	1892.3
N4	32	0.97	0.97	0.03	0.03	1558.5
N5	31	0.94	0.94	0.06	0.06	1128.7
AD	29	0.88	0.00	1.00	0.88	489.3
EG	351					
T = 10.64						

Table S4: Life table of the cohort of 33 *Zelus renardii* reared with D0 (beef liver and egg yolk-based oligidic diet). Abbreviations: EG = egg; N1 = first instar; N2 = second instar; N3 = third instar; N4 = fourth instar; N5 = fifth instar; AD = adult; *x* = number of living specimens; *lx* = cohort survival rate; *px* = age-specific mortality rate; *qx* = age-specific mortality rate; *dx* = distribution of deaths per *x*; *ex* = life expectancy per instar in ADD (Accumulate Degree-Days); *T* = population trend.

D0						
Instar (x)	**Number living(N)**	**Fraction surviving (lx)**	**Age-specific survival (px)**	**Age-specific mortality (qx)**	**Deaths distibution (dx)**	**Expectation of life (ADD) (ex)**
EG	33	1.00	1.00	0.00	0.00	3552.2
N1	33	1.00	0.97	0.03	0.03	3424.2
N2	32	0.97	1.00	0.00	0.00	3039.7
N3	32	0.97	0.97	0.03	0.03	2437.6
N4	31	0.94	0.94	0.06	0.06	2117.5
N5	29	0.88	0.93	0.07	0.06	1860.4
AD	27	0.82	0.00	1.00	0.82	1266
EG	648					
T = 19.64						

Table S5: Life table of the cohort of 33 *Zelus renardii* reared with D1 (holidic diet based on Meritene MOBILIS®). Abbreviations: EG = egg; N1 = first instar; N2 = second instar; N3 = third instar; N4 = fourth instar; N5 = fifth instar; AD = adult; *x* = number of living specimens; *lx* = cohort survival rate; *px* = age-specific mortality rate; *qx* = age-specific mortality rate; *dx* = distribution of deaths per *x*; *ex* = life expectancy per instar in ADD (Accumulate Degree-Days); *T* = population trend.

D1						
Instar (x)	**Number living(N)**	**Fraction surviving (lx)**	**Age-specific survival (px)**	**Age-specific mortality (qx)**	**Deaths distibution (dx)**	**Expectation of life (ADD) (ex)**
EG	33	1.00	1.00	0.00	0.00	2647.6
N1	33	1.00	0.94	0.06	0.06	2519.6
N2	31	0.94	1.00	0.00	0.00	2158.4
N3	31	0.94	0.94	0.06	0.06	1907.2
N4	29	0.88	0.97	0.03	0.03	1476.7
N5	28	0.85	1.00	0.00	0.00	1038.2
AD	28	0.85	0.00	1.00	0.85	593.9
EG	378					
T = 11.45						

Table S6: Life table of the cohort of 33 *Zelus renardii* reared with D3 (holidic artificial diet based on Nidina® 2 OPTIPRO®). Abbreviations: EG = egg; N1 = first instar; N2 = second instar; N3 = third instar; N4 = fourth instar; N5 = fifth instar; AD = adult; *x* = number of living specimens; *lx* = cohort survival rate; *px* = age-specific mortality rate; *qx* = age-specific mortality rate; *dx* = distribution of deaths per *x*; *ex* = life expectancy per instar in ADD (Accumulate Degree-Days); *T* = population trend.

D3						
Instar (x)	**Number living(N)**	**Fraction surviving (lx)**	**Age-specific survival (px)**	**Age-specific mortality (qx)**	**Deaths distibution (dx)**	**Expectation of life (ADD) (ex)**
EG	33	1.00	1.00	0.00	0.00	3028.7
N1	33	1.00	0.97	0.03	0.03	2900.7
N2	32	0.97	0.97	0.03	0.03	2334.6
N3	31	0.94	0.94	0.06	0.06	2052.8
N4	29	0.88	0.93	0.07	0.06	1668.8
N5	27	0.82	1.00	0.00	0.00	1282
AD	27	0.82	0.00	1.00	0.82	842.8
EG	486					
T = 14.73						

Table S7: Life table of the cohort of 33 *Zelus renardii* reared with D4 (meridic artificial diet based on Meritene MOBILIS®, KCl and OB). Abbreviations: EG = egg; N1 = first instar; N2 = second instar; N3 = third instar; N4 = fourth instar; N5 = fifth instar; AD = adult; *x* = number of living specimens; *lx* = cohort survival rate; *px* = age-specific mortality rate; *qx* = age-specific mortality rate; *dx* = distribution of deaths per *x*; *ex* = life expectancy per instar in ADD (Accumulate Degree-Days); *T* = population trend.

D4						
Instar (x)	**Number living(N)**	**Fraction surviving (lx)**	**Age-specific survival (px)**	**Age-specific mortality (qx)**	**Deaths distibution (dx)**	**Expectation of life (ADD) (ex)**
EG	33	1.00	1.00	0.00	0.00	2760.1
N1	33	1.00	0.94	0.06	0.06	2632.1
N2	31	0.94	0.94	0.06	0.06	2054.2
N3	29	0.88	0.97	0.03	0.03	1736.4
N4	28	0.85	0.96	0.04	0.03	1337.3
N5	27	0.82	1.00	0.00	0.00	901.4
AD	27	0.82	0.00	1.00	0.82	492.1
EG	351					
T = 10.64						

Table S8: Welch's ANOVA of ADD with Diet as fixed factor.

Factor	Df	Sum Sq	Mean Sq	F value	*P.value*
Diet	4	1650274	412568	3.382	0.0113*
Residuals	137	16710964	121978		

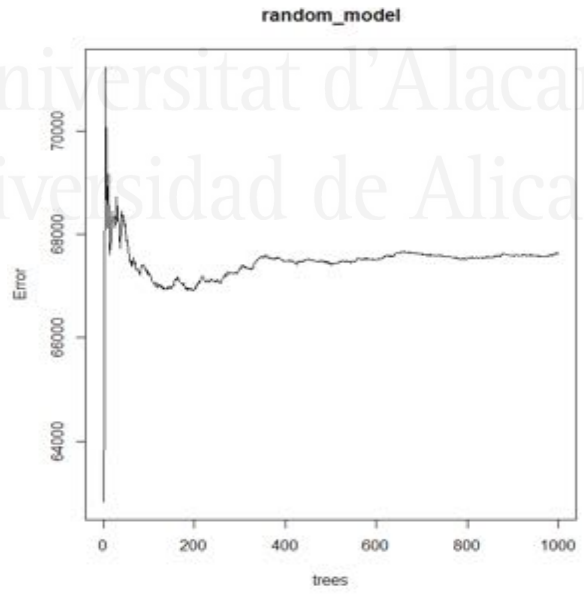

Figure S1: MSE of the random forest model by number of trees.

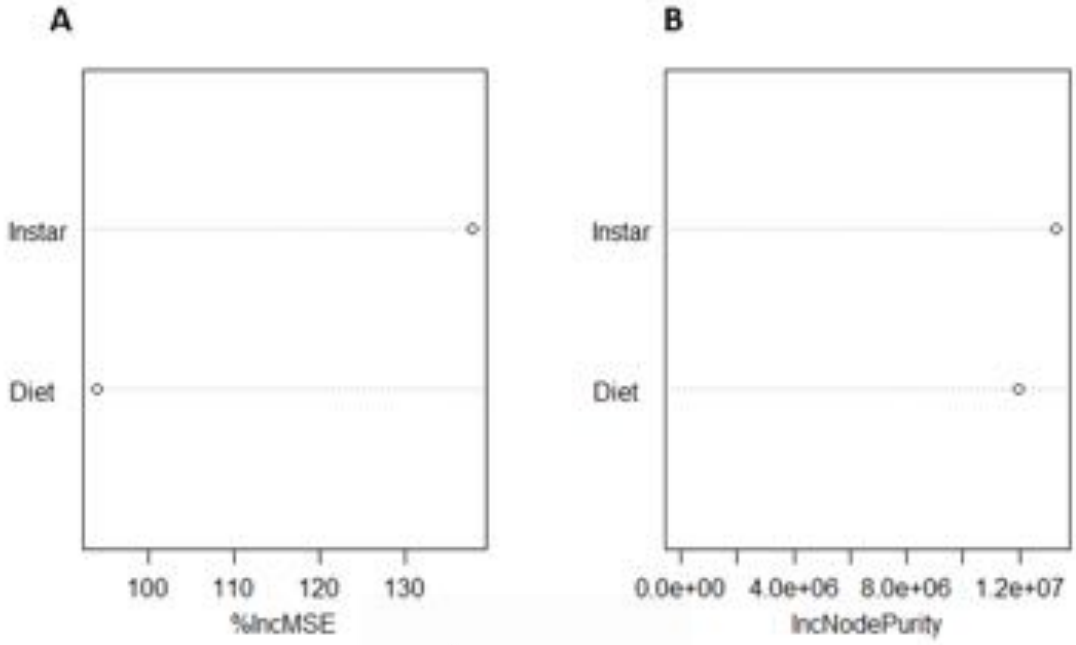

Figure S2: Dotchart of variable importance as measured by a Random Forest. A) Per cent increase in mean square error and B) variable importance plot.

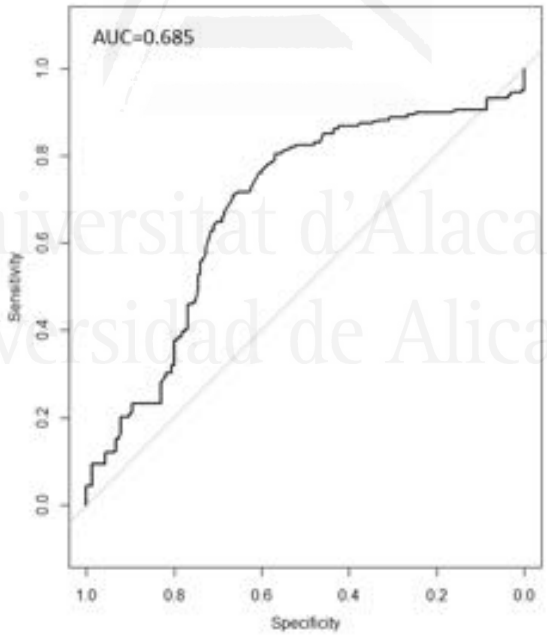

Figure S3: Specificity vs sensitivity plot showing the area under the curve.

Chapter 5 – General Conclusions

Recent estimates show that more than 6,500,000 olive trees are dead in southern Italy due to *X. fastidiosa* infection and consequent OQDS disease, and the affected area continues to expand. OQDS affects millions of olive trees, threatening three-quarters of the world's olive oil production. Most impacts fall on consumers since olive oil prices rise and stocks shorten. Further pathogen invasion would worsen this scenario, causing significant agricultural, environmental, social, and health impacts. OQDS imposes a reduction in the supply of ecosystem services derived from olive orchards of 30–34% and a decrease in associated biodiversity of 28%, in addition to the impacts on productivity and the entire olive oil supply chain. OQDS has a significant economic impact, and future projections do not suggest good olive yields. Slowing down the pathogen spread in *Xylella*-free areas and implementing OQDS mitigation measures in affected areas could help reduce the disease impact, thus restituting benefits.

In a worst-case lattice model, integrated infection management suggests robust options to reverse the hitherto unstoppable invasion of *Xylella fastidiosa pauca* ST53 epidemics. The model stresses the importance of the 'number of vectors per olive tree' parameter in effectively controlling the infection. Reducing one vector per olive tree will reduce *X. fastidiosa* expansion to economically and environmentally acceptable limits. Consequently, scoring the vector field population is crucial to implementing an effective IPM strategy before significant transmissions occur. Quantitative *ex-ante* sampling is essential to trigger the intensity and timing of control actions, as it is the *ex-post* sampling to assess pest management efficacy. *Xylella fastidiosa* can only infect olive trees if efficient vectors acquire the pathogen to infect the susceptible plants available. Reducing vector populations before interacting with *X. fastidiosa* significantly reduces the probability of pathogen acquisition, thus minimising the chances of infecting the target plant.

Insects and microorganisms have undergone long-standing interactions. A deeper understanding of this phenomenon will help to have a complete/multi-layered perspective of the actors involved. These interactions act as an evolutionary engine, enabling actors (guest and host) to cope with environmental stresses such as anthropisation and climate change. Interactions with certain microorganisms may favour optimal niche conditions for the post-embryonic development of insects, as in the case of bacteria living in foams produced by Aphrophoridae juveniles. The microbiota inhabiting the Aphrophoridae juveniles' foamy "nest" may protect juveniles' instars from adverse conditions. Bacterial metabolites could influence the foam or provide antibiotic or repellent effects. Bacterial symbiosis disruption may significantly change the niche feature, deleting the functional association between pests and microorganisms and reducing the target insect's fitness. Therefore, changes in the interaction with ectosymbionts in Aphrophoridae foams could pave the way for developing biological or biotechnological control means targeting the juvenile

instars, proposing a further tool to minimise the vector population before they can spread the *Xylella fastidiosa*.

Strategies against *Xylella* transmission require more biocontrol actions in an olive orchard IPM strategy. However, the currently available guild of vector antagonists is less effective than requested. Intense *P. spumarius* biocontrol action will strengthen infection management, offering new organic or agroecological crop production options. The recent appearance of *Zelus renardii* (Zr) in the Palaearctic Region offers the opportunity for its evaluation for olive pest biocontrol, including *Xylella* vectors. *Zelus renardii* showed remarkable predatory capacity against *Xylella* vectors. Numerical simulations also validated the efficacy of *Z. renardii* in reducing feral *P. spumarius* populations. The reduviid could play several roles in controlling bacterial invasion in infection management, reducing the overall vector populations, preventing egg laying, and killing pre-reproductive females. Assuming reducing 1,000,000 *P. spumarius* individuals to 90,000 *P. spumarius* adults per hectare, after eggs and juveniles mechanical control action, the introduction of about 4,000 Zr per hectare would be sufficient to reduce the incidence of infection to 10% in only two years without using synthetic chemical insecticides.

A further advantage of using *Z. renardii* is its ability to manage many olives' secondary pests at the cost of infection biocontrol. Nevertheless, the predation probability for *Z. renardii* is a function of the prey abundance (frequency of encounters) and, thus, the Reduviid will prey pest, preferably. *Zelus renardii* prefers larger pest prey than smaller hymenopteran parasitoids such as *Metaphycus* spp. (Encyrtidae), *Eurytoma* spp. (Eurytomidae), or *Pnigalium* spp. (Eulophidae). Olive trees are wind-pollinated, and since common pollinators do not collect pollen from olive flowers, they are not likely to be affected by an inundative biological control strategy in olive groves, including *Z. renardii*.

Zelus renardii has been considered a generalist predator capable of disrupting the natural enemy action by intra-guid predation. The suspect originates from simplified experiments in small cages exerting physical interference over a limited number of species and individuals forced in a limited volume. Overall evidence suggests that *Z. renardii* is stenophagous towards Hemiptera. *Zelus*' prey choice uses various factors, including habitat, host plant, abundance and mobility of the species encountered.

The promising results from laboratory experiments and the suggestions from numerical modelling support the potential efficacy of using *Z. renardii* to counteract *Xylella* infection, containing the devastating OQDS affecting olive production in southern Italy. Therefore, further *Z. renardii* ecological and physiological data will help to fully understand its role in reducing bacterial pathogen transmission to olive trees.

Zelus renardii can regulate the secretion and release of alarm pheromones from its own Brindley's glands in response to stress or the presence of prey. The interaction between *Z. renardii* and *P. spumarius* leads to

a decrease in the production of alarm pheromones that would reduce the capability of prey to detect the predator. This regulation of alarm pheromones can allow the predator to mark its preying territory, driving out conspecifics. This action may also reduce competition and cannibalism, thus improving *Z. renardii*'s natural enemy efficacy, and opening opportunities for the predator's behavioural manipulation.

Furthermore, adequate artificial diets for *Z. renardii* mass rearing will help to test this predator under controlled conditions. Such experiences would allow the inundative release of *Z. renardii* into the field for improving the existing *X. fastidiosa* IPM strategy.

The proposed biological and biotechnological control actions can contribute significantly to the IPM strategy of olive groves, leading to a substantial reduction (by several orders of magnitude) of *P. spumarius* populations and actively influencing the infective capacity of *Xylella* vectors. From a farm IPM perspective, this would result in a gradual decrease in the intensity of physical, biological, and chemical *X. fastidiosa* control measures over time, with a consequent reduction in the management costs of olive groves not yet affected by *X. fastidiosa*. These actions would act in preventive (avoiding future infections by the vector, as the insect dies during or immediately after acquiring the bacterium) and protective ways (limiting the action of the vector to a single infection, preventing repeated transmissions on the same or other plants).

Finally, considering that the olive tree hosts a range of pests equally problematic for crop yield, modelling a holistic management strategy through the combined use of physical, chemical, and biological measures ensures a better overall integrated production of the olive agroecosystems. These control actions undergo the TARDIS (Time And Relative Dimension In Space, BBC®) factors ruling pest invasions, making the strategy manageable in different olive productive contexts.

Many studies have accompanied the *Xylella* invasion since the beginning of the OQDS epidemics in Italy. This PhD Thesis adds new knowledge toward an effective IPM strategy for OQDS, trying to subdue the magmatic topic argument within a rational, experimental pathway. We suggest a reasonable posture minimising the interactions among the vector, the pathogen, and the susceptible plant, lowering the number of infections and, thus, the pathogen's massive spread. Disrupting the vector juvenile symbiosis with their microbiota introduces a new yet unexplored management preventive option during the pre-acquisition vector lifetime.

The availability of *Z. renardii* widens the biocontrol action to secondary pest management within an IPM strategy that cannot focus only on vectors. Artificial diets provide mass-rearing options to deepen our knowledge of the predators, including the new chances of behavioural manipulation offered by the secretion of insect Brindley's glands.

We might not be close to solving completely the OQDS epidemics, but we have progressed in modulating *Xylella*-vectors' infective abilities.

CONCLUSIONES GENERALES

Según estimaciones recientes, más de 6.500.000 olivos han muerto en el sur de Italia debido a la infección por *X. fastidiosa* y la consiguiente enfermedad que produce (SiDRO, Síndrome del Declive Rápido del Olivo). Además, la zona afectada sigue en expansión. El SiDRO afecta a millones de olivos, amenazando a tres cuartas partes de la producción mundial de aceite de oliva. La mayor parte de los impactos recaen sobre los consumidores, ya que el precio del aceite de oliva sube y la producción se reduce. Una mayor incidencia del patógeno empeoraría este escenario, causando un importante impacto agrícola, medioambiental, social y sanitario. El SiDRO impone una reducción de la oferta de servicios ecosistémicos derivados de los olivares del 30-34% y una disminución de la biodiversidad asociada del 28%, además del impacto en la productividad y en toda la cadena de suministro del aceite de oliva. El SiDRO tiene un impacto económico significativo, y las proyecciones futuras sugieren que el rendimiento del olivar se verá muy afectado. Frenar la propagación del patógeno en las zonas libres de *Xylella* y aplicar medidas de mitigación del SiDRO en las zonas afectadas podría ayudar a reducir el impacto de la enfermedad, restituyendo así los beneficios que generaba el cultivo.

En un modelo reticular, en el peor de sus escenarios, la gestión integrada de la infección sugiere opciones viables para revertir la, hasta ahora imparable, epidemia de *Xylella fastidiosa pauca* ST53. El modelo subraya la importancia del parámetro "número de vectores por olivo" para controlar eficazmente la infección. La reducción a un vector por olivo reducirá la expansión de *X. fastidiosa* hasta límites aceptables desde el punto de vista económico y medioambiental. En consecuencia, el censo de la población de vectores en el campo es crucial para aplicar una estrategia eficaz de gestión integrada de la enfermedad antes de que se produzcan transmisiones significativas. El muestreo cuantitativo *ex ante* es esencial para activar la intensidad y el calendario de las acciones de control, como lo es el muestreo *ex post* para evaluar la eficacia de la gestión de plagas. *Xylella fastidiosa* sólo puede infectar a los olivos si sus vectores eficientes adquieren el patógeno para infectar a las plantas susceptibles disponibles. La reducción de las poblaciones de vectores antes de interactuar con la *X. fastidiosa* reduce significativamente la probabilidad de adquisición del patógeno, minimizando así las posibilidades de infectar la planta huésped.

Los insectos y los microorganismos mantienen interacciones desde hace mucho tiempo. Una comprensión más profunda de este fenómeno ayudará a tener una perspectiva completa/multicapa de los actores implicados. Estas interacciones actúan como un motor evolutivo que permite a los actores (huésped y hospedador) hacer frente a tensiones medioambientales como la antropización y el cambio climático. Las interacciones con determinados microorganismos pueden favorecer unas condiciones de nicho óptimas para el desarrollo post-embrionario de los insectos, como en el caso de las bacterias que viven en las espumas producidas por los juveniles de Aphrophoridae. La microbiota que habita en el "nido" espumoso

de los juveniles de Aphrophoridae podría proteger a los juveniles frente a condiciones adversas. Los metabolitos bacterianos podrían influir en la formación de la espuma o quizá tener efectos antibióticos o repelentes. La alteración de la simbiosis bacteriana podría cambiar significativamente las características del nicho, eliminando la asociación funcional entre plagas y microorganismos y reduciendo la aptitud del insecto diana. Por lo tanto, los cambios en la interacción con los ectosimbiontes en las espumas de Aphrophoridae podrían allanar el camino para desarrollar medios de control biológico o biotecnológico dirigidos a los juveniles generando otra herramienta para minimizar la población de vectores antes de que puedan propagar a *X. fastidiosa*.

Las estrategias contra la transmisión de la *Xylella* requieren nuevas acciones de biocontrol en una estrategia de GIT (Gestión Integrada de las Trasmisiones) del olivar. Sin embargo, los antagonistas del vector disponibles son menos eficaces de lo que se sería necesario. Una acción intensa de biocontrol de *P. spumarius* reforzará el manejo de la infección, ofreciendo nuevas opciones de producción de cultivos orgánicos o agroecológicos. La reciente aparición de *Zelus renardii* (Zr) en la región Paleártica ofrece la oportunidad de evaluarlo para el control biológico de plagas del olivo, incluidos los vectores de *Xylella*. *Zelus renardii* mostró una notable capacidad depredadora sobre los vectores de *Xylella*. Las simulaciones numéricas también han mostrado la eficacia de *Z. renardii* para reducir las poblaciones de *P. spumarius*. El reduviido podría desempeñar varias funciones para el control de la infección bacteriana. La redución las poblaciones globales de vectores reduciría la puesta de huevos, que se eliminaría si se tratase de hembras pre-reproductoras. Suponiendo la reducción de 1.000.000 de individuos de *P. spumarius* a 90.000 adultos por hectárea, tras la acción de control mecánico de huevos y juveniles, la introducción de unos 4.000 Zr por hectárea bastaría para reducir la incidencia de la infección al 10% en sólo dos años sin utilizar insecticidas químicos sintéticos.

Otra ventaja del uso de *Z. renardii* es su capacidad para controlar, además, muchas plagas secundarias del olivo. No obstante, la probabilidad de depredación de *Z. renardii* está en función de la abundancia de presas (frecuencia de encuentros). *Zelus renardii* prefiere presas grandes, como los fitófagos, antes que himenópteros parasitoides que son más pequeños como *Metaphycus* spp. (Encyrtidae), *Eurytoma* spp. (Eurytomidae) o *Pnigalium* spp. (Eulophidae). Los olivos son polinizados por el viento y, puesto que los polinizadores comunes no recogen el polen de las flores del olivo, no es probable que se vean afectados por una estrategia de control biológico por inundación en los olivares con *Z. renardii*.

Se ha considerado que *Z. renardii* es un depredador generalista capaz de perturbar la acción de los enemigos naturales de plagas. La sospecha procede de experimentos simplificados en microcosmos, que ejercen interferencia física sobre un número limitado de individuos al encontrarse un volumen limitado. Las pruebas globales sugieren que *Z. renardii* es estenófago sobre los Hemípteros. La elección de presas por

parte de *Zelus* depende del hábitat, la planta huésped, la abundancia y la movilidad de las especies que encuentre.

Los prometedores resultados de los experimentos de laboratorio y las sugerencias de las modelizaciones apoyan el uso de *Z. renardii* para contrarrestar la infección por *Xylella*, que genera la devastadora SiDRO que afecta a la producción del olivar del sur de Italia. Por lo tanto, la obtención de más datos ecológicos y fisiológicos sobre *Z. renardii* ayudará a comprender plenamente su papel en la reducción de la transmisión de patógenos bacterianos a los olivos.

Zelus renardii puede regular la secreción y liberación de feromonas de alarma de sus propias glándulas de Brindley en respuesta al estrés o a la presencia de presas. La interacción entre *Z. renardii* y *P. spumarius* provoca una disminución de la producción de feromonas de alarma que reduciría la capacidad de las presas para detectar al depredador. Esta regulación de las feromonas de alarma permite al depredador marcar su propio territorio de depredación, expulsando competidores de su misma especie. También puede reducir la competencia y el canibalismo, mejorando así la eficacia de *Z. renardii* y abriendo oportunidades para la manipulación del comportamiento del depredador.

Además, las dietas artificiales adecuadas para la cría masiva de *Z. renardii* ayudarán a probar este depredador en condiciones controladas. Estos experimentos permitirían la liberación por inundación de *Z. renardii* en el campo para mejorar la estrategia actual de control integrado de plagas de los olivares.

Las acciones de control biológico y biotecnológico propuestas pueden contribuir significativamente a la estrategia de GIT de los olivares, conduciendo a una reducción sustancial (en varios órdenes de magnitud) de las poblaciones de *P. spumarius* que afectaría la capacidad transmisiva de estos vectores de *Xylella*. Desde la perspectiva de la gestión integrada de plagas en las explotaciones, esto se traduciría en una disminución gradual de la intensidad de las medidas físicas, biológicas y químicas de control de la *X. fastidiosa* a lo largo del tiempo, con la consiguiente reducción de los costes de gestión de los olivares aún no afectados por el SiDRO. Estas acciones actuarían de forma preventiva (evitando futuras infecciones por el vector, ya que el insecto muere durante o inmediatamente después de adquirir la bacteria) y protectora (limitando la acción del vector a una única infección, evitando transmisiones repetidas en la misma o en otras plantas).

Por último, teniendo en cuenta que en el olivo alberga otras plagas igualmente problemáticas para el rendimiento del cultivo, la modelización de una estrategia de gestión holística mediante el uso combinado de medidas físicas, químicas y biológicas garantiza una mejor producción global integrada de los agroecosistemas olivares. Estas acciones de control se someten a los factores TARDIS (Time And Relative Dimension In Space, BBC®) que rigen las invasiones de plagas, haciendo que la estrategia sea manejable en diferentes contextos productivos olivareros.

Numerosos estudios han acompañado la invasión de *Xylella* desde el inicio de las epidemias del SiDRO en Italia. Esta Tesis Doctoral añade nuevos conocimientos hacia una estrategia eficaz de GIT para el manejo del SiDRO. Sugerimos herramientas que minimicen las interacciones entre el vector, el patógeno y la planta susceptible, reduciendo el número de infecciones y, por tanto, la propagación masiva del patógeno. La interrupción de la simbiosis juvenil del vector con su microbiota introduce una nueva opción preventiva de gestión, aún inexplorada, durante la vida del vector previa a su adquisición.

La disponibilidad de *Z. renardii* amplía la acción de biocontrol a la gestión secundaria de plagas dentro de una estrategia de gestión integrada que no puede centrarse únicamente en los vectores. Las dietas artificiales proporcionan opciones de cría en masa para profundizar el conocimiento de los depredadores, incluidas las nuevas posibilidades de manipulación del comportamiento que ofrece la secreción de las glándulas de Brindley.

Puede que no estemos cerca de resolver por completo las epidemias de SiDRO, pero hemos progresado en la modulación de la capacidad infectiva de los vectores de *Xylella*.

CONCLUSIONI GENERALI

Stime recenti mostrano che più di 6.500.000 ulivi sono morti nel Sud Italia a causa dell'infezione da *X. fastidiosa* e della conseguente malattia OQDS, e l'area colpita continua ad espandersi. L'OQDS colpisce milioni di ulivi, minacciando tre quarti della produzione mondiale di olio d'oliva. La maggior parte dell'impatto ricade sui consumatori, poiché i prezzi dell'olio d'oliva aumentano e le scorte si riducono. Un'ulteriore invasione del patogeno peggiorerebbe questo scenario, causando impatti significativi a livello agricolo, ambientale, sociale e sanitario. L'OQDS impone una riduzione dell'offerta di servizi ecosistemici derivati dagli oliveti del 30-34% e una diminuzione della biodiversità associata del 28%, oltre agli impatti sulla produttività e sull'intera filiera dell'olio d'oliva. L'OQDS ha un impatto economico significativo e le proiezioni future non indicano una buona resa delle olive. Rallentare la diffusione del patogeno nelle aree esenti da *Xylella* e implementare misure di mitigazione dell'OQDS nelle aree colpite potrebbe aiutare a ridurre l'impatto della malattia, restituendo così i benefici.

In un modello reticolare del caso peggiore, la gestione integrata dell'infezione suggerisce opzioni solide per invertire l'invasione finora inarrestabile delle epidemie di *Xylella fastidiosa pauca* ST53. Il modello sottolinea l'importanza del parametro 'numero di vettori per olivo' per controllare efficacemente l'infezione. La riduzione di un vettore per olivo ridurrà l'espansione di *X. fastidiosa* a limiti economicamente e ambientalmente accettabili. Di conseguenza, la valutazione della popolazione di campo dei vettori è fondamentale per attuare una strategia IPM efficace prima che si verifichino trasmissioni significative. Il campionamento quantitativo *ex-ante* è essenziale per attivare l'intensità e la tempistica delle azioni di

controllo, così come il campionamento *ex-post* per valutare l'efficacia della gestione dei parassiti. La *Xylella fastidiosa* può infettare gli olivi solo se vettori efficienti acquisiscono il patogeno per infettare piante suscettibili disponibili. Ridurre le popolazioni di vettori prima che essi interagiscano con *X. fastidiosa* riduce significativamente la probabilità di acquisizione del patogeno, minimizzando così le possibilità di infettare la pianta bersaglio.

Gli insetti e i microrganismi hanno interazioni di lunga data. Una comprensione più approfondita di questo fenomeno aiuterà ad avere una prospettiva completa/multilivello degli attori coinvolti. Queste interazioni agiscono come un motore evolutivo, consentendo agli attori (ospite e ospitante) di far fronte a stress ambientali come l'antropizzazione e il cambiamento climatico. Le interazioni con alcuni microrganismi possono favorire condizioni di nicchia ottimali per lo sviluppo post-embrionale degli insetti, come nel caso dei batteri che vivono nelle schiume prodotte dai giovani di Aphrophoridae. Il microbiota che abita il 'nido' schiumoso dei giovani Aphrophoridae può proteggere gli stadi giovanili da condizioni avverse. I metaboliti batterici potrebbero influenzare la produzione della schiuma o fornire effetti antibiotici o repellenti. L'interruzione della simbiosi batterica può cambiare significativamente la caratteristica della nicchia, eliminando l'associazione funzionale tra parassiti e microrganismi e riducendo la fitness dell'insetto bersaglio. Pertanto, i cambiamenti nell'interazione con gli ectosimbionti nelle schiume di Aphrophoridae potrebbero aprire la strada allo sviluppo di mezzi di controllo biologici o biotecnologici mirati agli stadi giovanili, proponendo un ulteriore strumento per ridurre al minimo la popolazione di vettori prima che possano diffondere la *Xylella fastidiosa*.

Le strategie contro la trasmissione della *Xylella* richiedono più azioni di biocontrollo in una strategia IPM per gli oliveti. Tuttavia, il gruppo di antagonisti dei vettori attualmente disponibile è meno efficace di quanto sperato. Un'intensa azione di biocontrollo di *P. spumarius* rafforzerà la gestione dell'infezione, offrendo nuove opzioni di produzione di colture biologiche o agroecologiche. Il recente arrivo di *Zelus renardii* (Zr) nella Regione Paleartica offre l'opportunità di valutarlo per il biocontrollo dei parassiti dell'olivo, compresi i vettori di *Xylella*. *Zelus renardii* ha mostrato una notevole capacità predatoria contro i vettori di *Xylella*. Le simulazioni numeriche hanno anche convalidato l'efficacia di *Z. renardii* nel ridurre le popolazioni di *P. spumarius*. Il reduviide potrebbe svolgere diversi ruoli nel controllo dell'invasione batterica nella gestione delle infezioni poiché in grado di ridurre le popolazioni dei vettori, impedendo la deposizione delle uova e uccidendo le femmine pre-riproduttive. Ipotizzando di ridurre da 1.000.000 di individui di *P. spumarius* giovani a 90.000 adulti per ettaro, dopo l'azione di controllo meccanico delle uova e degli stadi preimmaginali, l'introduzione di circa 4.000 Zr per ettaro sarebbe sufficiente a ridurre l'incidenza dell'infezione al 10% in soli due anni, senza utilizzare insetticidi chimici sintetici.

Un ulteriore vantaggio dell'utilizzo di *Z. renardii* è la sua capacità di gestire anche parassiti secondari dell'olivo al costo del biocontrollo dell'infezione. Tuttavia, la probabilità di predazione per *Z. renardii* è una funzione dell'abbondanza della preda (frequenza degli incontri) e, quindi, il reduviide prederà preferenzialmente i parassiti. *Zelus renardii* preferisce le prede più grandi rispetto agli imenotteri parassitoidi più piccoli, come *Metaphycus* spp. (Encyrtidae), *Eurytoma* spp. (Eurytomidae) o *Pnigalium* spp. (Eulophidae). Gli ulivi sono impollinati dal vento e, poiché i comuni impollinatori non raccolgono il polline dai fiori di ulivo, è improbabile che vengano colpiti da una strategia di controllo biologico inondativo negli uliveti comprensiva di *Z. renardii*.

Zelus renardii è stato considerato un predatore generalista in grado di interrompere l'azione del nemico naturale attraverso la predazione intra-gilda. Il sospetto deriva da esperimenti semplificati in piccole gabbie che esercitano un'interferenza fisica su un numero limitato di specie e individui costretti in un volume limitato. Le prove generali suggeriscono che *Z. renardii* è stenofago nei confronti degli Emitteri. La scelta delle prede di *Z. renardii* si basa su vari fattori, tra cui l'habitat, la pianta ospite, l'abbondanza e la mobilità delle specie incontrate.

I risultati promettenti degli esperimenti di laboratorio e i suggerimenti della modellazione numerica supportano la potenziale efficacia dell'uso di *Z. renardii* per contrastare l'infezione da *Xylella*, contenendo la devastante OQDS che colpisce la produzione di olive nel Sud Italia. Pertanto, ulteriori dati ecologici e fisiologici di *Z. renardii* aiuteranno a comprendere appieno il suo ruolo nel ridurre la trasmissione del patogeno batterico agli ulivi.

Zelus renardii può regolare la secrezione e il rilascio di feromoni di allarme dalle proprie ghiandole di Brindley in risposta allo stress o alla presenza di prede. L'interazione tra *Z. renardii* e *P. spumarius* porta a una diminuzione della produzione di feromoni di allarme che ridurrebbe la capacità della preda di individuare il predatore. Questa regolazione dei feromoni di allarme può consentire al predatore di marcare il suo territorio di predazione, allontanando i conspecifici. Questa azione può anche ridurre la competizione e il cannibalismo, migliorando così l'efficacia di *Z. renardii* e aprendo opportunità per la manipolazione comportamentale del predatore.

Inoltre, una dieta artificiale adeguata all'allevamento di massa di *Z. renardii* aiuterà a testare questo predatore in condizioni controllate. Tali esperienze consentirebbero il rilascio inondativo di *Z. renardii* sul campo per migliorare l'attuale strategia IPM di *X. fastidiosa*.

Le azioni di controllo biologico e biotecnologico proposte possono contribuire in modo significativo alla strategia IPM degli oliveti, portando a una riduzione sostanziale (di diversi ordini di grandezza) delle popolazioni di *P. spumarius* e influenzando attivamente la capacità infettiva dei vettori di *Xylella*. Dal punto di vista dell'IPM aziendale, ciò comporterebbe una graduale diminuzione dell'intensità delle misure di

controllo fisico, biologico e chimico di *X. fastidiosa* nel tempo, con una conseguente riduzione dei costi di gestione degli oliveti non ancora colpiti dal batterio. Queste azioni agirebbero in modo preventivo (evitando future infezioni da parte del vettore, in quanto l'insetto muore durante o subito dopo l'acquisizione del batterio) e protettivo (limitando l'azione del vettore a una singola infezione, evitando trasmissioni ripetute sulla stessa o su altre piante).

Infine, considerando che l'olivo ospita una serie di parassiti ugualmente problematici per la resa della coltura, la modellazione di una strategia di gestione olistica attraverso l'uso combinato di misure fisiche, chimiche e biologiche assicura una migliore produzione integrata complessiva degli agroecosistemi olivicoli. Queste azioni di controllo subiscono i fattori TARDIS (Time And Relative Dimension In Space, BBC®) che governano le invasioni dei parassiti, rendendo la strategia gestibile in diversi contesti produttivi olivicoli.

Molti studi hanno accompagnato l'invasione di *Xylella* dall'inizio delle epidemie di OQDS in Italia. Questa tesi di dottorato aggiunge nuove conoscenze verso un'efficace strategia di IPM per l'OQDS, cercando di sottomettere l'argomento magmatico all'interno di un percorso razionale e sperimentale. Suggeriamo una postura ragionevole che minimizza le interazioni tra il vettore, il patogeno e la pianta suscettibile, riducendo il numero di infezioni e, quindi, la diffusione massiccia del patogeno. L'interruzione della simbiosi giovanile del vettore con il suo microbiota introduce una nuova opzione preventiva di gestione, ancora inesplorata, durante la vita del vettore prima dell'acquisizione.

La disponibilità di *Z. renardii* amplia l'azione di biocontrollo alla gestione secondaria dei parassiti, nell'ambito di una strategia IPM che non può concentrarsi solo sui vettori. Le diete artificiali offrono opzioni di allevamento di massa per approfondire la nostra conoscenza dei predatori, comprese le nuove possibilità di manipolazione comportamentale offerte dalla secrezione delle ghiandole di Brindley degli insetti.

Forse non siamo vicini a risolvere completamente le epidemie di OQDS, ma abbiamo fatto progressi nella modulazione delle capacità infettive dei vettori *Xylella*.