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Bioecology and ethology of Auchenorrhyncha potential vectors of *Xylella fastidiosa*

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Declaration

I hereby declare that this thesis represents my own work and contains the PhD results I personally obtained during the doctoral period (2019-2022) at the University of Florence. Where I have consulted the published work of others, this is always clearly cited, and the source is always given. Where other people have collaborated with me on aspects concerning my work, their contribution has been clearly stated and they have been included as co-author, acknowledged, or cited.

I also declare that this thesis has not been submitted for the award of any other degree or diploma to another University or Institution.

This is a true copy of the thesis, including final revisions.

Anita Nencioni

Abstract

This research work was aimed at expanding the general knowledge on two common spittlebug species, recently reconsidered as agricultural pests after being proved as vectors of the plant-pathogenic bacterium *Xylella fastidiosa* subsp. *pauca* in Europe: *Philaenus spumarius* L. (1758) and *Neophilaenus campestris* (Fallen, 1805). Several aspects of the bioecology and the ethology of these two spittlebugs were investigated to clarify their role in the spread of the subspecies *multiplex* of the bacterium, newly detected in Tuscany in an area characterized by Mediterranean maquis vegetation. The results of the conducted studies are reported in the five chapters of this thesis. In the first chapter, a long-term field survey carried out in the *Xylella*-infected area of Monte Argentario (Tuscany, Italy) has been presented; the survey allowed to verify the distribution and the seasonal abundance of *P. spumarius* and *N. campestris* in the Monte Argentario promontory and to outline the phenology of these two spittlebug species. Moreover, the host plant preference has been studied and the role of *P. spumarius* and *N. campestris* in the transmission of *X. fastidiosa* subsp. *multiplex* has been discussed. The second chapter include the outcomes of laboratory trials aimed at elucidating the protective fuction of the froth produced by spittlebug nymphs. The crab spider *Synema globosum* (Fabricius, 1775) and the ant *Crematogaster scutellaris* (Olivier, 1792) were used as model species to investigate the role of the froth in the protection against generalist predators. Our findings suggest that the froth could be considered as an antipredatory trait, at least in case of predators that use mainly olfactory cues to localize their preys, like ants. The froth of *P. spumarius* nymphs was also studied from a microbiological point of view and the structure of the associated microbial community was explored performing a denaturing gradient gel electrophoresis (DGGE) analysis. The same procedures were used also to study the microbial community associated to the gut and the Malpighian tubules of *P. spumarius* nymphs. Samples of froth, gut, and Malpighian tubules of the spittlebug *Lepyronia coleoptrata* (Linnaeus, 1758) were also analized to compare two different Aphrophoridae species. Results reveal that nymphs harbor a large and diverse bacterial community in their gut and froth, providing new accounts to the knowledge on facultative symbionts of spittlebugs. In the fourth chapter the results of a morphological and behavioral study on *P. spumarius* nymphs are reported. The gross morphology of the nymphs' antenna was studied through scanning electron microscopy, highlighting the presence of sensory structure with a putative olfactory function. Moreover, the behavioral response of nymphs of different age groups to olfactory stimuli was tested in a Y-tube olfactometer. The results of these experiments led to hypothesize the ability of *P. spumarius* nymphs to perceive volatile substances, altough such stimuli didn't seem sufficient to elicitate a clear

directional response in the tested insects. The role of the olfaction in the host location was carefully discussed in this chapter. Finally, the preliminary results of behavioral experiments aimed at evaluating the response of *P. spumarius* and *N. campestris* adults to different visual stimuli are reported in the fifth chapter. The ability to perceive and discriminate different wavelengths was assessed for both species and differences in the behavioral response of males and females were highlighted. Based on these findings, visual stimuli appear to be important in several aspects of spittlebugs ethology suggesting that the visual ecology of these insects deserves further investigation.

In conclusion, this research explored several aspects of the spittlebugs *P. spumarius* and *N. campestris* enlarging the general knowledge of these two species and improving the available information needed to develop effective and sustainable control strategies against these pests. Moreover, the preliminary outcomes obtained in this work could represent the basis for further and deepen investigations.

Introduction

Vector transmission of bacterial plant pathogens

Biotic diseases of plants are caused by a wide range of living organisms that comprise fungi, bacteria, phytoplasmas, viruses and viroids, nematodes and parasitic higher plants (Agrios, 2008).

Pathogens can infect one or more host plants, actively penetrating plant tissues or passively entering through natural openings such as stomata and wounds (Herman and Williams, 2012). Moreover, many of the microscopic agents of plant diseases can be transmitted by animals with piercing-sucking mouthparts, especially by insects, either accidentally or as a result of a specific vector-pathogen interaction (Agrios, 2008). Sap-feeding Hemiptera is one of the significant groups responsible for the transmission of plant pathogenic viruses, bacteria and phytoplasmas (Orlovskis *et al*., 2015; Eigenbrode *et al*., 2018) due to a specialized feeding mechanism that allows the acquisition and the delivery of pathogens directly from and to plant tissues (Fereres, 2020). Indeed, hemipterans are characterized by mouthparts modified in stylets that can penetrate plant epidermis and allow to suck the sap from the vascular system or cells. When pathogens colonize plant sap, insects can acquire these microorganisms while probing or feeding. Then, after moving to healthy plants, the emission of saliva preceding the liquid suction enables the injection of pathogenic cells and particles in plant tissues, resulting in the infection of new hosts. (Ng and Falk, 2006). Regarding vector-borne pathogenic bacteria, they are transmitted mainly by members of two hemipteran families, namely Psyllidae and Cicadellidae. However, it is essential to point out that species belonging to the Cercopidae and Aphrophoridae families are potentially involved in transmitting the xylem-limited bacterium *Xylella fastidiosa* (Wells *et al*., 1987) (Eigenbrode *et al*., 2018, Redak *et al*., 2004).

Vector-mediated transmission of plant pathogens requires three main phases: 1) the Acquisition Access Period (AAP), which is the time needed to acquire the pathogen by the vector; 2) the retention time, which is the period the vector remains competent for the transmission of the acquired pathogen; 3) the inoculation access period (IAP), namely the time needed by the vector to transmit the pathogen. Moreover, the time interval between the vector's acquisition and infective phase is called the latent period (Purcell, 1982).

The pathogen-vector relationship is non-persistent, semi-persistent or persistent, depending on the retention time duration. Non-persistent pathogens adhere to the cuticle of insect mouthparts or the alimentary canal during probing or feeding and can be transmitted soon after their acquisition without a latent period, showing a very brief retention time (Ng and Falk, 2006; Whitfield *et al*., 2015; Perilla-Henao and Casteel, 2016). In semi-persistent interactions, the retention time can last for days or weeks

because the vector ingests the pathogen that settles in alimentary canal This type of interaction requires a longer AAP to acquire the pathogen. Adult vectors can remain infectious for their entire life after the acquisition, while when preimaginal stages acquire the pathogen, the infectivity ends after moult (Ng and Zou, 2015). Finally, persistent relations are characterized by prolonged AAP and IAP, and the effective transmission of the pathogen can occur only after a latency period. In this case, the retention time coincides with the vector's life since the pathogen spreads from the alimentary canal and colonizes the hemolymph (Eigenbrode *et al*., 2018; Fereres, 2020). Vector-pathogen persistent associations typically show the most remarkable specificity rate (Eigenbrode *et al*., 2018). Vector-borne pathogens can also be classified as non-circulative and circulative depending on their retention site in the insect body. Hence, non-circulative pathogens do not enter the hemocoel of their vectors but usually colonize mouthparts and foregut, while circulative microorganisms pass through the gut walls entering the circulatory system. Once in the hemolymph, the pathogen can reach the vector salivary glands, prompt to be inoculated in a new host plant via the insect feeding activity (Perilla-Henao and Casteel, 2016).

Non-circulative pathogens can be involved in non-persistent or semi-persistent relationships with their vectors. At the same time, circulative pathogens always establish persistent associations and can multiply inside the insect body. Therefore, circulative pathogens are further classified as non-propagative when they don't replicate in vectors and propagative when they circulate and multiply within the insect (Gray *et al.*, 2014).

While viruses can be transmitted through all the described mechanisms, vector-borne bacterial pathogens are involved only in circulative propagative relationships, except for the xylem specialist bacterium *X. fastidiosa,* which show a non-circulative semi-persistent interaction with its hemipteran vectors (Purcell and Finlay, 1979; Purcell, 1982; Almeida *et al*., 2005; Killiny and Almeida, 2009). *Xylella fastidiosa* is the only vector-borne xylem-limited bacterial pathogen known so far, and it shows unique transmission characteristics, like the capability to be persistently transmitted by its vectors without the occurrence of a latency period (Almeida *et al*., 2005; Killiny *et al*., 2012).

Although plant-bacteria-vector interactions are far from being understood, some shared features could be highlighted for vector-borne plant pathogenic bacteria. For instance, they can propagate in both plant and insect hosts (Perilla-Henao and Casteel, 2016). This ability is remarkable, considering the reduced genomes of vector-borne plant bacteria (Chatterjee *et al*., 2008; Perilla-Henao and Casteel, 2016). Despite many advances in recent years, research on insect vectors involved in the transmission of plant pathogenic bacteria needs to be enhanced for bacteria such as *X. fastidiosa,* even though it has been investigated extensively (Perilla-Henao and Casteel, 2016).

Xylella fastidiosa (Wells *et al*., 1987)

Xylella fastidiosa is a vector-borne xylem-inhabiting gamma-Proteobacteria that causes severe vascular diseases in many plants (Purcell, 1997; Janse and Obradovich, 2010). Currently, this bacterium is associated with 679 plant species, 304 genera and 88 families (EFSA, 2023). It has been proven to be the causal agent of some economically significant plant diseases such as Pierce's disease (PD) of grapevine, Citrus Variegated Chlorosis (CVC), Almond Leaf Scorch (ALS), and the Oleander Leaf Scorch (OLS) (Janse and Obradovich, 2010).

Xylella fastidiosa is characterized by a broad genetic heterogeneity, showing several subspecies, among which *fastidiosa*, *multiplex* and *pauca* are recognized as valid taxa. At the same time, strains *sandyi* and *morus* are currently included in the *fastidiosa* subspecies (Denancè *et al*., 2019). Moreover, several sequence types have been identified so far (Chen *et al*., 1992; Cariddi *et al*., 2014; Denancé *et al*., 2017; Saponari *et al*., 2019).

Native to North America, *X. fastidiosa* was first reported in Europe in 2013, in Apulia (Italy), in association with the Olive Quick Decline Syndrome (OQDS) (Saponari *et al*., 2013), which has caused the loss of about 20 million of olive trees in the Salento peninsula. The subsp. *pauca* ST53 was ascertained as the pathogenic agent of the outbreak in South Italy. Subsequently, other *X. fastidiosa* outbreaks, originating from different subspecies of the bacterium, have been recorded in Spain, France, and Portugal (Trkulja *et al*., 2022) as well as in the Italian regions of Tuscany and Latium (Marchi *et al*., 2018; EPPO, 2022). These last outbreaks of *X. fastidiosa* in Central Italy were caused by the subspecies *multiplex* ST87, which mainly infects spontaneous plants and abandoned fruit trees (Marchi *et al*., 2018; Falsini *et al*., 2022; EPPO, 2022).

Xylella fastidiosa extensively colonize host plant xylem vessels producing a sap-blocking biofilm that causes leaf scorch as a primary and typical disease symptom (Chatterjee *et al*., 2008, Janse and Obradovich, 2010). Symptoms initially concern only a few branches, although the course of the disease can lead to the host plant death (Rapicavoli *et al*., 2017). On the other hand, *X. fastidiosa* can remain latent in several symptomless hosts (Hopkins, 1989; Rapicavoli *et al*., 2017). The bacterium produces many pathogenicity factors to better settle in plant and insect hosts, enabling, for instance, the attachment to host tissues and biofilm production (Chatterjee *et al*., 2008; Perilla-Henao and Casteel, 2016).

The infection of healthy plants and the spread of *X. fastidiosa* in the environment are mediated only by insect vectors (Purcell, 1990) that transmit *X. fastidiosa* in a non-circulative semi-persistent way. No transstadial or transovarial transmission of the acquired bacterium occurs, and only adult vectors retain the capacity to transmit *X. fastidiosa* (Almeida *et al*., 2005). The bacterium adheres to the insect

foregut, and a few days after the acquisition, it starts to multiply in the portion of the precibarium proximal to the cibarium (Almeida and Purcell, 2006; Backus and Morgan, 2011; Cornara *et al*., 2020). During the colonization of the insect foregut, *X. fastidiosa* forms a biofilm that is supposed to protect the bacterium "from the disruption of fast-moving currents of ingested fluid and to aid its nutrient extraction from the dilute xylem sap" (Redak *et al*., 200; Backus and Morgan, 2011). Inoculation of bacterial cells in host plants appears to result from the salivation–ingestion–egestion mechanism in which the insect firstly ingests a mixture of saliva and xylem sap directed to the precibarium to be sensed by the pre-cibarial sensilla. Then, the turbulent passage of this liquid mixture in the precibarium can cause the detachment of bacterial cells that were inoculated in plant xylem vessels during the subsequent egestion of the fluid from the insect's alimentary canal (Backus *et al*. 2012). Both the acquisition and inoculation efficiency increase proportionally to the vector access period to the host plants (Almeida and Purcell, 2006). Although only a few bacterial cells are required for transmission, the efficiency of this process varies greatly depending on the vector and host plant couple (Purcell, 1990; Redak *et al*., 2004; Janse and Obradovich, 2010).

Hemipteran xylem sap-feeding insects belonging to the superfamily Cicadoidea and Cercopoidea, as well as to the subfamily Cicadellinae, are responsible for the transmission of the bacterium from plant to plant (Redak *et al*., 2004). Since the lack of specificity in the relationship with its vectors, *X. fastidiosa* could potentially be transmitted by all the xylem sap feeders. However, according to the geographic area where the bacterium occurs, two leading groups are considered potential vectors: sharpshooter leafhoppers in the Americas and spittlebugs in Europe (Redak *et al*., 2004; Sicard *et al*., 2018, Cornara *et al*., 2019).

So far, the primary vector identified for the OQDS is the spittlebug *Philaenus spumarius* L. (1758) (Hemiptera: Aphrophoridae). The key role played by this species in *X. fastidiosa* diffusion is due to its capacity to acquire and transmit *X. fastidiosa* and its wide distribution in olive groves and surrounding habitats (Cornara *et al*., 2016; Cornara *et al*., 2018). In addition to *P. spumarius*, two other aphrophorids, *Neophilaenus campestris* (Fallen, 1805) and *Philaenus italosignus* Drosopoulos and Remane 2000, were proved to be able to transmit *X. fastidiosa*. Still, they were considered to play a secondary role in the infection of healthy olive trees due to their less abundance in olive agroecosystems (Cavalieri *et al*., 2019).

Spittlebugs as European vectors of *Xylella fastidiosa*

In Italy, research on spittlebugs was boosted after the outbreak of *X. fastidiosa* subsp. *pauca* in the Apulia region, focusing mainly on their biology, control methods and role as vectors, all related to olive groves. However, the two spittlebug species proved to be the main vectors of *X. fastidiosa* in South Italy, *P. spumarius* and *N. campestris,* are very common in Mediterranean areas where *Xylella fastidiosa* subsp. *multiplex* outbreaks occurred.

Taxonomical classification

Philaenus spumarius and *N. campestris* belong to the order Hemiptera, suborder Auchenorrhyncha, superfamily Cercopoidea, and family Aphrophoridae. The suborder Auchenorrhyncha comprises more than 40,000 species that are distinguished from other Hemiptera for having beak-like modified mouthparts expanding from the posterior part of the head, antennae with a hair-like flagellum, forewings entirely membranous or leathery and a sound-producing tymbal apparatus usually located on the first abdominal segment (Dietrich, 2009; Larivière *et al*., 2010). Approximately 3,000 species have been described in the superfamily Cercopoidea (Cryan and Svenson, 2010), which comprises four taxa, namely the family Cercopidae, Aphrophoridae, Clastopteridae and Machaerotidae (Peck and Thompson, 2008). Among them, the family Aphrophoridae includes about 820 species mainly distributed in temperate climates, although many also occur in tropical zones (Peck and Thompson, 2008, Dietrich, 2009). Cercopoidea species are commonly called spittlebugs - the common name of *P. spumarius* is meadow spittlebug - due to the peculiar habit of nymphs to develop inside a self-produced saliva-like frothy mass. Moreover, the term “froghoppers” is often used to refer to Cercopoidea, given their extraordinary jump ability and aspect resembling little frogs.

Geographical distribution and host plants

Spittlebugs (Hemiptera: Aphrophoridae) are spread in many terrestrial ecosystems, and some species are known to be important phytophagous (Thompson, 2004; Chen and Liang, 2012; Schöbel *et al*., 2021). *P. spumarius* is a widespread polyphagous species commonly found in natural, agricultural, and anthropic environments (Yurtsever, 2000). Native to Paleartic, *P spumarius* was accidentally introduced in North America in the nineteenth (Weaver and King, 1954); currently, its occurrence has also been reported for the Afrotropical, Neotropical and Australian ecozones (EFSA, 2015). Differently, *N. campestris* shows a narrower geographical range to *P. spumarius* since its range is

restricted to the Palearctic, the Near East and North Africa (Holzinger, 2003, EFSA, 2015, Demir, 2019).

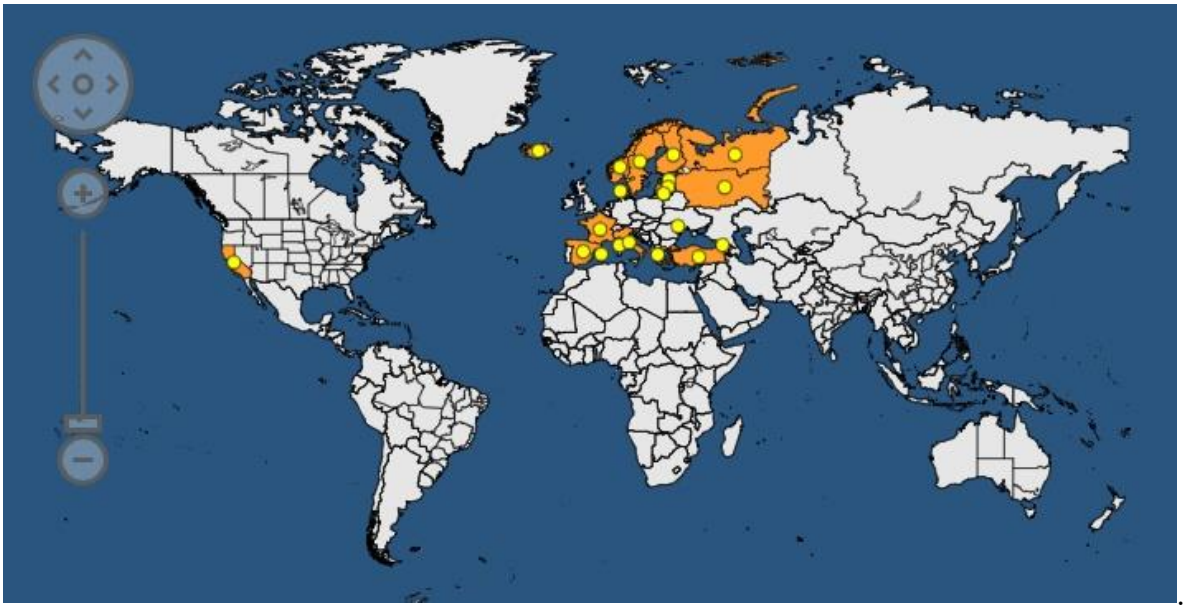

Figure 1. Geographic distribution of the meadow spittlebug *Philaenus spumarius*; source EPPO, (January 26th, 2023).

Morphology

Taxonomical characters that identify all Cercopoidea species are the convex frontoclypeus, the absence of the median ocellus, scutellum about as long as wide, hind tibia without rows of setae but with laterally fixed spines and forewings held tent-shaped on the abdomen. Classification at the species level requires the preparation of male genitalia (Dietrich, 2005; EPPO, 2019).

Philaenus spumarius adult – The body length range between 5.3-6.0 mm in males and 5.4-6.9 mm in females. Adults are 2-2.5 mm large, with females generally broader than males. The body colour is highly variable, from whitish yellow to black, with several colour morphs described so far (Yurtsever, 2000; Drosopoulos *et al*., 2010; Lahbib *et al*., 2022). In general, morphs are divided according to the presence, extension and intensity of marks on the dorsal side of the body (Yurtsever, 2000; Lahbib *et al*., 2022) The distal tibial and first tarsus ends bear 7-8 spurs (Elbeaino *et al*., 2014; EPPO, 2019).

Philaenus spumarius nymph – Nymphs body length range from 1.35 mm in the first instar to 6.25 in the fifth instar. During the development, the body length and the head capsule width increase.

Nymphs change their body colour after each moult: first and second instar nymphs are yellow/light orange tiny insects. Third-, fourth and fifth instar nymphs ranged from pale to brilliant green. An accurate description of the external morphology of *P. spumarius* nymphs is provided in Weaver and King (1954) and Yurtsever (2000).

Neophilaenus campestris adult – Adults are smaller and slimmer than *P. spumarius*. The body length range between 5.0-5.3 mm in males and 5.4-5.7 mm in females. Usually greyish-yellow, with a longitudinal dark streak from vertex to scutellum. The forewings have two pale marks on their outer margin. Distal tibial and first tarsus ends with 11-12 spurs on a double row (Elbeaino *et al*., 2014; EPPO, 2019).

Figure 2. Adults of *Philaenus spumarius* (A) and *Neophilaenus campestris* (B). (Source: https://www.britishbugs.org.uk/index.html)

Neophilaenus campestris nymph – No detailed description of *N. campestris* nymphs is available. In general, immatures are beige or orange with a black pattern on the dorsal part of the thorax.

Egg – *P. spumarius* eggs are elongated (about 1 mm long and 0.35 mm wide) and pale yellow. An orange-pigmented spot is visible in the shell at one end of the egg. If the egg has been fertilized, the spot gets more extensive and darker, migrating towards the opposite side of the egg. This migration indicates the embryo's initial involution. During its development, the embryo is enveloped by a hatching membrane on which the egg burster grows (Weaver and King, 1954; Yurtsever, 2000). The egg burster is a dark formation positioned on the embryo's vertex that be seen from the egg's shell. At hatching, the insect perforates the shell with the burster and wriggles out of the egg also leaving its embracing membrane.

No detailed description of the egg of *N. campestris* is available. However, our observations didn't show significant differences from that of *P. spumarius*. Both species laid eggs in groups covered by protective frothy cement.

Life cycle

P. spumarius and *N. campestris* are univoltine species that overwinter as eggs laid in batches in herbs, stubble, or residuals of herbaceous plants (Weaver and King, 1954; Whittaker, 1965a). The number of eggs per group varies from 2 to 30, with an average number of 7 elements, case of single egg exists (Weaver and King, 1954). The pre-imaginal development of those spittlebugs passes through five instars. Nymphs show a peculiar behaviour developing inside a self-produced saliva-like frothy mixture. This foam is composed of the excretion of the alimentary canal, mainly represented by water added with mucopolysaccharides and proteins produced by specialized cells of the Malpighian tubules (Marshall, 1966; Marshall, 1973). Through telescopic movements of the abdomen, the nymph can introduce air bubbles in this mixture, conferring the typical frothy state to its excreta. The foam protects the nymphs against dehydration, temperature fluctuations and natural enemies (Weaver and King, 1954; del Campo *et al*., 2011; Chen *et al*., 2018; Tonelli *et al*., 2019). Nymphs continuously produce their froth during feeding. The production ceases at the time of the last moult, and the froth dehydrates, forming a chamber where the adults can complete their development (Weaver and King, 1954).

At the beginning of spring, nymphs hatch from overwintering eggs and immediately pierce the closest suitable plant, starting froth production (Whittaker, 1965a, Yurtsever, 2000, Bodino *et al*., 2019). First-instar nymphs are tiny and delicate soft-bodied insects usually located in little froths on the basis of plant stems, where they can benefit from the site's protection (Weaver and King, 1954; Yurtsever, 2000). Early instar nymphs tend to aggregate inside the same spittle with conspecifics or even with nymphs belonging to other spittlebug species (Halkka *et al*., 1977). A widely accepted explanation of this phenomenon is that the aggregation allows the nymphs to better exploit the food source and save energy in producing the spittle mass (Whittaker, 1965b; Biedermann, 2003, Chen and Liang, 2015; Bodino *et al*., 2019). Late instar nymphs are more mobile and move towards the plant apex or crawl to reach other suitable host plants nearby (Cornara *et al*., 2018). Although temperature and humidity are considered the most critical factors affecting the growth and the presence of *P. spumarius,* discordant data are present in the literature regarding the correlation between temperature

and spittlebug phenology. According to Zajac *et al*. (1989), 2.8 and 26.7°C represent the lower and upper-temperature thresholds for *P. spumarius* nymphal development. A recent study by Bodino and colleagues (2019) reports that first instar nymphs of *P. spumarius* appear after an accumulation of 78-198 Degree Days (DD) in Southern Italy and 94-100 DD in Northern Italy.

Moreover, the seasonal peak of nymphs in the studied sites occurs in the first half of April, both in the Northern and Southern regions. Finally, it has been reported that the complete preimaginal development takes from 30 to 50 days in South Italy and 50-70 days in Northern Italy. The same study describes a similar pattern for *N. campestris* development, highlighting only a slight delay in the phenological timing of this second species.

Adults appear between mid-April and May, depending on latitude and climatic conditions (Yurtsever, 2000; Cornara *et al*., 2018; Bodino *et al*., 2019) and initially feed and rest on herbaceous plants until the vegetation dries up for the temperature rising. Adults start to mate soon after their emergence, although females show ovarian parapause, which delays egg maturation (Whitsak, 1973). The photoperiod regulates this ovarian diapause, that is ovarian development accelerates when the days become shorter (Whitsak, 1973; Morente *et al*., 2021). Thus, egg maturation starts at the end of summer, when mating increases (Cornara *et al*., 2018).

Both *P. spumarius* and *N. campestris* show two mass-movements events during their life: when herbaceous plants start to dry up because of the increase in temperature, adults move to shrubs and trees to exploit these plants as food sources and shelters during the aestivation period (Cornara *et al*., 2018, Cornara *et al*., 2021, Lago *et al*., 2021a). In the epidemiology of the Olive Quick Decline Syndrome, the migration of adults from herbaceous plants to olive trees appears to be a crucial moment. In that period, newly emerged adults inhabiting herbs in olive groves shift to olive trees, transmitting *X. fastidiosa* from plant to plant, then move to surrounding areas where they spend summer mainly on spontaneous plant species (Cornara *et al*., 2016). Since spittlebug adults were found to be infective soon after their emergence, control measures currently aim to reduce the adult movement to olive trees (Morelli *et al*., 2021).

The second mass-movement occurs at the end of summer because adults return to herbaceous vegetation to mate and lay eggs (Weaver, 1951; Lavigne, 1959). Adults' life ends in winter, naturally or due to low temperature.

Habits

Both juveniles and adults of spittlebug species are xylem-sap feeders (Wiegert, 1964; Malone *et al*., 1999; Redak *et al*., 2004). *P. spumarius* is highly polyphagous during their entire life, with hundreds

of reported host plants (Weaver and King, 1954; Di Serio *et al*., 2019; Bodino *et al*., 2020). Nymphs feed on herbaceous plants, mainly dicotyledonous species belonging to the family Asteraceae and Fabaceae (Morente *et al.*, 2018; Bodino *et al*., 2019, 2020; Antonatos *et al*., 2021). Nitrogen-fixing legumes and plant species characterized by xylem sap rich in amino acids are usually preferred hosts (Yurtsever, 2000). On the other hand, the host plant range in *N. campestris* is relatively narrow compared to the diversity of plants where *P. spumarius* can feed. *Neophilaenus campestris* nymphs are strictly associated with monocotyledons (Whittaker, 1971), with species of the Poaceae family as preferred hosts, while adults feed preferably on conifers (Mazzoni, 2005; Lago *et al*., 2021a). Polyphagy in spittlebugs appears to be related to the poor nutritional quality of the xylem sap, so a broad host range provides more opportunities to exploit food resources (Novotny and Wilson, 1997). The feeding behaviour of spittlebugs is described as a sequence of stereotypical actions after landing on a potential host plant. Firstly, the insect explores the plant surface by dabbing with the tip of its labium, which possesses numerous sensory structures. During dabbing, a variable amount of saliva is secreted and reingested to be sensed by the precibarial chemosensilla. Subsequently, a series of stylets probing precedes the insertion of the stylets into the preferred feeding tissue. Spittlebugs usually produce a salivary sheath around the stylets to prevent the leakage of sap or air during feeding. Once the suitable feeding site has been identified, the ingestion of xylem sap can last from a few seconds to several hours (Backus, 1988; Crews *et al*., 1998; Dietrich, 2009; Markheiser *et al.*, 2022). Although spittlebugs usually move by jumping or crawling (Burrows, 2003, 2006), they can also fly or glide. Recent studies found that *P. spumarius* in mark–release–recapture experiments can be retrieved from places at a distance not exceeding 32 m, with an estimated mean daily dispersal of 1.5 m. In comparison, *P. spumarius* can fly an average of 102 m in flight-mills experiments (Casarin *et al*., 2022). However, other flight-mills experiments proved different performances, such as the ability to fly 500 m in 30 min (Lago *et al*., 2021a) and to disperse up to hundreds of meters in the environment (Bodino *et al*., 2021). In similar experiments, *N. campestris* proved to be able to fly longer distances since it was retrieved from places distant more than 2 km and in flight, mills reached 1.4 km in an 82-min single flight (Lago *et al*., 2021b). *P. spumarius* females show a higher flight potential than males, at least in spring (Lago *et al*., 2021b). However, the extent of *P. spumarius* dispersal probably varies according to the availability of a suitable food plant and landscape composition, as evidenced in studies carried out in Italy (Bodino *et al*., 2020, 2021). Contrariwise, the almost exclusive association of *N. campestris* adults to conifers likely elicit a more accentuated dispersal movement since spittlebugs need to reach their specific summer hosts (Bodino *et al*., 2021, Casarin *et al*., 2022). Spittlebugs were also found to passively disperse over long distances due to transportation by wind or by humans (Weaver and King, 1954). This aspect increases the risk that

these vectors could introduce plant pathogens in new environments. The vector dispersal ability is a crucial aspect in spreading plant pathogens. Thus, understanding the movement patterns of vectors is of great importance in planning effective pest management programs (Power, 1992).

Intraspecific communication in spittlebugs is mediated by vibrational substrate-borne signals produced by specialized organs (tymbals) located in the proximal part of the abdomen. These acoustic signals are essential during courtship but mediate intrasexual communication (Avosani *et al*., 2020; Akassou *et al*., 2021). In *P. spumarius*, females emit calling signals at the beginning of ovarian maturation, intensifying this behaviour according to ovarioles' development and maturation of eggs. Males respond to calling signals produced by receptive females, reaching them on the plant and establishing the couple (vibrational duet) (Avosani *et al*., 2020; 2021).

Association with *Xylella fastidiosa* and control strategies

Until recently, spittlebugs have never been considered a severe pest in Europe since the direct damages they could inflict on plants are considered negligible. However, after the outbreak of *X. fastidiosa* on olive trees and other crops across Europe, spittlebugs were proved to be the primary vectors involved in the spread of this dangerous plant-pathogenic bacterium (Cornara *et al*., 2016; Cavalieri *et al*., 2019). Hence, they assumed the role of major pests, especially in the Mediterranean basin (Cornara *et al*., 2019).

As leafhopper vectors of leaf scorch diseases in the Americas, spittlebugs transmit *X. fastidiosa* in a semi-persistent non-circulative way (Redak *et al*., 2004; Almeida *et al*., 2005). The bacterium adheres to the insect foregut e starts to multiply, forming a biofilm. Bacterium cells are detached from this biofilm due to the passage of the mixture of saliva, and xylem sucked during dabbing. Then, removed cells are inoculated in the xylem vessels during insect feeding (Backus *et al*., 2012). Since *X. fastidiosa* colonize the insect's precibarium and cibarium, anchoring itself to the cuticle, the bacterium is lost during moults and persists only in adult individuals (Redak *et al*., 2004).

The transmission efficiency is influenced by many factors, including the dynamic of xylem ingestion, the number of bacterial cells in the foregut, the insect-host relationships, and the environmental conditions (Redak *et al*., 2004; Bodino *et al*., 2021b; Markheiser *et al*., 2022).

Due to the lack of therapeutic strategies against *X. fastidiosa* infection in olive trees, vectors of *X. fastidiosa* are the main target of integrated pest management. Control strategies could be summarized as monitoring the vector presence and phenology, reducing the nymphal population and avoiding contact between spittlebug adults and olive trees (Morelli *et al*., 2021). The monitoring of vectors population, especially in the first part of their life, is quite complicated for the absence of practical

and effective methods for surveying. The sampling of immature insects is essentially visual and requires experienced operators and a significant amount of time. Additionally, adults are mainly surveyed manually by a sweeping net, also because we lack effective trapping methods. So, the timing for the application of control measures in olive groves is mainly based on the available ecological and phenological data and the periodic information released by local Plant Protection Services. Agronomic and chemical interventions are applied against juvenile stages to reduce population size. Managing soil and ground cover plants is crucial to significantly minimize spittlebugs early in life (Sanna *et al*., 2021). Moreover, insecticides could enhance the effect of the abovementioned strategies (Morelli *et al*., 2021). Control measures against spittlebug immatures must be applied at the peak of the fourth-instar nymphs to maximize the effects on the entire nymphal population before the emergence of the adults (Cornara *et al*., 2018; Morelli *et al*., 2021).

Using insecticides on olive trees to reduce as much as possible spittlebug visits is currently the primary control strategy applied against adults. Pyrethroids and neonicotinoids are considered effective products (Dongiovanni *et al*., 2019; Morelli *et al.*, 2021), although their sustainability could be questionable (Blacquiere *et al*., 2012; Maund *et al*., 2012; Pisa *et al*., 2015).

Unfortunately, sustainable, efficient control strategies against spittlebug adults in olive groves still need to be increased.

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Aims of the thesis

The main aim of the thesis was to improve the knowledge on the ecology of *P. spumarius* and *N. campestris* in the Mediterranean maquis in order to better understand their possible role in the transmission of *X. fastidiosa* subsp. *multiplex* ST87. To accomplish this aim, a long-term field survey has been conducted in the *Xylella*-positive area of Monte Argentario to collect data on the distribution, seasonal abundance and the host plant preference of these two putative vector species. Increasing the available information on spittlebug ecology in natural environments is essential to develop effective control strategies and to limit the spread of a quarantine bacterium like *X. fastidiosa*.

A secondary aim of the thesis was to deepen traits in the biology of *P. spumarius*, focusing on pre-imaginal stages. So that the bacterial symbionts of nymphs's gut and foam were investigated applying molecular techniques. Moreover, behavioral assays to test the role of the foam in protecting spittlebug juveniles were carried out. Scanning electron microscopy combined with behavioral assays were used to explore the gross morphology of the antennae of nymphs and their response to olfactory cues. Finally, preliminary behavioral assays were conducted to test the adult behavioral response to visual stimuli.

1. Ecology of spittlebugs potential vectors of *Xylella fastidiosa* in Tuscany

Personal contribution: Conceptualization, data collection, taxonomical identification of the collected specimens, data analysis.

Dott. Roberto Guidi[1] has collaborated with data collection; Dott.ssa Marzia Cristiana Rosi[1] has performed the statistical analysis. Prof.ssa Patrizia Sacchetti[1] participated in the conceptualization, data collection and manuscript preparation.

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Abstract

Spittlebugs have received increasing attention in the last decade after being proved as vector of the plant-pathogenic bacterium *X. fastidiosa* in Europe. The role of *Philaenus spumarius* in the spread of the pathogen in olive groves is crucial and its action appear to be reinforced by other spittlebugs species that occasionally feed on olive trees, like *Neophilaenus campestris*. Although the Olive Quick Decline Syndrom (OQDS) pathosystem was almost completely clarified, other *Xylella* pathosystems, such the one characterized by the subsp. *multiplex* in Tuscany (Italy), are far to be understood. Particularly, the possible role of *P. spumarius* and *N. campestris* in the spread of this bacterium subspecies is not clear. So that, this work aimed at studying the ecology of these two spittlebug species in the *Xylella*-infected area of Monte Argentario, focusing on the relationship between *P. spumarius* and *N. campestris* potential vectors and their host plants. Moreover, the phenology of these two Aphrophoridae was outlined and their possible role in the transmission of *X. fastidiosa* subsp. *multiplex* was carefully discussed.

Background

The family Aphrophoridae comprises about 820 species mainly distributed in temperate climates, although many also occur in tropical areas (Peck and Thompson, 2008; Dietrich, 2009). Members of the family Aphrophoridae are commonly called spittlebugs due to the peculiar habit of nymphs developing inside a self-produced frothy mass (Peck and Thompson, 2008). This bubble nest protects the nymph against dehydration, temperature fluctuations, UV light and natural enemies (Weaver and King, 1954; del Campo *et al*., 2011; Chen *et al*., 2018; Tonelli *et al*., 2019).

In the past, research on Aphrophoridae was almost exclusively focused on the taxonomy and the polymorphism of the most common species, the meadow spittlebug *Philaenus spumarius* L. (1758). Thus, when spittlebugs were recognized as the main European vectors of the plant-pathogenic bacterium *Xylella fastidiosa* (Wells *et al*., 1987), the gap in the knowledge of biology and the ecology of this taxonomic group of insects was dramatically highlighted.

Xylella fastidiosa is a vector-borne polyphagous pathogen that can infect over 600 plant species, including economically important crops such as grapevine, citrus, peach, almond and olive (Janse and Obradovich, 2010, EFSA, 2023). Since 2013, the subspecies *pauca* of the bacterium was proven to be the causal agent of the Olive Quick Decline Syndrome (OQDS), a severe disease that has caused the loss of thousands of olive trees in Apulia (Italy) (Martelli *et al*., 2016; Morelli *et al*., 2021). The spittlebug *P. spumarius* was found to be the major vector of this pathogen. In addition, *Neophilaenus campestris* (Fallen, 1805) and *Philaenus italosignus* Drosopoulos and Remane, 2000 could also transmit *X. fastidiosa*. However, they are considered to play a secondary role in infecting olive trees since their less abundance in olive agroecosystems (Cavalieri *et al*., 2019).

In Italy, apart from the outbreak of the subspecies *pauca* in Apulian olive groves, another *X. fastidiosa* subspecies was reported in Tuscany. In 2018, *X. fastidiosa* subsp. *multiplex* ST87 was detected in Monte Argentario, where it has occurred in areas mainly characterized by sclerophyllous vegetation. The bacterium has been infecting many trees and shrubs typical of the Mediterranean maquis (Macchia Mediterranea), like *Spartium junceum* L., *Rhamnus alaternus* L. and *Cistus monspeliensis* L. (Marchi *et al.*, 2018, Saponari *et al.*, 2019). The pathosystem involving *X. fastidiosa* subsp. *multiplex* in Monte Argentario resembles the situation currently established in Corsica, where different strains of the same *Xylella* subspecies were found infecting *Polygala myrtifolia* and other species typical of the Mediterranean maquis (Chauvel *et al*., 2015; Cruaud *et al*., 2019).

Previous studies in Corsica and Tuscany on the distribution of *P. spumarius* and *N. campestris* have suggested their involvement in transmitting *X. fastidiosa multiplex* (Albre and Gibernau, 2019; Albre *et al*., 2021; Gargani *et al*., 2021). Even if *P. spumarius* and *N. campestris* adults surveyed in these

Xylella-positive areas were positive for the bacterium (Cunty *et al*., 2020; Gargani *et al*., 2021), their role in the transmission of *X. fastidiosa* in Tuscany is still largely unclear.

Vectors play a crucial role in the spread of *X. fastidiosa* and could affect the occurrence and establishment of the bacterium in a given area. Although a large amount of data has been gathered regarding the biology and the ecology of insects involved in the transmission of *X. fastidiosa* to cultivated plants, including information on spittlebugs that are vectors of the OQDS, the complexity of *X. fastidiosa* epidemiology does not allow the generalization of information between different pathosystems (Sicard *et al*., 2018; Desprez-Loustau *et al*., 2021).

Mediterranean ecosystems are considered significant "biodiversity hot spot" for their high environmental heterogeneity and unique geological, climatic, floristic and faunistic characteristics (Vogiatzakis *et al.*, 2006). These ecosystems are the results of the millenary action of man and their survival is strictly associated to the human intervention. So that, these biotopes are considered fragile since they are very susceptible to degradation, mainly due to the abandonement of the traditional human activities like agriculture and pastoralism. Their frequent exposition to stress (deforestation, drought, fire etc.) and their climate suitability make Mediterranean ecosystems high-risk areas for the establishment of *X. fastidiosa,* which could potentially cause severe damage to these environments (Desprez-Loustau *et al*., 2021). Since then, studying *X. fastidiosa* epidemiology in areas characterized by Mediterranean vegetation appears important in protecting this high-value ecosystem.

In this study, a long-term field survey has been carried out to collect data on the ecology of *P. spumarius* and *N. campestris* in the Monte Argentario area, where these insects are the putative vectors of *X. fastidiosa* subsp. *multiplex*. The association between spittlebugs and typical Mediterranean plant species usually infected by *X. fastidiosa* has been investigated to elucidate the role of *P. spumarius* and *N. campestris* in transmitting the bacterium.

Materials and Methods

Study area

The Monte Argentario promontory is located in central Italy, in the southern part of Tuscany. The Tyrrhenian Sea surrounds three sides of this peculiar area. At the same time, the fourth is connected to the western coast of Tuscany by two narrow sandy isthmi named "tomboli". The northernmost isthmus is called "Tombolo of Giannella", and the southernmost is named "Tombolo of Feniglia". These two strips of land delimit the Orbetello lagoon (about 26 km^2) that separates the Monte

Argentario promontory from the mainland. A median incomplete isthmus departing from the Tuscany coast (in front of the city of Orbetello) is connected to the promontory by an artificial dam and divides the lagoon into two sectors. The total surface of Monte Argentario is about 60 km^2 and shows a high lithological and topographical heterogeneity. The climate is typically Mediterranean, with mild and rainy winters and hot and dry summers (Arrigoni and Di Tommaso, 1997).

A longitudinal ridge consisting of several mountainous elevations (oriented NW-SE), with Punta Telegrafo (632 m a.s.l.) as the highest point, ideally divides the promontory into two sides that are characterized by different topographical, meteorological and vegetational features. The southwest side of the promontory displays steep slopes, lower annual precipitation, and xeric vegetation. On the contrary, the northeast side is characterized by gradual slopes, significant yearly rainfall, and mesophilic vegetation (Baldini, 1995, Arrigoni and Di Tommaso, 1997). The Monte Argentario flora is a mosaic of patches derived from thousands of anthropic disturbances. Indeed, recurrent fires and clearances for obtaining agricultural and grazing lands have strongly affected the original vegetation. In the last century, the abandonment of farming has led to a partial natural regeneration of the native forests. However, the south-west side of the promontory still shows the traits of intense degradation since its warmer climate has favoured the development of extensive and monotonous garrigues, slowing down the reconstitution of the native sclerophyllous wood (Arrigoni and Di Tommaso, 1997). However, the flora of Monte Argentario could be ascribed to two main zonal vegetation areas: the broad-leaved deciduous wood on the north-east side, and the evergreen sclerophyllous woods, with many degraded landscapes, on the south-west side. Finally, well-differentiated azonal vegetation could be observed on sandy dunes and rocky coastal slopes (Arrigoni and Di Tommaso, 1997).

Sampling of insects

Philaenus spumarius and Neophilaenus campestris preimaginal development

The population dynamics of spittlebug juveniles were monitored in 2019 and 2021, with the 2020 interruption due to the COVID-19 pandemic limitations. In spring 2019, the preimaginal development of *P. spumarius* and *N. campestris* was monitored in two sampling sites in the Monte Argentario area. Furthermore, two additional sampling sites were surveyed in the spring of 2021. Sampling locations differed in altitude, exposure, and vegetation type (Table 1). However, two associations of herbaceous ground cover were surveyed: mixed herbaceous plants were sampled in “Cannelle” and “L’acqua dolce” sites. At the same time, a meadow was observed in “Stadium” and “Forte Stella”. In each

sampling site, an area of about 2,000 m^2 was examined using the quadrat sampling method, frequently applied to quantify the density of spittlebug nymphs (Holzinger *et al*., 2003; Morente *et al*., 2018; Avosani *et al*., 2022). At each sampling date, a rectangular frame (110x25cm, 0,25 m^2), considered the sampling unit (SU), was randomly placed on the ground 20 times, checking the presence of spittlebug nymphs in all the plants enclosed by the frame. The spittlebug host plants, the number of spittles per plant, the position of the spittle on the plant and the number of nymphs per spittle were recorded. The survey was conservative, so individuals were returned to their host plant after being counted.

The surveyed area of the "L'acqua dolce" site was characterized by a high abundance of *Cistus monspeliensis* L. plants that are known to host a great number of *P. spumarius* nymphs during spring (Albre *et al*., 2021). Since then, we decided to integrate the quadrat method sampling 20 randomly chosen spittles occurring on *C. monspeliensis* plants to avoid underestimating *P. spumarius* nymphs' population and development.

Sites were visited weekly from March to June. Nymphs' species and instar were identified based on previously described morphological features (Yurtsever, 2000; Stöckmann *et al*., 2013). Finally, species other than *P. spumarius* and *N. campestris* were identified based on morphological feature described in Stöckmann *et al*., (2013) and registered.

Adults spittlebug sampling

Random sampling was conducted throughout the Monte Argentario area to identify aestivation sites and summer hosts of *P. spumarius* and *N. campestris*. Sampling sites were randomly chosen on both sides of the Monte Argentario promontory (Arrigoni and Di Tommaso, 1997). However, *X. fastidiosa*-susceptible host plants (only shrubs and trees were considered) were always present in the selected sampling localities. This condition was necessary to allow the evaluation of the association between potential *X. fastidiosa* subsp. *multiplex* vectors and susceptible host plant species occurred in the Monte Argentario area. Sampling localities were georeferenced. The sampling was conducted every fifteen days during the period June-October 2021. In each site, two trained operators collected insects for 15 minutes by sweeping nets, randomly choosing the plants to sample (herbaceous plants, shrubs, and trees). The canopy of five plants was swept for five times in each sampling site. At least one *X. fastidiosa*-susceptible host plant (shrub or tree) was sampled in each sampling site. Collected insects were individually stored in 1.5 ml micro vials in 96% ethanol and brought to the laboratory for identification. Taxonomical identification at the species level was carried out according to Drosopoulos and Remane (2000), Holzinger *et al*., (2003) and Biedermann and Niedringhaus (2009).

The plant species on which the insect was captured was recorded, and all the other plant species were surveyed.

Table 1. – Geographical and vegetational features of the four sampling sites in the Monte Argentario area, visited for the survey of spittlebug nymphs in spring 2019 and 2021

Sampling site	**Latitude and longitude**	**Altitude (m a.s.l.)**	**Exposure**	**Side**	**Vegetation type**	**Sampling year**
Cannelle	42.386333N 11.145210E	93	North-West	South-West	Sclerophyllous vegetation	2019
L'acqua dolce	42.377010N 11.186200E	120	North-East	North-East	Maquis of sclerophyllous vegetation. *Cistus* spp. maquis	2021
Forte Stella	43.484587N 11.194481E	105	North-East	North-East	Meadow surrounded by sclerophyllous vegetation. Scattered unmanaged olive trees and *Pinus* sp. trees	2021
Stadium	42.4293100N 11.1791820E	2	Plain area	North-East	Meadow with scattered *Quercus suber* trees	2019 and 2021

Since L'acqua dolce and Cannelle sampling site was characterized by the presence of several *Xylella fastidiosa* host plant species (e.g., *R. alaternus*, *S. junceum*, *C. monspeliensis*, *Cistus creticus*, *Cercis siliquastrum* and *Rosmarinus officinalis*), a transect sampling was conducted in 2021 (from June to October) to monitor spittlebugs movement from herbaceous vegetation to shrub and trees. The sampling was carried out applying the protocol described by Gargani *et al*. (2021) with the addition of six yellow sticky traps (0.80 m above the ground) on *X. fastidiosa* susceptible hosts plants that were present in both sampling localities: *R. alaternus*, *C. monspeliensis* and *S. junceum*.

Statistical analysis

Data collected during the monitoring of spittlebug preimaginal development were analyzed by performing a Multiple Factor Analysis (MFA). MFA is a multivariate data analysis method used to analyze data tables in which observations are described by several heterogeneous (quantitative and qualitative) inter-correlated dependent variables. This analysis enables a summary and graphical display of complex data sets, showing patterns of similarity between observation and variables (Abdi and Williams, 2010).

In the MFA analysis, the number of *P. spumarius* and *N. campestris* nymphs per plant was considered the dependent variable. In contrast, the species, the host plant family, and the position of the spittle on the host plant were considered factors. For the comparison, host plant species that accounted for less than 2% of the recorded species were grouped in a single factor class defined as "other spp.". Finally, shrubs of the Cistaceae family were excluded from this analysis since they were not sampled by the quadrat sampling method.

The MFA analysis aimed to explore the association between all the considered variables and highlight which factors determined the variability of the presence of *P. spumarius* and *N. campestris*.

MFA was performed with the open-source software RStudio (RStudio Version 1.3.1093, 2009–2020, PBC, Boston, MA; http://www.rstudio.com/) using the packages FactoMineR (Lê *et al*., 2008) and FactoExtra (Kassambara and Mundt, 2017). All variables were considered active, and the Kaiser criterion (average eigenvalue < 2) was applied to decide the number of retained dimensions.

Results

Philaenus spumarius and Neophilaenus campestris preimaginal development

Species composition, relative abundance, and phenology - During the 2019-2021 survey in Monte Argentario, 1.250 spittlebug nymphs were monitored in the sampling sites. Sampled individuals belong to five species: *P. spumarius*, *N. campestris*, *Lepyronia coleoptrata* (L., 1758) and *Aphrophora alni* (Fallen, 1805) included in the family Aphrophoridae and *Cercopis sanguinolenta* (Scopoli, 1763) that is a member of the family Cercopidae. *P. spumarius* and *N. campestris* were the two most frequent species in all the sampling sites, accounting respectively for 62.33% (778 individuals) and 30.65% (383 individuals) of the total amount of sampled nymphs. *Philaenus spumarius* was always present in the highest number except in the site of Cannelle, where *N.*

campestris was the most abundant. Among the three less represented species, *L. coleoptrata* was the most frequent, especially in those sampling sites where dicotyledonous species were predominant in the herbaceous ground cover (e.g., Stadium). First instar nymphs of *L. coleoptrata* and *A. alni* occurred on herbaceous plants from the end of March, while only late instar nymphs (from third to fifth) of *C. sanguinolenta* were recorded in April in the Cannelle sampling site. However, the overall amount of specimens of *L. coleoptrata*, *A. alni* and *C. sanguinolenta* reached only 0.07% of the total number of sampled nymphs.

First instar nymphs of *P. spumarius* and *N. campestris* appeared approximately in the 12th week (about the third week of March), with slight differences among sampling sites (Figure 1, 2, 3). Also, the two species' hatching period and developmental time did not vary notably among sampling years. The mean density of nymphs in both *P. spumarius* and *N. campestris* varies from 2 to 12 individuals per m^2 (Figure 2). At the Stadium site, the mean density of *P. spumarius* was always higher than that of *N. campestris* in both sampling years. The seasonal peak of the nymphal population for *P. spumarius* usually occurred in the 16th week. At the same time, that for *N. campestris* varied between the beginning of April and mid-April (14-16th week), depending on the sampling site and year. Generally, all nymphal instars were present at the seasonal peak in *P. spumarius*, while *N. campestris* showed a higher variability. In 2019, nymphal instars from first to fourth were monitored at the seasonal peak. In 2021, the peak occurred earlier in Stadium, and Forte Stella sites and only first to third-instar nymphs were present during the seasonal rise. Differently, in the L'acqua dolce site, the peak occurred in mid-April, and all the nymphal instars were recorded on that date.

Newly emerged *P. spumarius,* and *N. campestris* adults were recorded from the end of April/beginning of May (17-18th week) in all the sampling years. The preimaginal development of both species, from the disclosure of first-instar nymphs to the adult emergence, takes 30-40 days depending on the year, without remarkable variations.

In the "L'acqua dolce" site, *P. spumarius* was the predominant species, and nymphs were observed almost exclusively on *C. monspeliensis* shrubs. This fact might explain the low density of nymphs resulting from the quadrat sampling (Figure 4). Nymphs generally group on *C. monspeliensis*, forming numerous clusters, up to 5 nymphs per spittle, and a much froth, compared to other herbaceous host plant species.

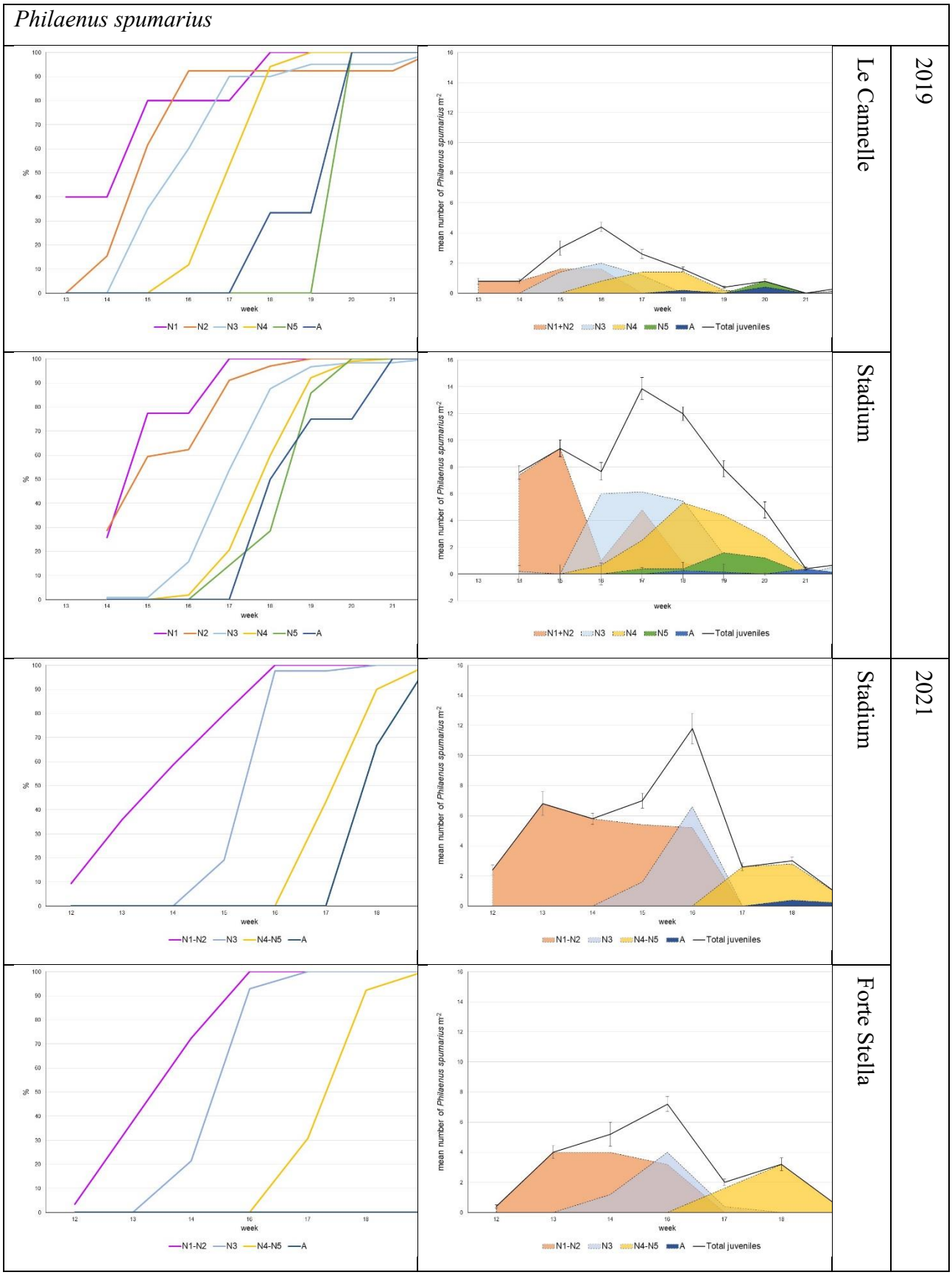

Figure 1. Life stage structure and observed cumulative populations of the juveniles and newly emerged adults of *Philaenus spumarius* in three locations of Monte Argentario (Tuscany, Italy) as recorded in 2019 and 2021. The area curves of nymphal instars and adults and the curve of total juveniles (graphs in the right column) are based on the mean densities estimated by quadrat sampling on each sampling date.

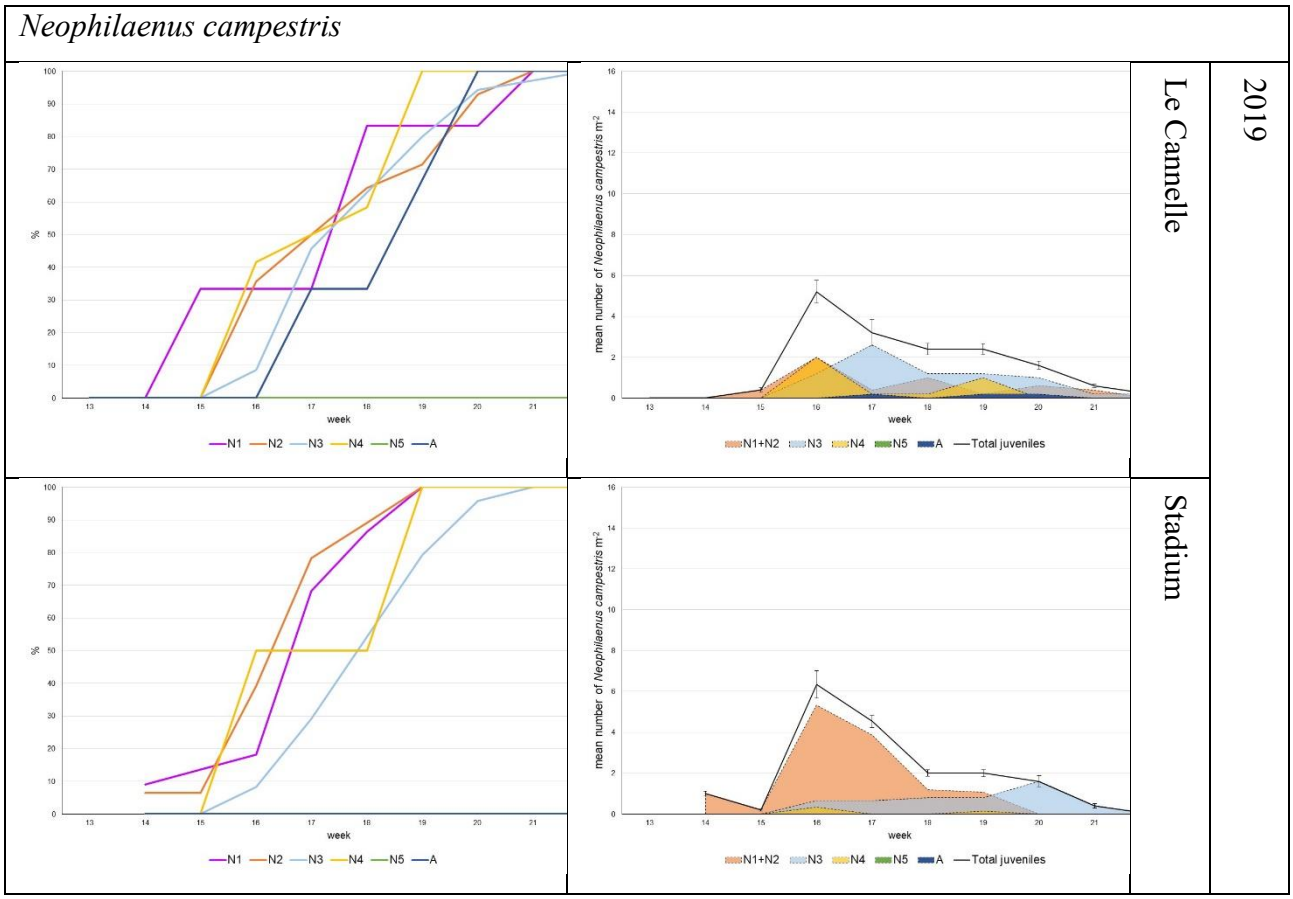

Figure 2. Life stage structure and observed cumulative populations of the juveniles and newly emerged adults of *Neophilaenus campestris* in two locations of Monte Argentario (Tuscany, Italy) as recorded in 2019. The area curves of nymphal instars and adults and the curve of total juveniles (graphs in the right column) are based on the mean densities estimated by quadrat sampling on each sampling date.

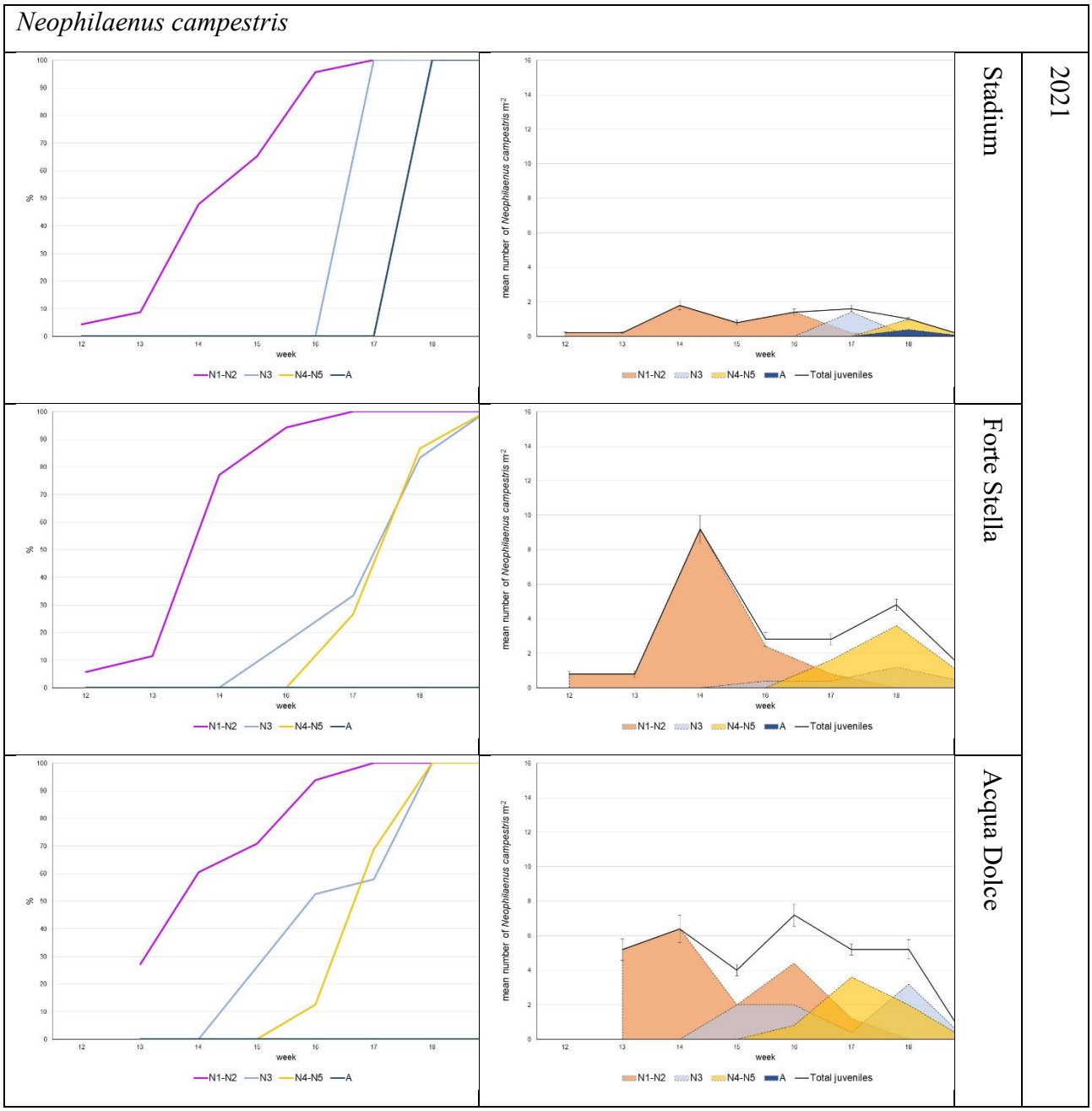

Figure 3. Life stage structure and observed cumulative populations of the juveniles and newly emerged adults of *Neophilaenus campestris* in three locations of Monte Argentario (Tuscany, Italy) as recorded in 2021. The area curves of nymphal instars and the curve of total juveniles (graphs in the right column) are based on the mean densities estimated by quadrat sampling on each sampling date.

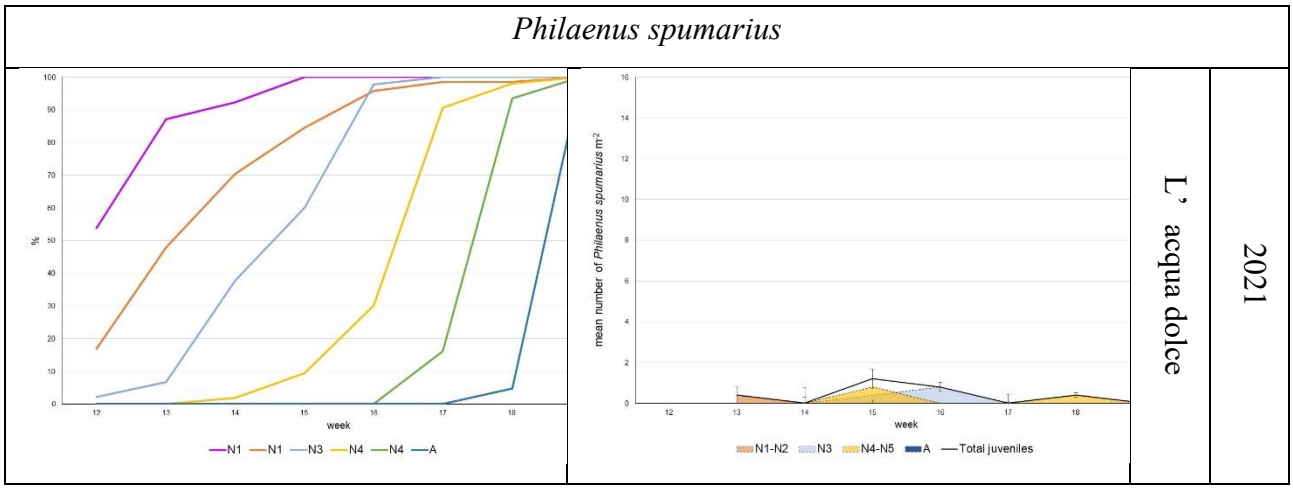

Figure 4 Life stage structure and observed cumulative populations of the juveniles and newly emerged adults of *Philaenus spumarius* in the "L'acqua dolce" sampling site of Monte Argentario (Tuscany, Italy) as recorded in 2021. The area curves of nymphal instars and the curve of total juveniles (graph in the right column) are based on the mean densities estimated by quadrat sampling on each sampling date.

Host plants – P. spumarius nymphs were recorded on many plant species, but *C. monspeliensis* appear to be the favourite host when it is present in the sampling site. The number of nymphs observed on this plant species (239 individuals), only in the area of L'acqua dolce and only in one year of sampling, is greater than that observed on *Foeniculum vulgare* Mill, which is the second most selected host plant. A large number of juveniles were also found on plant species belonging to the families Apiaceae, Fabaceae, Asteraceae, Poaceae and Plantaginaceae (Figure 5). *Foeniculum vulgare* was the preferred host among the Apiaceae species, plants of the genus *Medicago* were the preferred hosts among Leguminosae, *Dittrichia viscosa* L. among Asteraceae, *Plantago lanceolata* L. among Plantaginaceae and *Poa* spp. among Poaceae. Many nymphs were also observed on Geraniaceae, especially on *Geranium molle* L., and Ranunculaceae, mainly on *Ranunculus* spp. Some strictly Mediterranean species, such as *Psoralea bituminosa* L. and *Calamintha nepeta* (L.), were also found to be hosts of *P. spumarius* nymphs.

Our data highlights a stage-specific host plant preference when we relate host plants to different juvenile instars. Notably, early instar nymphs were monitored more frequently on plants of the Geraniaceae family and Apiaceae species (Figure 6). The stage specificity is less noticeable in fourth and fifth instar nymphs, which seem to be more recurrent on Asteraceae species (Figure 6).

As expected, *N. campestris* showed a clear preference for Poaceae species with a few sporadic records on dicotyledonous species (Table 3). The majority of *N. campestris* nymphs were observed on *Poa* spp., *Bromus* spp. and *Avena barbata*.

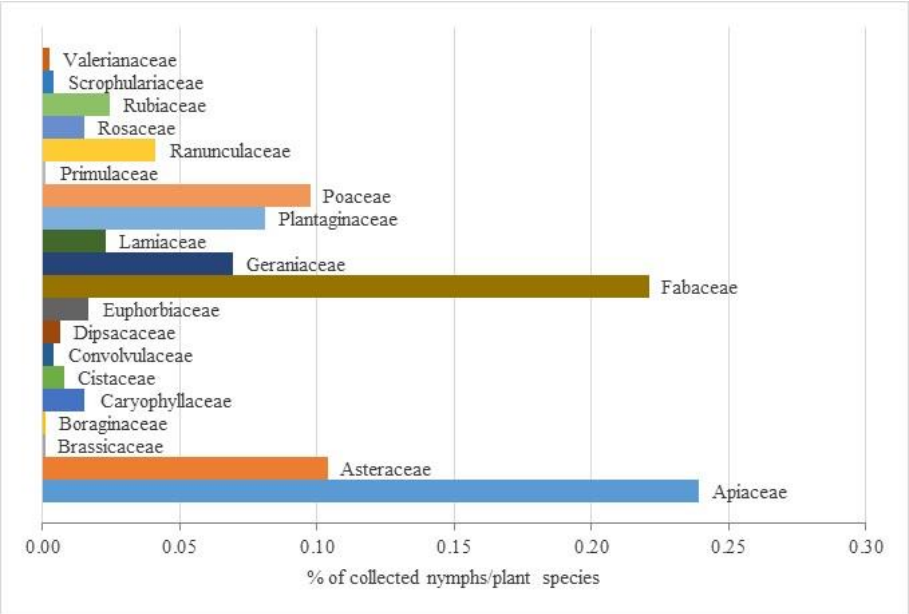

Figure 5. Distribution of *P. spumarius* nymphs on host plants in the Monte Argentario area (percentage over the total number of nymphs recorded in 2019 and 2021). Nymphs visually inspected on *C. monspeliensis* shrubs were excluded from this data to avoid biases due to the differences in the sampling method. The category Cistaceae in this graph comprises only small plants enclosed in the quadrat sampling.

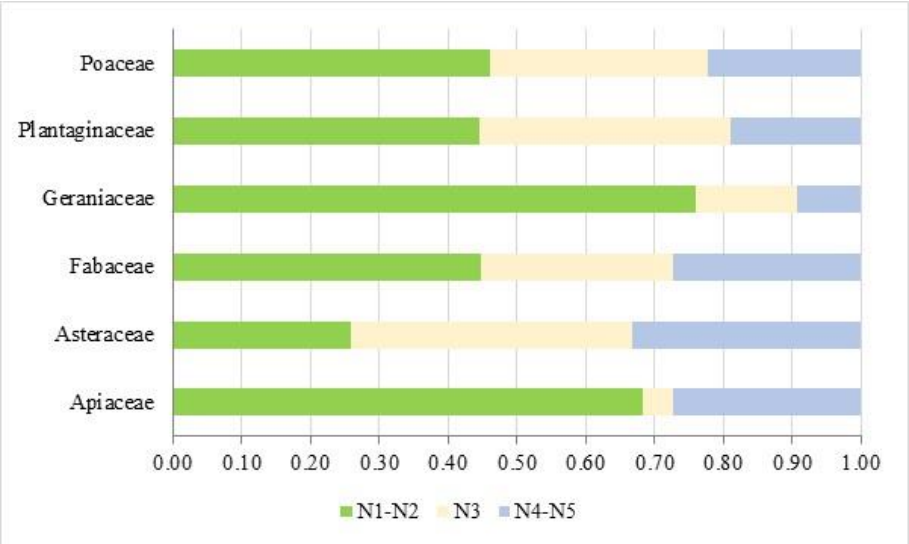

Figure 6. Host plants stage-specificity of *P. spumarius* nymphs observed in the Monte Argentario area (percentage over the total number of nymphs recorded in 2019 and 2021)

Table 2. Host plants of *Philaenus spumarius* juveniles. Numbers of monitored specimens of different instars were reported for each plant's family/species.

Host plants	**Number of nymphs**			**Total**
	N1-N2	**N3**	**N4-N5**	
Apiaceae				**186**
Daucus carota	0	0	1	1
Foeniculum vulgare	125	5	48	178
Scandix pecten-veneris	1	2	2	5
Torilis sp.	1	0	0	1
Tordylium apulum	0	1	0	1
Asteraceae				**81**
Bellis sp.	0	0	1	1
Calendula arvensis	2	0	1	3
Carduus nutans	1	1	2	4
Centaurea sp.	1	0	0	1
Cichorium intybus	2	0	0	2
Cirsium vulgare	0	3	0	3
Dittrichia viscosa	11	14	9	34
Leontodon sp.	0	4	0	4
Pallenis spinosa	0	0	4	4
Sonchus asper	4	10	6	20
Taraxacum officinale	0	0	1	1
Urospermum delechampii	0	1	3	4
Brassicaceae				**1**
Sinapis arvensis	0	0	1	1
Boraginaceae	0	0	0	**1**
Borago officinalis	0	0	1	1
Caryophyllaceae				**12**
Silene latifolia	2	9	0	11
Stellaria media	0	1	0	1
Cistaceae				**6**
Cistus creticus	1	0	0	1
Cistus monspeliensis	0	3	2	5
Convolvulaceae				**3**
Convolvulus sp.	1	0	2	3
Dipsacaceae				**5**
Scabiosa columbaria	0	2	3	5
Euphorbiaceae				**13**
Euphorbia cuneifolia	8	4	1	13
Fabaceae	4	0	3	**172**
Psoralea bituminosa	12	7	1	20
Lathyrus sp.	1	0	0	1
Medicago lupolina	0	4	12	16
Medicago sp.	30	17	15	62
Trifolium sp.	11	7	7	25
Trifolium stellatum	0	1	0	1
Vicia sp.	19	12	9	40
Geraniaceae				**54**
Erodium sp.	2	3	0	5

Geranium molle	39	5	5	49
Lamiaceae				**18**
Calamintha nepeta	1	2	15	18
Plantaginaceae				**63**
Plantago lanceolata	26	21	12	59
Veronica sp.	2	2	0	4
Poaceae	34	12	14	**76**
Avena barbata	0	2	2	4
Poa spp.	1	9	1	11
Bromus sp.	0	1	0	1
Primulaceae				**1**
Lysimachia sp.	0	0	1	1
Ranunculaceae				**32**
Anemone hortensis	3	0	0	3
Ranunculus sp.	19	4	6	29
Rosaceae				**12**
Rubus ulmifolius	3	2	0	5
Potentilla reptans	3	3	1	7
Rubiaceae				**19**
Galium sp.	3	6	0	9
Sherardia arvensis	10	0	0	10
Scrophulariaceae				**3**
Bellardia trixago	3	0	0	3
Valerianaceae				**2**
Centranthus sp.	1	1	0	2
Unidentified plant species	10	6	2	**18**

Table 3. Host plants of *Neophilaenus campestris* juveniles. Numbers of collected specimens of different instars were reported for each plant's family/species.

Host plants	**Number of nymphs**			**Total**
	N1-N2	**N3**	**N4-N5**	
Apiaceae				1
Foeniculum vulgare	1	0	0	1
Brassicaceae				2
Hirschfeldia incana	0	1	1	2
Fabaceae				4
Medicago lupulina	0	1	0	1
Medicago spp.	1	0	0	1
Psoralea bituminosa	0	0	1	1
Trifolium sp.	0	1	0	1
Plantaginaceae				1
Plantago lanceolata	1	0	0	1
Poaceae	164	70	46	374
Aegilops geniculata	4	2	2	8
Avena barbata	12	7	1	20
Avena sterilis	2	4	0	6

Bromus sp.	9	13	3	25
Dactilys glomerata	2	3	0	5
Lagurus ovatus	1	0	0	1
Panicum repens	0	1	0	1
Poa spp.	23	4	1	28
Ranunculaceae				1
Ranunculus spp.	1	0	0	1

Overall evaluation of the species occurrence - The eigenvalue to explain the total variance of data was selected according to the criteria defined by Kaiser (1958) (average eigenvalue < 2). Dimension 1 (Dim. 1) satisfied the criterion and summarized most information, but Dimensions 1 to 5 must be considered to explain at least 50% of the overall variability. However, to simplify the visualization of the results, we only considered Dim. 1 and Dim. 2, which explained 25.98% of the overall variability. Although it was less than 50%, the representation was valid for describing the variance distribution within the dataset. The results of the MFA analysis are reported in Table 4.

The two variables, the "number of nymphs per plant family" and the "spittlebug species", are linked with Dim. 1 (Figure 7) and explain most of its total variability, contributing to 32.99% and 34.31%, respectively. Moreover, these two variables positively correlate to the first dimension (Corr. = 0.79 and 0.64, respectively). Considering the two components of the factor "spittlebug species" separately, they contribute to the construction of Dim. 1. Still, they show a different correlation: *P. spumarius* (Contr = 8.58%) is positively correlated to the Dim. 1 while *N. campestris* displays a negative correlation (Corr. = -1.27) to the same dimension. Observations positively correlated to Dim. 1 may be characterized by more sampled individuals. Factors *P. spumarius* (Contr. = 0.77%) and *N. campestris* (Contr. = 2.31%) slightly contribute also to the variability of the Dim. 2, although in an opposite way (Figure 7). The variable "host plant family" contributes both to Dim.1 (Contr. = 21.86%) and Dim.2 (Contr. = 59.35%), with a higher contribution to the second one. Moreover, although dimensions from 3 to 5 were not considered, it could be interesting to highlight that the "host plant family" strongly contribute to these three dimensions (Contr. = 46.90%, to Dim. 3; Contr. = 99.00%, to Dim. 4; Contr. = 100.00%, to Dim. 5).

Among host plant families, *Poaceae* is mainly linked and positively correlated to Dim. 2 (Contr. = 14.28; Corr. = 1.16). Moreover, the *Poaceae* family also contributes to Dim. 1, although negative correlates to this dimension (Contr. = 6.20%; Corr. = -1.34). Indeed, the factor *Poaceae* ensues in an opposite position to Dim. 1, close to Dim. 2 (Figure 7), emphasizing the importance of this plant family for *N. campestris*.

Table 4. Variable and factor contributions to the Dimension from 1 to 5. Although only Dim. 1 and Dim. 2 were considered to describe the variance distribution, results were also reported for dimensions 3 to 5 to show the complete information.

Variables and Factors	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5
Number of nymphs/plant family	32.99	0.00	0.72	0.64	0.00
Spittlebug species	34.31	3.08	0.34	0.36	0.00
Philaenus spumarius	8.58	0.77	0.09	0.09	0.00
Neophilaenus campestris	25.73	2.31	0.26	0.27	0.00
Plant family	21.86	59.35	46.90	99.00	100.00
Apiaceae	0.15	28.29	2.30	3.37	0.38
Asteraceae	3.25	0.79	1.77	39.54	6.14
Fabaceae	2.75	1.71	17.24	0.23	0.47
Geraniaceae	4.37	0.79	1.06	2.41	13.35
Plantaginaceae	0.12	4.24	6.53	6.17	5.77
Poaceae	6.20	14.28	2.66	4.02	0.56
Ranunculaceae	0.01	4.23	7.88	16.27	31.59
Other families	0.10	4.23	6.67	3.12	10.36
Position of the spittle on the plant	10.84	37.57	52.03	0.00	0.00
Top	5.43	24.46	4.67	0.00	0.00
Middle	0.08	1.06	35.86	0.00	0.00
Bottom	5.33	12.05	11.50	0.00	0.00

Among the other considered host plant families, the Apiaceae family strongly contributes to Dim. 2 with a positive correlation (Contr. = 28.29%; Corr. = 1.94). Its intermediate position between Dim. 1 and Dim. 2 indicates that this plant family is an essential host for *P. spumarius,* like for the family Poaceae for *N. campestris* (Fig. 6). The representation of the other host plant families that are condensed in the Dim. 1 together with *P. spumarius*, suggests the presence of a similar number of *P. spumarius* nymphs on each one of them. Finally, the factor "position of the spittle on the host plant" contributes mainly to the Dim. 2 (Contr. = 37.57%; Corr. = 0.44) and together with the factor "host plant family" explains the 96.92% of this dimension. As for the relationship between the "position of the spittle" and Dim.1., this dimension is positively correlated with the apical position (Contr. = 5.43%; Corr. = 0.73), while it is negatively correlated with the basal position (Contr. = 5.33%; Corr. = -0.76). This result suggests that *P. spumarius* nymphs were most frequently recorded on the apical part of the plant, while *N. campestris* was mainly observed at the plant bottom. Results of the MFA analysis emphasize a difference in the feeding habits of the nymphs of the two spittlebug species.

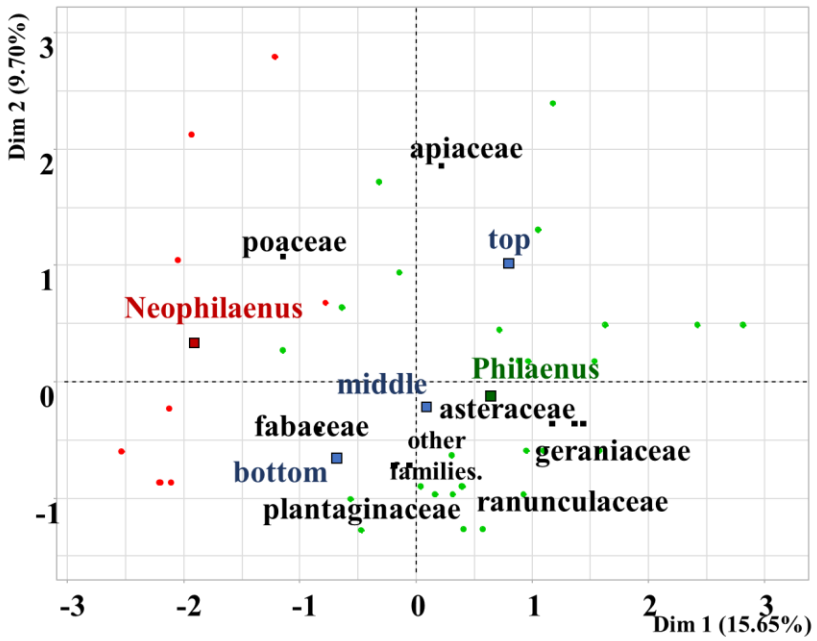

Figure 7. MFA results: correlation of qualitative variables to Dim. 1 and Dim. 2. For each category, points indicate the barycenter of the observations.

Sampling of spittlebug adults

199 *P. spumarius* and 60 *N. campestris* adults were collected from June to October 2021 in the Monte Argentario area (random sampling plus transect sampling). Transect- and trap-sampling resulted ineffective to track the seasonal pattern of spittlebug adults and verifying the association of *P. spumarius* and *N. campestris* to *X. fastidiosa*-susceptible hosts. Indeed, very few spittlebug captures were accomplished during transect sampling, and those achieved were concentrated in late summer when spittlebug adults returned to herbaceous plants to mate and lay eggs. Specifically, in October, one *N. campestris* female was collected on grasses in the L'acqua dolce site, while three males and one female were collected on grasses in Cannelle. In September, one *P. spumarius* male was collected in the Acqua dolce site on herbs. In October, one male and one female were collected on *Pistacia lentiscus* and *C. monspeliensis,* respectively. Yellow sticky traps registered only one capture in September: one *P. spumarius* female on a trap positioned on *C. monspeliensis* in the Acqua Dolce site.

Table 5. Dimensions description of the variables and factors

Variables and Factors*	Dim. 1	Dim. 2	Dim. 3	Dim. 4
Number of nymphs/plant family	0.79	-	-	-
Spittlebug species	0.64	-	-	-
Philaenus	1.27	-	-	-

Neophilaenus	-1.27	-	-	-
Plant family	0.41	0.69	0.53	0.99
Poaceae	-1.34	1.16	-	-
Apiaceae	-	1.94	-	-
Ranunculaceae	-	-	-	1.24
Fabaceae	-	-	-1.28	-
Asteraceae	-	-	-	-2.17
Position on plant	0.20	0.44	0.59	-
Top	0.73	0.98	-	-
Middle	-	-	-1.16	-
Bottom	-0.76	-0.71	0.67	-

*Values for the continuous variables refer to the correlation coefficient. In contrast, the categorical variables refer to the square of the correlation ratio (T-test to compare the category average with the general mean). All results were reported when the value was significantly different ($p < 0.05$).

Transect- and trap-sampling did not enable to clarify the seasonal movement of spittlebugs adults but highlighted the almost complete absence of *P. spumarius* and *N. campestris* from *Spartium junceum* and *Rhamnus alaternus,* which are the two more frequently *Xylella*-infected plant species in Monte Argentario. Only one *N. campestris* male was collected in June from *R. alaternus* at Cannelle. On the other hand, members of the families Issidae and Cicadellidae were the more abundant Auchenorrhyncha collected along the transects.

A random sampling of spittlebug adults throughout Monte Argentario promontory permitted the collection of them from their summer food plants. *P. spumarius* was frequently collected from conifers, especially from *Cupressus sempervirens*. However, figure 8 shows that more specimens between June and August were collected on *Laurus nobilis*. Interestingly, all the 51 *P. spumarius* individuals reported in the graph were collected from a single large *L. nobilis* plant during a 15-minutes survey. This plant was in the " Cacciarella " site, the same reported by Gargani *et al*., (2021). Although we excluded these data from the descriptive analysis, we would say that other 102 adult specimens were successively captured from this unique plant using the sweeping net on three consecutive sampling dates (15th July, 29th July and 31st of August). In addition, *P. spumarius* adults were abundantly collected from *Pistacia lentiscus*, *Quercus ilex,* and *C. sempervirens* near this *L. nobilis* plant. In September, *P. spumarius* adults were mainly collected on *Dittrichia viscosa*; in October, adults occurred mainly on meadows.

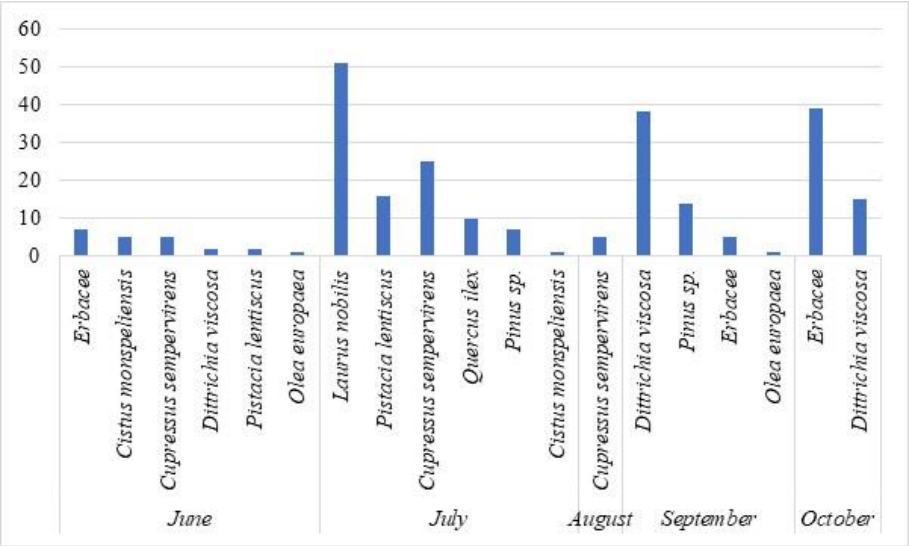

Figure 8. Number of *Philaenus spumarius* adults collected by sweeping net from different shrubs and trees in Monte Argentario (June-September 2021)

N. campestris adults were primarily collected from *Pinus* species and show a narrower host plants range, as evidenced for juvenile stages (Figure 9). As in the case of *P. spumarius*, no *N. campestris* adults were collected from *R. alaternus* and *Spartium junceum* from June and October, except for the single specimens captured during transect sampling.

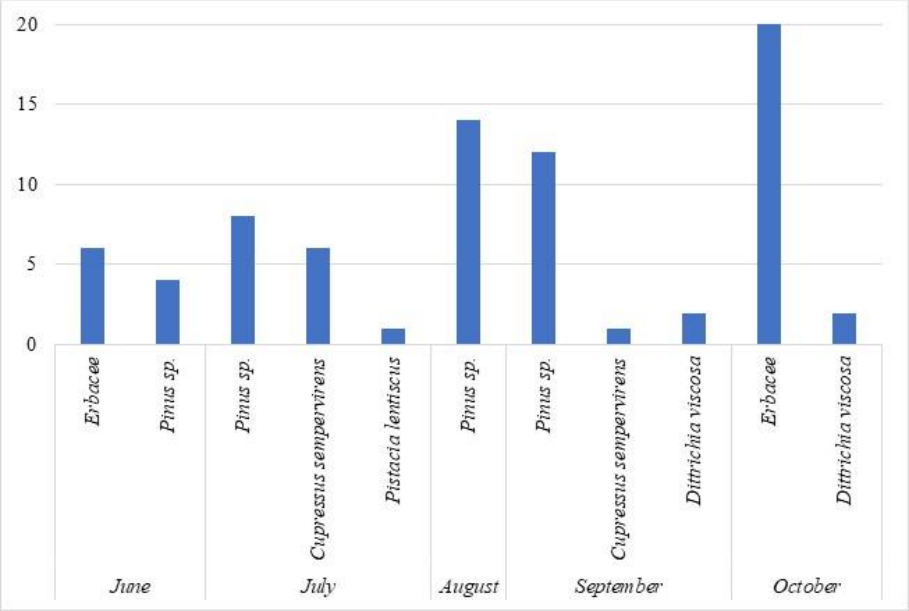

Figure 9. Number of *Neophilaenus campestris* adults collected by sweeping net from different shrubs and trees in Monte Argentario (June-September 2021)

Adult surveys have also evidenced two peaks of abundance on herbaceous plants. The first peak occurred in late May-early June, and it is very restricted in time, besides being less obvious. After

this peak, adults were rarefied, almost disappearing from most sites where juveniles were observed. The second peak occurred in late September when adults of both species heavily increased their abundance of herbs.

Finally, among other spittlebug species observed during the sampling of preimaginal stages, *A. alni* was the only one collected from shrubs and trees during summer (June-August). Six individuals were captured: *A. unedo* (1 specimen), *P. lentiscus* (4 specimens) and *C. sempervirens* (1 specimen). *L. coleoptrata* was collected from herbaceous plants in summer (less frequently) and autumn. *Aphrophora alni* adults have never been collected on herbs in autumn. A few individuals of *C. sanguinolenta* (8) were collected in June from grasses.

Discussion

The long-term field survey was conducted in the Monte Argentario area to study Aphrophoridae potential vectors of *X. fastidiosa* subsp. *multiplex* showed the presence of five spittlebugs species. *P. spumarius* and *N. campestris* were the most abundant species, representing 99% of the surveyed Cercopoidea fauna. The other identified spittlebugs, namely *L. coleoptrata*, *A. alni* and *C. sanguinolenta,* were sporadically collected on herbs, shrubs, and trees as nymphs and adults. *A. alni* and *L. coleoptrata's* life history appears similar to that of *P. spumarius* and *N. campestris,* and these species partially share habitat and host plants. *L. coleoptrata* nymphs were observed only in spring, according to that ascertained for populations monitored in southern Italy (Trotta *et al.*, 2021) and contrarily to that observed in northern Italy by Barro and Pavan (1999), that described *L. coleoptrata* as a polyvoltine species. Consistently to previous studies, nymphs of *L. coleoptrata* and *A. alni* appear on herbaceous plants from the end of March and are mainly associated with dicotyledons (Bodino *et al*., 2019; 2020; Trotta *et al*., 2021). In April, the fourth and fifth instar nymphs of *Cercopis sanguinolenta* were observed only in the “Cannelle” site. This froghopper is a univoltine species found in dry and wet grasslands (Biedermann, 2002). Preimaginal stages feed on grass roots below the ground or just above the soil surface, generally close to the root neck (Biedermann, 2002; Dietrich, 2009). *C. sanguinolenta* overwinters as nymphs and concludes its preimaginal development in spring. For this reason, our springtime survey of preimaginal stages has recorded only late-instar nymphs of this species. Although *C. sanguinolenta* could be considered a relatively common species in Italy (D’Urso, 1995; Guglielmino *et al*., 2005; Mazzoni, 2005; Jach, 2014), our findings integrate a previous study on Auchenorrhyncha of Monte Argentario that did not report this species (Gargani *et al*., 2021).

Both *P. spumarius* and *N. campestris* nymphs appear on herbs starting in the second half of March. Although some variation could occur depending on the climate conditions, only a few differences were observed among sampling years and sites. Our observations were consistent with those reported in the literature in recent years (Bodino *et al*., 2019, Di Serio *et al*., 2019).

First and second-instar nymphs of *P. spumarius* and *N. campestris* were consistently more abundant than successive stages and showed a greater density in the field. This fact was particularly evident for *N. campestris* in 2021 when the population peak in Forte Stella and Stadium sites corresponded to the rise of early instar nymphs. The tendency of small nymphs of both species to aggregate inside the same foam increases the registered density per square meter (Weaver and King, 1954; Halkka *et al*., 1977). Besides that, decreasing population abundance might be correlated to a high natural mortality rate often observed in spittlebugs (Wiegert, 1964; Whittaker, 1965; 1973).

Cistus monspeliensis appear to be a key host for *P. spumarius* juveniles, as previously observed in the Ajaccio region of Corsica (Abre *et al*., 2021). Indeed, where this plant species occurs, most of the meadow spittlebug nymphs were collected on it. *Cistus monspeliensis* is a dominant shrub species in Mediterranean areas, typically distributed on degraded and poor soils (Zalegh *et al*., 2021). Its survival strategies mainly rely on producing efficient secondary metabolites that allow the exploitation of resources in extreme ecosystems (Saracini *et al*., 2005). *Cistus* species have a quantity of polyphenols that protect plants from biotic and abiotic environmental stress. For example, these compounds are crucial in reducing nitrogen's mobility in soil, enhancing the availability of this essential element for *Cistus* plants (Sarcini *et al.*, 2005). This fact might explain the close association between this plant and *P. spumarius* since the meadow spittlebug is known to feed preferentially on plant species with high nitrogen content in their xylem sap, such as Fabaceae species (Thompson, 1994). In addition, *P. spumarius* is primarily associated with mycorrhizal plants (Thompson, 1999; 2022), and this could explain the relationship between this insect and *C. monspeliensis* plants, which are known to establish symbiosis with ectomycorrhizal fungi (Maremmani *et al*., 2003; Carvalho *et al*., 2003).

Besides *C. monspeliensis*, *P. spumarius* nymphs prefer some genera of Apiaceae (*Foeniculum*), Asteraceae (*Dittrichia*), and Fabaceae (*Medicago*), corroborating what has been observed in previous studies conducted in Italy (Dongiovanni *et al*., 2019; Bodino *et al*., 2020; Trotta *et al*., 2021). On the other hand, *N. campestris* was confirmed to be almost exclusively associated with Poaceae (Nickel and Remane, 2002; Cornara *et al*., 2021). This outcome was also evidenced by the MFA analysis that emphasized the contribution of Poaceae to the presence and abundance of *N. campestris* nymphs.

Host association in *P. spumarius* nymphs change over time. Nymphs, especially later instars, have good dispersal capability and crawl to search for new hosts (Weaver and King, 1954; Albre *et al*.,

2021). Although the mechanism of host plant selection in *P. spumarius* juveniles is poorly understood, nymphs seem driven by host plant phenology and feed on actively growing plants (Cornara *et al*., 2018). In this study, early instar nymphs are mainly spotted on Geraniaceae and Apiaceae, while older nymphs did not show a clear preference.

Several plant species on which *P. spumarius* and *N. campestris* nymphs feed are reported to be hosts of *X. fastidiosa* (EFSA, 2023). For example, *Medicago sativa*, *Plantago lanceolata*, *Dittrichia viscosa* and *C. monspeliensis* were found to be naturally infected by the subspecies *multiplex* of the bacterium. Juvenile spittlebugs are not considered a vector of *X. fastidiosa* since the bacterium is lost with moult (Redak *et al*., 2004). However, these plants could represent a reservoir of the pathogen, and newly emerged adults could acquire *X. fastidiosa* before dispersing towards estivation sites.

Adult spittlebugs appear at the end of April and show two peaks of abundance, a first one after their emergence and a second and more evident one in autumn when they return to herbaceous plants for mating. Dispersal events in *P. spumarius* and *N. campestris* have been observed several times (Weaver, 1951; Lavigne, 1959; Cornara *et al*., 2016; Morente *et al*., 2018; Lago *et al*., 2021). In general, *N. campestris* tend to move for longer distances because it needs to reach its specific summer hosts since it shows a restricted host plant range compared to *P. spumarius* (Lago *et al.*, 2021; Bodino *et al*., 2021). Differently, the meadow spittlebug can exploit several plant species, so its dispersal range can change depending on the environmental condition and the availability of food resources (Bodino *et al.*, 2019).

In Mediterranean areas, the hot and dry summer triggers a strong dispersal movement in spittlebug adults that leave the place of their development in search of more sheltered and cool sites. Our observations are consistent with that observed in the Ajaccio region of Corsica, where adults completely rarefied during summer, reappearing when the temperature decreases (Albre *et al*., 2021). In our survey, from June to August, *P. spumarius* and *N. campestris* adults were mainly collected on conifers, which are already known to be summer hosts of these species (Mazzoni, 2005; Cornara *et al*., 2018; Lago *et al*., 2021). In addition, *P. spumarius* was abundantly found on a single large plant of *L. nobilis*, showing an interesting aggregation pattern. The sampled *L. nobilis* plant was in the " Cacciarella " site, which Gargani *et al*., (2021) describe as the site where the most significant number of spittlebugs were collected throughout the year. The Cacciarella site was on the southwest side of Monte Argentario, north exposed and at 183 m a.s.l. The studied area is characterized by an olive grove with a meadow grass soil coverage, surrounded by Mediterranean maquis vegetation, mainly consisting of *P. lentiscus*, *S. junceum* and *C. monspeliensis*. During our summer survey (June-August), we noted that spittlebugs were relatively abundant in the olive grove and their surroundings, different from all the other sampled sites.

Further investigations focusing on the peculiar microclimatic characteristic of this site could offer the possibility of understanding the *P. spumarius* dispersal drivers.

Since *P. spumarius* and *N. campestris* were considered as the most probable vector of *X. fastidiosa* subsp. *multiplex* in Monte Argentario promontory, we expected that *Xylella*-infected plant species were shared food of these two spittlebugs, at least for a limited period. On the contrary, our survey has confirmed what Gargani *et al*., (2021) observed, that spittlebugs (including *A. alni* and *L. coleoptrata*) seem to not feed on the two plant species more frequently found infected by *X. fastidiosa*: *S. junceum* and *R. alaternus* (Fitosirt database, https://fitosirt.regione.toscana.it/). However, *P. spumarius* was collected on *C. monspeliensis*, *L. nobilis* and *Q. ilex*, also infected in Monte Argentario. Moreover, the meadow spittlebug has also been captured on *D. viscosa,* a proven host of *X. fastidiosa*. Differently, *N. campestris* was found on *D. viscosa* in autumn and only once on *R. alaternus,* but not on the other mentioned *X. fastidiosa* host plants.

Sampling spittlebug adults seems to be a hard task. A recent study in which various sampling methods were compared assessed the inefficacy of many tested tools, including yellow sticky traps, confirming the sweeping net as the most valid method (Morente *et al*., 2018). However, sweeping nets do not allow the adequate sampling of tree canopies, especially when the branches are firm, as in the case of *R. alaternus*. An ineffective sampling method could explain the little informative value of the collected data. However, we were able to sample insects from shrubs and trees, indicating that the absence of spittlebug records from some plant species could not be ascribed to the failure of the sampling method.

Our observation posed an important question: are *P. spumarius* and *N. campestris* involved in transmitting *X. fastidiosa* subsp. *multiplex* in Monte Argentario?

Based on samplings of Auchenorrhyncha fauna in Monte Argentario, these two spittlebug species are the most represented xylem-feeders in the area. Moreover, several individuals of both species were positive for the bacterium (Gargani *et al*., 2021).

Among the factors that affect the successful transmission of *X. fastidiosa* by xylem-sap feeding species, feeding behaviour plays a key role. The egestion of saliva during the probing is a crucial step in the inoculation of the bacterium (Backus and Morgan, 2011; Backus *et al*., 2012). Frequent probing is usually associated with a lesser acceptance of the host plants but, at the same time, a greater probability of *X. fastidiosa* transmission (Daugherty and Almeida, 2009; Cornara *et al*., 2020a; Markheiser *et al*., 2022). So, we can speculate that *S. junceum* and *R. alaternus,* although unsuitable hosts for spittlebugs, just need a marked probing behaviour for *X. fastidiosa* subsp. *multiplex* infection. Considering the information in this study, *P. spumarius* seems to be the main species potentially involved in the transmission of the bacterium since its more comprehensive host range

and the possibility to acquire *X. fastidiosa* from shrubs and trees like *C. monspeliensis* or *L. nobilis,* which frequently host *P. spumarius* adults. However, the role of *N. campestris* should not be overlooked. More focused investigations on its host plants, ecology and dispersal are needed to clarify its contribution to the spread of *X. fastidiosa*.

On the other hand, the presence of the bacterium within potential vectors cannot lead to the conclusion that such insects are the actual vector. Moreover, the complexity of *X. fastidiosa* pathosystems prevents the generalization of knowledge derived from other studied situations (Sicard *et al*., 2018). Since that, other hypotheses should be evaluated. For example, cicadas are widespread in Monte Argentario and the Mediterranean basin. Cicadas are challenging to sample, so their occurrence on *X. fastidiosa* host plants could be underestimated. Although the role of cicadas in transmitting *X. fastidiosa* to olive trees was negligible (Cornara *et al.,* 2020b), these insects could play a different role in spreading the bacterium in natural environments like the Mediterranean maquis. Again, *Aphrophora* species have been reported as a potential vector of *X. fastidiosa*, especially in forest ecosystems (Desprez-Loustau *et al.,* 2021; Casarin *et al*., 2022), and although a few specimens of *A. alni* were collected on shrubs and trees in this survey, its ecology should be better investigated.

Another hypothesis is phloem-feeders' involvement in *X. fastidiosa* transmission that could accidentally access the xylem. If this is the case, we must investigate planthoppers like Issidae - especially *Latilica maculipes* and *Agalmatium flavescens* - frequently detected on *S. junceum*, *R. alaternus* and other species of the Mediterranean maquis along the year. Since the feeding behaviour of these species has never been described, their role in the transmission of the bacterium deserves further investigation.

Our results have provided first accounts on the ecology of spittlebug potential vectors of *X. fastidiosa* subsp. *multiplex* in the Mediterranean maquis. This information could be employed in managing the *X. fastidiosa* outbreak in Monte Argentario and in planning future and more focused studies aimed at clarifying the role of *P. spumarius* and *N. campestris* in the epidemiology of *X. fastidiosa* subsp. *multiplex* in Tuscany.

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2. Spittlebug invisibility cloak: experimental tests on the antipredatory effect of the froth of *Philaenus spumarius*

Personal contribution: Conceptualization, insect rearing, laboratory trials and data collection, data analysis, manuscript preparation.

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Abstract

In Europe, the meadow spittlebug *Philaenus spumarius* (L.) (Hemiptera: Aphrophoridae) is the main vector of the bacterium *Xylella fastidiosa* (Wells *et al.*), the etiological agent of the Olive Quick Decline Syndrome. The froth produced by spittlebug nymphs has a primary function in protecting the insect from dehydration and thermal stress. It is also accepted that the froth protects nymphs from predators, although the underlying mechanism is not completely clear. We investigated such a process using the crab spider *Synema globosum* (Fabricius) and the ant *Crematogaster scutellaris* (Olivier) as model species. Nymphs of *P. spumarius* were divided into two groups, one whose froth was left and one whose froth was removed. The nymphs were then exposed to predators and their survival recorded. The survival of defrothed nymphs was considerably lower than controls with both spiders and ants, though this could be due to increased motility of defrothed nymphs. Moreover, to test the chemical properties of the froth and exclude any physical hindrance effect, *P. spumarius* nymphs and dead *Sarcophaga carnaria* (L.) larvae (maggots) under three different conditions (defrothed, water-coated, and centrifuged froth-coated) were offered to workers of *C. scutellaris*. The survival of the nymphs and ants' bites to both prey were recorded. Again, defrothed nymphs showed a lower survival probability compared to those moistened with water and froth, while no differences were found between these two treatments, suggesting a chemical deterrence or mimicry of the froth. The highest number of ants' bites towards nymphs and maggots was recorded in the defrothed group, while the

lowest in the froth-coated nymphs. A significant difference between the water- and froth-coated treatments was only found in nymphs and not in maggots, suggesting the presence of some residual substances on the nymph's integument that could have a deterrent or masking effect. Additionally, our direct observations of ants drinking the froth reinforce chemical mimicry as a more plausible explanation than deterrence. In conclusion, our findings suggest that the froth plays an antipredatory role, at least for predators that use mainly olfactory cues to localise their preys, through a chemical mimicry mechanism.

Introduction

The meadow spittlebug, *Philaenus spumarius* (L.) (Hemiptera: Aphrophoridae), is a polyphagous xylem fluid-feeding species widespread in the Palaearctic and Nearctic realms (Drosopoulos and Remane, 2000; Cornara *et al.*, 2018) where it occurs at different latitudes and altitudes (Yurtsever, 2000). It is also present in the Azores, Hawaii and New Zealand, where it was probably introduced in the second half of twentieth century (Yurtsever, 2000). The common name "spittlebug" refers to the nymphs' habit to develop inside a self-produced froth excreted from the anus. Although spittlebugs have attracted the attention of naturalists for centuries, it is still not fully understood how the froth is produced, what it is composed of and what its exact role is.

The main constituent of spittlebug froth is the liquid derived from xylem sap of the plant on which the insect feeds (Wilson and Dorsey, 1957). Both nymphs and adults are xylem feeders, ingesting large amounts of sap to compensate for the poor nutritional value of this nourishment, and excreting the excess from the anus (Weaver and King, 1954; Wilson and Dorsey, 1957). Nymphs introduce air bubbles into the expelled fluid, by means of telescopic movements of the abdominal urites, giving the typical frothy appearance to the spittle mass (Weaver and King, 1954). In addition to the alimentary canal, Malpighian tubules are involved in the production of mucopolysaccharides that probably act as surfactants, giving stability to the froth (Marshall, 1966). Moreover, the Batelli glands, described only in nymphs of species belonging to the Aphrophoridae family, seem to produce a lipoid substance that prevents froth desiccation (Marshall, 1965).

It is widely accepted that the froth protects spittlebug nymphs against desiccation and natural enemies (Whittaker, 1970; del Campo *et al.*, 2011). A thermoregulatory function of the froth has been demonstrated for *Mahanarva fimbriolata* (Stål) (Tonelli *et al.*, 2018) and proposed for *P. spumarius* (Whittaker, 1970; Cornara *et al.*, 2018) and *Poophilus costalis* (Walker) (Sahayaraj *et al.*, 2021). Moreover, some examples of repellence, antibacterial, and antifungal activity of the froth have been

reported for various spittlebug species (del Campo *et al.*, 2011; Chang *et al.*, 2019; Tonelli *et al.*, 2019, 2020; Sahayaraj *et al.*, 2021) except for *P. spumarius*. For instance, Whittaker (1970) stated that the nymphs of *Neophilaenus lineatus* (L.) deprived of the froth were significantly more likely found and captured by predators than those with froth. However, despite that a few cases of predation and parasitization on spittlebug nymphs have been recorded, the effective role of the froth in protecting juveniles against predators is not fully understood yet. Observations of entomophagous insects, spiders and harvestmen preying on *P. spumarius* nymphs (Weaver and King, 1954; Yurtsever, 2000) could suggest that the froth is not involved in the protection of the insect against predators. In some cases, the froth seems to be even disadvantageous for nymphs, including those of *P. spumarius,* for example when the froth is used as a clue to locate the prey by some birds (Whittaker, 1970; Weaver and King, 1954) or arthropods (Weaver and King, 1954; del Campo *et al.*, 2011).

Recently, the meadow spittlebug has assumed remarkable importance as a crop pest, being proved as the main vector of the xylem-inhabiting bacterium *Xylella fastidiosa* (Wells *et al.*) in Europe (Cornara *et al.*, 2016). For this reason, improving the knowledge about the biology and ecology of *P. spumarius* is crucial. To date, the management of this pest is mandatory in areas where *X. fastidiosa* occurs and nymphs represent the most susceptible stage of the insect (Dáder *et al.*, 2019). Exploring the defensive strategies of *P. spumarius* nymphs could help in planning sustainable and efficient control strategies, as well as in assessing biological control feasibility, using natural enemies and/or entomopathogens.

Two model species were used in this study to evaluate the possible role of *P. spumarius* froth in protecting nymphs from arthropod generalist predators: the acrobat ant *Crematogaster scutellaris* (Olivier) and the crab spider *Synema globosum* (Fabricius), which are commonly found in Mediterranean olive groves (Santini *et al.*, 2011; Benhadi-Marin *et al.*, 2020; Picchi, 2020) where also *P. spumarius* usually live (Elbeaino *et al.*, 2014; Cornara *et al.*, 2016; Antonatos *et al.*, 2019). The aims of this study were to: 1) assess the potential antipredatory effect of the froth produced by *P. spumarius* nymphs; 2) test the chemical effect of the froth as an antipredatory mechanism. We hypothesised that: 1) the froth is an antipredatory trait; 2) the antipredatory mechanism has a chemical rather than a physical nature; and 3) the froth is not chemically repellent but rather it masks the odour of the prey.

Materials and methods

Animal collection

Spittlebugs were collected in the field from a variety of plants (Ranunculaceae, Apiaceae and Fabaceae) and placed on two-months old pot-growing chickpea plants (*Cicer arietinum* L. cultivar "Principe") for at least 48 h before being used in the experiments, to avoid potential biases due to differences in froth composition. The nymphs were divided into two age classes: class A, including first and second instar nymphs (N1-N2) and class B, comprising third, fourth and fifth instar nymphs (N3-N5), according to their size, colour and presence of wing pads (Yurtsever, 2000).

Adult specimens of *S. globosum* were collected in the field on *Ranunculus* sp. flowers, placed individually in plastic cups and provided with preys *ad libitum* consisting of adults of Mediterranean fruit fly *Ceratitis capitata* (Wiedemann). Before being used in the experiments, spiders were starved for seven days to standardize their conditions (Benhadi-Marin *et al.*, 2020).

Workers of the ant *C. scutellaris* were collected from natural colonies and divided in 20 pseudocolonies, each with about 100 workers, that were acclimated for one week and provided with water *ad libitum* but without food before being used in the experiments to make them more responsive to the tests. The ants were housed in plastic aquaria (10 × 20 × 30 cm) with the walls coated with Fluon (Whitford, Runcorn, UK) to prevent escape.

All animals were kept in the laboratory at ambient conditions (mean room temperature: 25 °C).

Spittlebug mortality after froth removal

To define the duration of experiments, preliminary trials on spittlebug mortality after froth removal (presumably due to dehydration) were carried out. Spittlebug nymphs (N1-N2, n = 20) were removed from their froth on the plant with a soft brush, positioned onto filter paper until the froth was completely absorbed, and introduced into 35 mm Petri dishes closed with Parafilm®, where their status (0 = dead or 1 = alive) was checked every 10 min. The first dead nymphs were recorded after 210 min (Figure S1), so that the experiment length was set well below, at 120 min. Since N1 and N2 nymphs were considered as more vulnerable than older instars, this experiment duration was evaluated as appropriate for all the bioassays.

Spittlebug mortality when challenged with predators

To test whether spittlebugs' froth acts as an antipredatory trait against generalist predators, we performed arena experiments.

Freshly collected spittlebug nymphs from both age classes (N1-N2 and N3-N5) were divided in two groups: one to which the froth was left (control) and one to which the froth was removed (treatment).

Before the beginning of the experiments, plastic jars with perforated caps and 1.5 ml upside-down Eppendorf® tubes perforated at the bottom were prepared. The basal end of a chickpea cut shoot was then inserted through these perforated containers. For the control group, shoots bearing nymphs wrapped in their froth were used, and the plastic jar (or the tube) contained water, in which the shoot base was immersed, to allow sap transport. For the treated group, nymphs were gently deprived from their froth on the plant with a soft brush and laid onto filter paper to remove froth residuals. The nymph defrothed was then transferred on an unoccupied shoot. In this case, an empty jar (or tube) was used, to prevent sap transport and, therefore, the production of new froth. Occasional froth formed from residual sap was removed with filter paper until no new froth was produced (about 10 min).

At the end of this period, each jar or upside-down tube containing the nymph was inserted into the experimental arena hosting the ant pseudocolony or the spider, so that the nymphs were accessible to predators. The status of nymphs (0 = dead or 1 = alive) was checked every 10 min for two hours. The experiment was observed for its whole duration to ensure that the observed mortality was in fact due to predation. For each group (age class:treatment) 10 nymphs were tested with spiders, for a total of 40 nymphs, and 20 nymphs per group were tested with ants, for a total of 80 nymphs.

Spittlebug froth deterrence

In this experiment, the potentially deterrent effect of the froth produced by *P. spumarius* nymphs towards *C. scutellaris* was tested. Specifically, the deterrent effect due to the chemical composition of the froth was tested by excluding the confounding effect of the frothy physical state.

Before the beginning of the experiment, froth was collected with a soft brush from lab-reared chickpea plants and from other plants in the field (belonging to the families Ranunculaceae, Apiaceae and Fabaceae). The collected froth was mixed and centrifuged at 448 g for 30 s, and the resulting liquid was preserved at -20 °C until used in the experiments.

Nymphs were removed from their froth from the chickpea plants with a soft brush and laid onto filter paper to blot froth residuals. Each nymph was then randomly assigned to one of the following three treatments: nymphs moistened with water (treatment W), nymphs moistened with froth (treatment F), and nymphs without water or froth (treatment N). W and F treated nymphs were wet pouring on them 2.5 µl of either water or froth with a micropipette.

Ten *C. scutellaris* workers were placed into 9 cm diameter Petri dishes with Fluon coated walls to acclimatize to experimental conditions. After one hour, we placed one spittlebug nymph into the Petri dish and video recorded the experiment for the following 30 min. The status of the nymph at the end of the experiment (0 = dead or 1 = alive) as well as the number of ants' bites towards the nymph were registered. Only nymphs attacked by ants were scored as "preyed". For each treatment, 20 replicates were performed, for a total of 60 replicate tests.

To discriminate if the deterrence was caused by the froth only or by other deterrent/unpalatable substances on the tegument of spittlebug nymphs, we repeated this latter experiment using larvae (maggots) of the dipteran *Sarcophaga carnaria* (L.) as bioassays. Maggots have been chosen as alternative preys because they are highly appreciated by ants, either dead or alive. Maggots were washed with water to remove any detritus and killed by freezing as their high motility could interfere with ants' behaviour. Maggots defrosted at ambient temperature were then offered to ants (one for each Petri dish) following exactly the same protocol described above.

Statistical analyses

The spittlebug survival (alive or dead at the end of the experiment) was assessed using generalized linear models (GLM with binomial distribution) using predator, age class and treatment as predictors. Models of different complexity (from the null to the full model) were evaluated using Akaike Information Criterion (AIC) and the best one was also tested using a χ^2 test. Then, using the best predictors found with GLM selection, Kaplan-Meier curves were drawn and the difference between them was tested using the Mantel-Haenszel test.

For the deterrence experiment, nymph survival (alive or dead at the end of the experiment) was assessed with binomial GLMs using the treatment as predictor, and the model was tested using a χ^2 test, followed by Tukey tests as multiple comparisons. Moreover, the number of ants' bites to nymphs and maggots were separately tested with a GLM (family = quasipoisson) using the treatment (levels: N, W, F) as predictor, and the model was tested using a χ^2 test, followed by Tukey tests as multiple comparisons.

All statistical analyses were performed using the software R (R Core Team 2020, version 4.0.5) using the packages 'survival' (Therneau and Lumley, 2015) and 'multcomp' (Hothorn *et al.*, 2016).

Results

The best GLM was the one considering the predator, the treatment, and the age class as predictors (Table 1). However, the predator (spiders or ants) and the age class of the nymphs (class A, N1-N2 or class B, N3-N5) had no significant effect (Table 2), therefore they were not considered in the subsequent analyses. The model showed that control spittlebugs died significantly less frequently than the treated ones (Table 2).

Table 1. Results of generalized linear model (GLM) selection for spittlebug survival using Akaike information criterion (AIC)

Predictor	**d.f.**	**AIC**
Null Model	1	150.26
Predator	2	148.81
Treatment	2	94.93
Age Class	2	150.34
Predator + Treatment	3	91.41
Predator + Age Class	3	148.83
Treatment + Age Class	3	93.71
Predator + Treatment + Age Class	4	89.88

At the end of the arena experiments, no control spittlebugs died, while for the treated group 8 out of 20 (40%) and 28 out of 40 (70%) died in presence of spiders and ants, respectively (Figure 1), and the difference between the two Kaplan-Meier curves was statistically significant (spiders: χ^2 = 9.8, d.f. = 1, P-value < 0.01; ants: χ^2 = 44.4, d.f. = 1, P-value < 0.001).

Table 2 Results of generalized linear models (GLMs) on spittlebug survival at the end of the experiment as a function of treatment (treatment: froth removed, control: froth not removed)

	d.f.	Deviance	Resid. d.f.	Resid. Deviance	Pr (>Chi)
Null model			119	148.26	
Predator	1	3.45	118	144.81	0.06
Treatment	1	59.40	117	85.41	1.288e-14 ***
Age Class	1	3.53	116	81.88	0.06

At the end of the deterrence experiment, 16 out of 20 (80%) nymphs of the treatment N, while only 2 (10%) in the treatment W and none in the treatment group F were predated. The effect of the experimental treatment was highly significant (Table 3) and, in particular, the number of survivors of group N was significantly lower than either those of treatments W and F (P-value < 0.001 for both), while no difference was observed between treatments W and F (P-value = 0.82).

Table 3. Results of the generalized linear model (GLM) on spittlebugs survival at the end of the experiment as a function of treatment (treatments: moistened with water, moistened with froth, moistened with nothing)

	d.f.	Deviance	Resid. d.f.	Resid. Deviance	Pr (>Chi)
Null model			59	74.92	
Treatment	2	33.96	57	40.96	4.224 e-08 ***

Table 4. Results of generalized linear models (GLMs) on ants' bites inflicted on spittlebug nymphs and *S. carnaria* maggots as a function of treatment (treatments: moistened with water, moistened with froth, moistened with nothing)

Prey		d.f.	Deviance	Resid. d.f.	Resid. Deviance	Pr (>Chi)
Spittlebug nymphs	Null model			59	853.51	
	Treatment	2	622.66	57	230.85	< 2.2 e-16 ***
S. carnaria maggots	Null model			59	171.91	
	Treatment	2	61.30	57	110.61	6.397e-08 ***

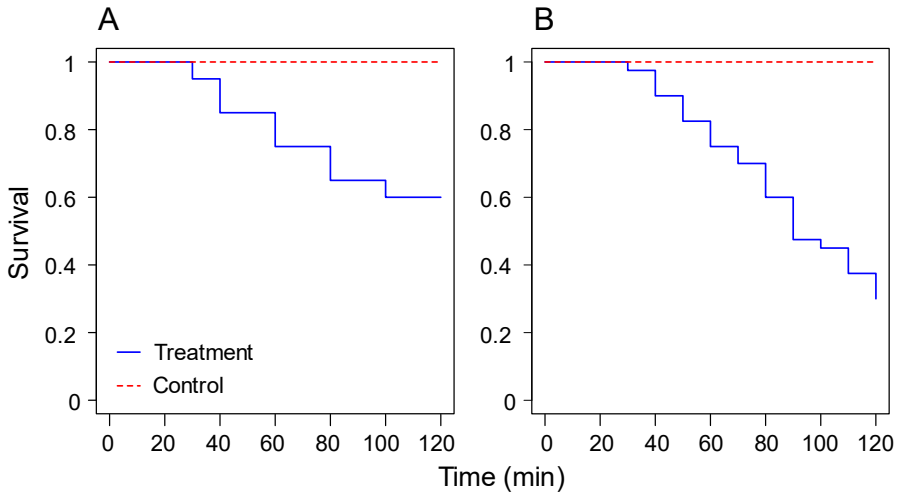

Figure 1. Kaplan-Meier curves showing the survival probability of spittlebugs as a function of time and treatment (treatment: froth removed, control: froth not removed). A = spittlebugs challenged with spiders, B = spittlebugs challenged with ants

The number of bites inflicted to the spittlebug nymphs and maggots differed among the three treatments (Table 4). In particular, for spittlebugs, the number of bites was higher in the group N compared to treatments W and F (P-value < 0.001 for both), but fewer bites were recorded in F-treated than W-treated nymphs (P-value < 0.01; Figure 2A). Similarly, in the bioassays with maggots, a significantly lower number of bites were inflicted toward the treatment F compared to the treatments W and N (P-value < 0.001), but no difference was found between the treatments W and C (P-value = 0.12; Figure 2B).

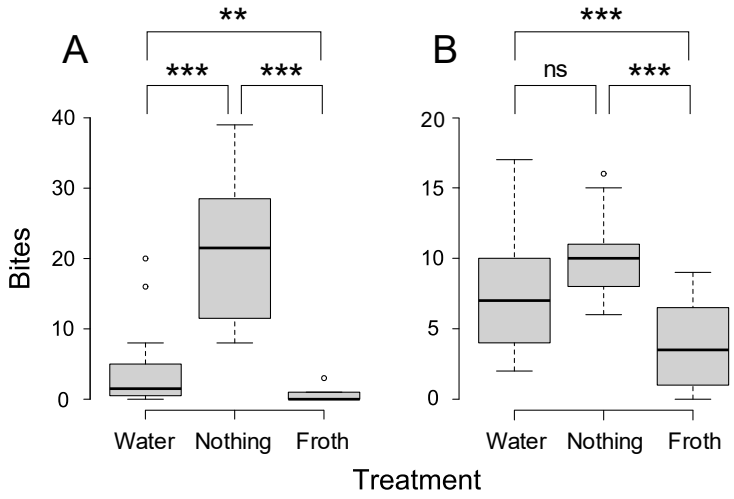

Figure 2. Number of ants' bites inflicted on spittlebug nymphs (A) and *S. carnaria* maggots (B) for the different treatments (treatments: moistened with water, moistened with froth, moistened with nothing). Significance levels: n.s.= not significant, **= P-value < 0.01, ***= P-value < 0.001

Discussion and conclusions

Spittlebugs are generally affected by a low predation rate (Whittaker, 1965; Biedermann, 2003), although there is evidence of predation by generalist spider or insect species (Harper and Whittaker, 1976; Callan, 1980), including ants (Hewitt and Nilakhe, 1986; Sujii *et al.*, 2002, 2004), as well as by specialized predators (Weaver and King, 1954; del Campo *et al.*, 2011). The froth produced by spittlebug nymphs has been usually associated with a thermoregulatory and dehydration-preventing function (Cornara *et al.*, 2018), acknowledging also an antipredatory role, among others (Whittaker, 1970).

Consistently with what has been reported for other spittlebug species (Nachappa *et al.*, 2006; del Campo *et al.*, 2011), in the first experiment we observed considerably lower survival probability in *P. spumarius* nymphs deprived of froth compared to the control nymphs. This result was consistent either when the nymphs were challenged with spiders or ants. However, this result should be taken cautiously, because it probably owed more to the behaviour of the nymphs than to the predators' ability. Indeed, the nymphs deprived of the froth moved to search (without finding it) a new point in the shoot where to feed and produce new froth (Weaver and King, 1954). Increased motility, however, also increased the probability of nymphs to run into the predators, for example because they fell from the shoot or moved around in the arena. In contrast, the number of intentional attacks by spiders and ants were very low, in line with previous studies (Henderson *et al.*, 1990). Similarly, in the control group, no death occurred at the end of the experiment, because the predators did not engage in direct attacks and the nymphs stationarily remained within their froth.

This result partially deviates from the one from Henderson *et al.* (1990), who observed a predation rate of 25% on *P. spumarius* nymphs within their froth by the ant *Formica montana* (Weelher). Nevertheless, it should be noted that their experiment lasted 72 h, much longer than ours, and involved a bigger species and a higher number of workers in the experiments. On the other hand, Nachappa *et al.* (2006) observed that nymphs of *Prosapia bicincta* (Say) in presence of colonies of the ant *Solenopsis invicta* Buren for 24 h were rarely killed when left within their froth compared to nymphs whose froth was removed. Hewitt and Nilakhe (1986) reported predation by several ant species belonging to the genera *Solenopsis*, *Pheidole* and *Conomyrma* on *Zulia entrerriana* (Berg). In particular, predation occurred on the eggs and newly hatched first instar nymphs before they produced froth. Yet another study found considerably higher number of workers of *S. invicta* on defrothed compared to froth-coated nymphs of *M. fimbriolata* in a cafeteria experiment, and the authors conclude that this was due to chemical repellence of the froth (Tonelli *et al.*, 2019). However, their conclusion seems not fully supported, given that their findings could simply reflect a preference for more readily available prey.

Several authors reported that spittlebugs can be killed by spiders or other arachnids (Weaver and King, 1954; Phillipson, 1960; Harper and Whittaker, 1976; Yurtsever, 2000). For example, the DNA of *P. spumarius* has been found in the gut content of the wolf spider *Alopecosa cuneata* (Clerck) collected in an olive orchard in spring (Lantero *et al.*, 2018), while the spiders *S. globosum* and *Araniella cucurbitina* (Clerck) showed type I and type II functional responses (respectively) towards *P. spumarius* adults (Benhadi-Marin *et al.*, 2020). In Mediterranean ecosystems, *S. globosum* can be commonly observed on flowers of several plant species (Ajuria and Reader, 2014; Benhadi-Marin *et al.*, 2019), often sharing the same plant with the nymphs of *P. spumarius* (pers. obs.). Nonetheless, no direct attack of *S. globosum* against *P. spumarius* juveniles is reported. Crab spiders (Thomisidae), such as *S. globosum*, are ambushing predators that stay motionless on flowers waiting for prey that they capture with their strong front legs (Gertsch, 1939). Therefore, the absence of spider attacks observed in our experiment could be explained both by the effect of the spittlebug froth and the hunting strategywhich mainly targets actively moving prey. However, the froth could be an adaptation to hide the nymph from predators that use visual cues to detect their prey, a strategy adopted also by some Hemipteran species (Moss *et al.*, 2006). Further studies involving spiders with different hunting strategies should be carried out to better explore this hypothesis.

Therefore, from the results of the first experiment we cannot conclude that froth has a deterrent effect, as we cannot exclude other possible explanations, such as a physical hindrance by the froth, increased mortality due to increased motility of nymphs when not covered by froth, or even an effect of the hunting strategy. However, this latter aspect seems to be not an issue here, since a lack of aggression towards nymphs was observed also with *C. scutellaris* ants, which are active predators (Frizzi *et al.*, 2016; Balzani *et al.*, 2020). Moreover, the effect of the age class was not significant, even if this factor, as well as the aggregation rate, could affect the mortality of spittlebug nymphs, as found for *Neophilaenus albipennis* (Fabricius, 1798) by Biedermann (2003).

Our second experiment allowed us to test the chemical effect of the froth by excluding the confounding effect of the physical hindrance (due to the foamy texture of the froth). Predation on defrothed nymphs by *C. scutellaris* was confirmed in almost all replicates, whereas nymphs moistened with water or with centrifuged liquid froth were more rarely predated. These results suggest that the froth could chemically mask the prey odour, or be repellent for ants. Also, it seems that even the defrothed nymphs would continue carrying residuals of some substance that, diluted with water, played a masking or repellent effect. The ants' bite counts provided support to this hypothesis. Indeed, the number of bites was greater in the defrothed group, intermediate in the water-coated group and lower in the froth-coated group. In particular, the ants' response to water-coated nymphs compared to the other treatments seems to confirm the deterrent or masking effect of some

residual substance on the nymph integument, as previously suggested by Henderson *et al.* (1990). Our findings support the ones found on another spittlebug species, *Aphrophora cribrata* (Walker), whose froth was analysed and its deterrence tested against the ant *Formica exsectoides* Forel (del Campo *et al.*, 2011). These authors found that *A. cribrata* nymphs coated with froth had a significantly lower mortality compared to the control nymphs, and both the natural froth and the artificially synthesized one had a deterrent effect towards ants in 90% of contacts. The authors concluded that the deterrence cannot be based on volatile chemicals, since the ants did not avoid the froth, but rather is due to some contact-deterrent substance in the froth, as proposed later also by Tonelli *et al.* (2019). While our observations confirmed this, we highlight that the authors did not consider the possibility that the froth could have an odour-masking effect, that seems a better explanation.

Moreover, ants that encountered the froth-coated nymphs were previously observed self-grooming their antennae (Henderson *et al.*, 1990; del Campo *et al.*, 2011; Tonelli *et al.*, 2019). All these authors interpreted this behaviour as a consequence of froth deterrence. We too observed sporadic cases of self-grooming of the antennae in ants after contacting the froth. However, this seemed more related to the need of cleaning the antennae after the contact with a sticky and frothy substance than a true deterrence. On the contrary, during the experiments, we could observe that sometimes the ants drank the froth, especially when it was liquefied, as already documented by a previous paper (Henderson *et al.*, 1990). This strongly reinforces our hypothesis that a chemical mimicry is a more plausible explanation than a chemical deterrence.

The results of maggot bioassays allow to further clarify the role of the froth in protecting nymphs from predators. Indeed, while the number of ants' bites to froth-coated larvae was lower than towards the other treatments, no significant difference was found between the control and the water-coated groups. This finding further supports the idea of a masking effect of froth and corroborates the assumption of residual substances on the spittlebug nymph body, substances not present on the maggot body.

To reduce the spread of *X. fastidiosa* in olive growing areas, research of effective biological control agents against its main vector *P. spumarius* has increased in the last years (e.g. Liccardo *et al*., 2020; Mesmin *et al*., 2020). The results of our experiments show that the hunting strategy and sensory ability of predators must be considered in evaluating nymphs' potential biocontrol agents. In particular, our results suggest that generalist predators using chemical cues to locate the prey, such as ants, could not be efficient biocontrol agents for nymphs of the meadow spittlebug. Thus, considering the importance of nymphs' control in the *X. fastidiosa* management (Dáder *et al.*, 2019), further studies aimed at clarifying the antipredatory trait of froth appear to be crucial.

In conclusion, we provided evidence that spittlebugs froth, could play a role as a multifaceted protection against generalist predators, beyond protecting the nymph from thermal stress and dehydration. While its frothy state might represent a physical hindrance also hiding the nymphs from visual predators, like spiders, its chemical composition could prevent detection by predators that rely most on olfactory stimuli, such as ants. Regarding the chemical effect of the froth, our results suggest that froth could contain specific odour-masking substances that hide nymphs from predators, rather than acting as a true deterrent. Further studies on the chemical composition of the froth are needed to clarify the role of this self-produced biofoam in the chemical mimicry of spittlebug nymphs.

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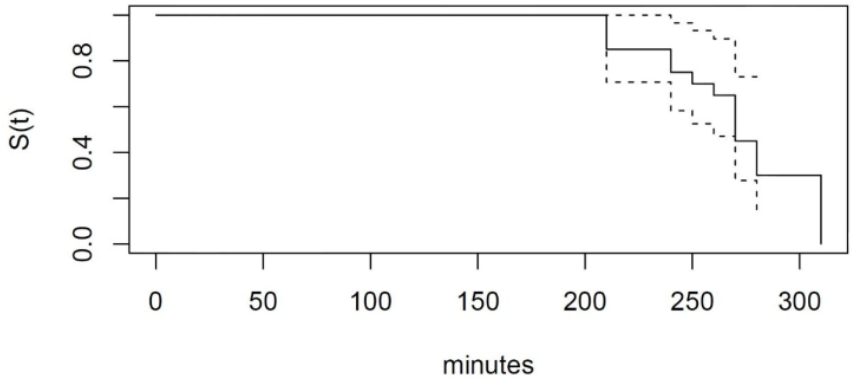
Supplementary materials

Figure S1 Kaplan-Meier curve shows spittlebug nymphs' natural survival probability after froth removal. The dashed lines indicate 95% confidence intervals.



3. Diversity of the bacterial community associated with hindgut, Malpighian tubules, and foam of nymphs of two spittlebug species (Hemiptera: Aphrophoridae)

Personal contribution: Conceptualization, data collection, molecular procedures, manuscript preparation.

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Abstract

Spittlebugs are xylem-sap feeding insects that can exploit a nutrient-poor diet thanks to mutualistic endosymbionts residing in various organs of their body. Although obligate symbioses in some spittlebug species have been quite well studied, little is known about their facultative endosymbionts, especially those inhabiting the gut. Recently the role played by spittlebugs as vectors of the phytopathogenetic bacterium *Xylella fastidiosa* aroused attention to this insect group, boosting investigations aimed at developing effective yet sustainable control strategies. Since spittlebug nymphs are currently the main target of applied control, the composition of gut bacterial community of the juveniles of *Philaenus spumarius* and *Lepyronia coleoptrata* was investigated using molecular techniques. Moreover, bacteria associated to their froth, sampled from different host plants, were studied. Results revealed that *Sodalis* and *Rickettsia* bacteria are the predominant taxa in the gut of *P. spumarius* and *L. coleoptrata* nymphs, respectively, while *Rhodococcus* was found in both species. Our investigations also highlighted the presence of recurring bacteria in the froth; in addition, the foam hosted several bacterial species depending on the host plant, the insect species, or on soil contaminant. Overall, first findings showed that nymphs harbor a large and diverse bacterial community in their gut and froth, providing new accounts to the knowledge on facultative symbionts of spittlebugs.

Introduction

Endosymbioses are widespread in insects and affect many aspects of their biology, ecology and evolution (Buchner, 1965; Moran and Baumann, 2000). Among heritable intracellular bacteria, three main categories of endosymbionts can be recognized: primary symbionts (P-symbionts), secondary symbionts (S-symbionts) and reproductive manipulators (Moran *et al*., 2008).

P-symbionts are obligate mutualists inhabiting specialized cells called bacteriocytes which may form organs known as bacteriomes (Moran and Baumann, 2000; Moran *et al*., 2008). They are vertically transmitted and provide essential nutrients to the host (Baumann, 2005). Since the association between P-symbionts and their hosts originates from an ancient infection, usually all the descendants of the infected ancestor have coevolved with the same P-symbiont.

On the other hand, S-symbionts and reproductive manipulators are facultative endosymbionts that can colonize several organs or even reside extracellularly in the hemocoel (Moran *et al*., 2008; Baumann, 2005). S-symbionts usually enhance the host fitness, for example offering protection against stress or natural enemies, while reproductive manipulators could be considered as parasites, since they affect host reproduction to favor its own spread (Moran *et al*., 2008).

Sap-feeding insects, as those belonging to the sub-order Auchenorrhyncha, are known to host large communities of symbionts and bacteriome-associated mutualistic organisms implicated mainly in the provision of nutrient lacking in the diet (Baumann, 2005; Koga *et al*., 2013). Particularly, the Bacteroidetes *Candidatus* Sulcia muelleri has long been recognized as the P-symbiont of leafhoppers, spittlebugs, cicadas and other Auchenorrhyncha species (Moran *et al*., 2005). In many cases, *Ca*. Sulcia muelleri is coupled with another S obligate symbiont that occupies a specific area of bacteriomes. Typically, co-resident endosymbionts show a complementary role in the biosynthesis of essential amino acids (McCutheon *et al.*, 2005; Koga *et al.*, 2014).

The Cercopoidea superfamily comprises xylem-feeding insects that are commonly known as froghoppers or spittlebugs, due to the habit of nymphs to develop inside a self-produced foam nest. This foam is formed by the excretion of the alimentary canal, mainly composed by metabolized xylem sap, added with mucopolysaccharides and proteins produced by specialized cells of the Malpighian tubules (Marshall, 1966; 1973; Yurtsever, 2000). Nymphs introduce air bubble in this mixture through telescopic movements of the abdomen, giving the typical frothy state to their excreta. The foam protects nymphs against dehydrations, temperature fluctuations and natural enemies (Cornara *et al.*, 2018; Tonelli *et al.*, 2018; Balzani *et al*., 2023).

Spittlebugs (Hemiptera: Aphrophoridae) are spread in many terrestrial ecosystems and some species are known to be important phytophagous (Cornara *et al.*, 2018; Schöbel *et al.*, 2021; Thompson, 2004; Chen *et al.*, 2012). The meadow spittlebug *Philaenus spumarius* L. 1758, is currently

considered a major pest in Europe due to its competence to transmit the xylem-inhabiting harmful bacterium *Xylella fastidiosa* subsp. *pauca* ST53 (Cornara *et al.*, 2016; 2018), which is the causal agent of the Olive Quick Decline Syndrome (Loconsole *et al.*, 2014).

In spittlebugs, *Candidatus* Zinderia insecticola and a *Sodalis*-like bacterium, allied to *Sodalis glossinidius*, are the two bacteriome-associated endosymbionts known so far to be co-residents of *Ca.* Sulcia muelleri (Koga *et al.*, 2013; 2014). In particular, *Ca.* Zinderia insecticola is associated to most of the spittlebugs, while the *Sodalis*-like bacterium is an endosymbiont of the species belonging to the tribe Philaenini, such as *P. spumarius* (Koga *et al.*, 2013).

Differently from P-symbionts, the microbial community of spittlebugs' facultative endosymbionts has received less attention. The genera *Rickettsia*, *Arsenophonus*, *Hamiltonella* and *Wolbachia* are reported to be facultative symbionts of *P. spumarius* and other Philaenini species (Kapantaidaki *et al.*, 2021). However, studies on the anatomical localization of these bacteria are lacking. Similarly, functions of mutualistic microorganisms other than P-symbionts are still little explored in spittlebugs. For instance, the hypothesis that the gut microbiota of aphrophorid nymphs could play a role not only in the hydrolysis and assimilation of food but also in the foam production has never been tested (Wilson and Dorsey, 1957). Furthermore, information on the microbial community associated with the froth is scarce and the origin and function of foam-inhabiting microorganisms is still poorly known. Recently, most of the bacteria found in the froth of *Mahanarva fimbriolata* (Stål, 1854) have been identified as alpha-Proteobacteria, microorganisms which presumably might play a defensive role against different natural enemies, although evidence supporting this hypothesis are lacking (Tonelli *et al.*, 2020).

Since it has been recognized the potential of novel, effective control strategies that could be developed on the basis of the knowledge on pests' microbiome (Arora *et al.*, 2017), the study of *P. spumarius* facultative endosymbionts appear to be of great importance in order to manage the *X. fastidiosa* emergency in Europe. Currently, spittlebug juveniles are considered the main target of applied control strategies because of their reduced mobility (Dáder *et al.*, 2019). Given that, analyzing the structure of microbial community associated to nymphs' gut and froth could be useful to develop effective and sustainable control strategies against aphrophorid nymphs.

So that, in this study, the endosymbionts harbored in the mid- and hindgut of *P. spumarius* nymphs were explored using molecular procedures. Moreover, nymphs of the spittlebug *Lepyronia coleoptrata* (Linnaeus, 1758) were examined to explore and compare the gut microbiota of a non-Philaenini species. Finally, in both species the structure of the bacterial community associated with the foam and the Malpighian tubules involved in the froth production were studied.

Materials and Methods

Collection of foam and insect samples

Fifth-instar nymphs of *P. spumarius* and *L. coleoptrata* were collected in the field from *Ranunculus* sp. and *Trifolium repens*, respectively, and brought alive to the laboratory to dissect the alimentary canal (Table 1). Specimens were cold-anesthetized, externally sterilized in a 2% sodium hypochlorite solution and then dissected in a sterile 0.8% NaCl solution. Dissections were carried out under a stereoscopic microscope in a laminar-flow hood.

Table 1. Sampling sites where nymphs of *Philaenus spumarius* and *Lepyronia coleoptrata* and/or their foams were collected from April to June 2021 (all sampling sites are located in Tuscany, Italy).

Site of collection	GPS coordinates	Species nymph or foam	Host plant	Number of collected nymphs	Quantity of collected foam (mL)
Monte Argentario (Grosseto)	42.37701 N 11.18620 E	*P. spumarius*	*Cistus monspeliensis*	-	12
Fabio, Vaiano (Prato)	43.939682 N 11.142821 E	*P. spumarius*	*Vicia sativa*	-	9
			Cirsium sp.	-	9
Gavigno, Cantagallo (Prato)	44.040865 N 11.104997 E	*P. spumarius*	*Euphorbia cyparissias*	-	10
			Ranunculus sp.	20	-
Montepaldi, San Casciano (Firenze)	43.667408 N 11.143868 E	*L. coleoptrata*	*Trifolium repens*	15	40

For *P. spumarius* the filter chamber linked to the conical segment, the posterior tubular midgut and the Malpighian tubules were taken. The ileum was added to the above-mentioned sections for *L. coleoptrata*. Anatomical regions were located and cut apart according to their morphological descriptions reported previously (Cecil, 1930; Zhong *et al.*, 2013). Insect samples were formed pooling five dissected portions of the same type; samples were stored in sterile 1.5 mL vials and maintained at -80°C until processed.

Foam samples were collected directly in the field (Table 1) by means of sterile spatulas and microscope slides. Each sample was formed by the foam produced by five fifth-instar nymphs which were feeding on the same plant species. Foams collected from different plant species were kept and

treated separately. Each foam sample was stored in sterile 1.5 mL vials and maintained at -80°C until processed.

Microbiological analyses

Bacterial DNA extraction was carried out using the FastDNA™ Spin Kit for Soil (MP Biomedicals, USA) according to the manufacturer's instructions. Frozen insect samples were crushed and mashed with a sterile pestle prior to the beginning of the extraction procedure. Foam samples were thawed, cleaned from impurities by centrifugation (14,000 g for 5 minutes) and then treated with the extraction kit. To assess the extraction quality and integrity, DNA preparations were visualized by electrophoresis in 1% (*w/v*) agarose gel at 4 V/cm for 1 h in TAE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA; pH 8.3), stained with Xpert green DNA stain (GriSP Research Solutions, Portugal) and observed under UV light.

Real Time PCR

Quantitative Real Time PCR was performed to quantify the bacterial biomass in each gut sample. Amplification reactions were carried out in an ABI StepOne™ Real Time PCR system (Applied Biosystems, USA) in a 10 µl mixture containing 1x BlasTaq™ qPCR MasterMix (Applied Biological Materials Inc., Canada), 400 nM of each primer (341F and 515R [Lawson *et al.*, 2001]), and 1 µl of template DNA. The amplification conditions consisted of incubation at 95°C for 10 m, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 60 s, and extension at 72°C for 45 s. All samples were run in triplicate in optical 96-well plates together with negative control and standard curve. Standard curve was created for absolute quantification of 16S rRNA gene by using a plasmid containing the target gene fragment from *Pseudomonas* sp. DSM1650 ten-fold diluted from 3.60×10^7 to 3.60×10^3 gene copy numbers μl^{-1}. Fluorescent light outputs were collected during each elongation step and analyzed with the ABI StepOne Real Time PCR system SDS software v. 2.3 (Applied Biosystems). Melting curves were generated after amplification by increasing the temperature of 0.5°C every 30 s from 65 to 95°C, to verify the absence of primer dimers or artifacts.

Denaturing Gradient Gel Electrophoresis

Denaturing Gradient Gel Electrophoresis (DGGE) analysis was performed by using the universal primers 986F-GC and 1401F (Felske *et al.*, 1996), designed to amplify the V6-V8 region of the 16S rRNA bacterial gene in order to explore the composition of the bacterial community associated to the gut, the Malpighian tubules and the foam of the two spittlebug species. Amplification reactions were carried out in a T100 Thermal Cycler (Bio-Rad Laboratories, UK) in a 25 µl volume containing 1x

Xpert Taq Reaction Buffer (GRISP Research Solution), 1.5 mM $MgCl_2$, 250 µM of deoxynucleotide triphosphate (dNTPs), 400 nM of each primer, and 1 U of Xpert Taq DNA (GRISP Research Solution). The reaction conditions consisted in an initial denaturation of 94°C for 5 m followed by 35 cycles of 94°C for 30 s, annealing at 55°C for 30 s, extension at 72 °C for 45 s and a final extension of 72 °C for 10 m. Successively, the amplification products were loaded onto a 6% polyacrylamide gel (acrylamide/bis 37.5:1), with a 42-68% linear denaturing gradient increasing in the electrophoretic run direction and obtained with a 100% denaturing solution containing 40% formamide (VWR, USA) and 7 M Urea (Promega, USA). The gels were run in a D-Code System (Bio-Rad) for 18 h in 1x TAE buffer at constant voltage (80 V) and temperature (60 °C) and stained with SYBR®GOLD (Molecular Probes, USA) diluted 1:1000 in 1x TAE. DGGE images were digitally captured under UV light using a Chemidoc XRS apparatus (Bio-Rad).

Sequence analysis

The dominant DGGE bands were aseptically excised from gels and sequenced to taxonomically identify bacterial symbionts (Figures S1, S2). The middle portion of each band was placed in 25 µl of distilled water and stored at -20°C overnight. Successively, DNA fragments were eluted from gel through freezing and thawing and 1 µl of each elution was used as template in an amplification reaction carried out as previously described for DGGE analysis. The re-amplified PCR products were purified using PureLinkTM Quick PCR Purification kit (Invitrogen-Life Technologies, USA) and sent to the center CIBIACI (Centro Interdipartimentale di Servizi per le Biotecnologie di Interesse Agrario, Chimico, Industriale) of the University of Florence for the sequencing service. The obtained sequences were edited using Chromas Lite software (v2.1.1; Technelysium Pty Ltd, AU; http://www.technelysium.com.au/chromas_- lite.htm) to verify the absence of ambiguous peaks and convert them to the FASTA format. The DECIPHER's Find Chimeras web tool (http://decipher.cee.wisc.edu) was used to uncover chimeras hidden in the 16S rDNA sequences. Nucleotide sequences were compared against all sequences stored within the NCBI database using the Web-based BLAST tool (http://www.ncbi.nlm.nih.gov/ BLAST) to find closely related nucleotide sequences. Taxonomical identification was achieved by means of different sequence similarity thresholds as described by Webster *et al*. (2010). The nucleotide sequences were deposited in the GenBank database under accession numbers OP012727-OP012755.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Fisher Least-Significant Difference (LSD) were applied to analyze data obtained from the real-time PCR using Statistica software (Palo Alto, USA).

The Gel Compare II software v 4.6 (Applied Maths, Belgium) was used to analyze the foam DGGE and to convert it into a matching table based on presence/absence and intensity of bands within each banding pattern to be imported into PAST4.03 software for subsequent multivariate statistical analysis (Hammer *et al.*, 2001). Non-metric multidimensional scaling (nMDS) analysis and one-way analysis of similarity (ANOSIM) were performed using the Bray-Curtis distance measure and 9,999 permutational tests to visualize the similarity/dissimilarity of bacterial communities hosted in the collected foams in a two-dimensional space and to determine the extent of these similarity/dissimilarity according to the different plant species, respectively. The accuracy of the nMDS plot was determined by calculating a 2D stress value. An ANOSIM R value of 1 indicates that the bacterial communities of foam collected from each plant species are more similar to each other than to any sample from another plant species, whereas an R value of 0 indicates that there is as much variation within a group as among groups being compared. More specifically, $0.5 < R \text{ values} < 0.75$ were interpreted as separated but overlapping (Ramette, 2007).

Results

Bacterial biomass quantification

A total of 20 *P. spumarius* and 15 *L. coleoptrata* nymphs and 40 mL of foam per each species were analyzed. Bacterial DNA was extracted from all the samples. The richness in endosymbionts (quantified by the real-time PCR) was expressed as number of 16S rRNA gene copies (Figure 1). The larger bacterial biomass ($6.00 \times 10^5 \pm 6.86 \times 10^5$ 16S rRNA gene copies) was harbored by the filter chamber joined to the conical segment of *L. coleoptrata*. Compared to *P. spumarius*, *L. coleoptrata* showed a higher average content of bacteria also in the posterior tubular midgut ($1.08 \times 10^5 \pm 1.52 \times 10^5$ 16S rRNA gene copies) and in the Malpighian tubules ($1.38 \times 10^5 \pm 2.17 \times 10^5$ 16S rRNA gene copies). Even the ileum, which was the poorest part as for symbionts abundance ($2.70 \times 10^4 \pm 2.44 \times 10^4$ 16S rRNA gene copies), contained a mean bacterial load higher than that measured for the posterior tubular midgut of *P. spumarius* ($2.40 \times 10^4 \pm 1.80 \times 10^4$ 16S rRNA gene copies). The greatest abundance of bacteria in *P. spumarius* was in the Malpighian tubules ($7.28 \times 10^4 \pm 5.57 \times 10^4$ 16S rRNA gene copies).

Statistical analyses showed that the filter chamber linked to the conical segment of *L. coleoptrata* was the only studied portion that contained a number of symbionts significantly higher than the other considered anatomical parts, including those of *P. spumarius*. No other significant differences have been highlighted among the analyzed gut portions and organs within each species (Figure 1).

Gut bacterial community composition

The DGGE profiles of gut dissections and Malpighian tubules of both *P. spumarius* and *L. coleoptrata* revealed three dominant bands, with two of them shared by the two spittlebugs, suggesting the presence of common endosymbiont species (Figure S3). Other less prominent bands were revealed for both insects. Sequencing analysis of excised DGGE bands of *P. spumarius* gut samples and Malpighian tubules revealed to share sequence identity with a *Sodalis*-like bacterium allied to *Sodalis glossinidius* (98% similarity to GenBank accession number LN854557), a species belonging to Enterobacteriaceae family (*Escherichia coli*: 100% similarity to GenBank accession number MN083301) and a *Rhodococcus* species (*Rhodococcus gingshengii:*100% similarity to GenBank accession number MN826591). The gene sequencing disclosed the presence of a *Rickettsia*-endosymbiont (*Rickettsia bellii*: 98.8% similarity to GenBank accession number KU586119) together with the Enterobacteriaceae species (*Escherichia coli:* 100% similarity to GenBank accession number MN083301) and a *Rhodococcus* bacterium *(Rhodococcus gingshengii:* 100% similarity to GenBank accession number MN826591) as likely putative endosymbionts of *L. coleoptrata* (Table S1).

Foam bacterial community composition

DGGE profiles of foam samples showed the presence of several dominant bands, together with other fainter ones (Figure S4). Some conspicuous bands were common to both *P. spumarius* and *L. coleoptrata* foams samples. All bands selected for sequencing showed high similarity to species belonging to Proteobacteria, with a majority of alpha-Proteobacteria such as *Brevundimonas mediterranea* (99.7% similarity to GenBank accession number MK250497), *Devosia oryziradicis* (97.2% similarity to GenBank accession number CP068047) and several Rhizobiaceae species. Members of Rhizobiaceae detected in the froth, displayed a distinct discrepancy in the association between insect and bacteria species. Indeed, *P. spumarius* foam samples seemed to harbor bacteria species referring to the genus *Ciceribacter*, while spittles of *L. coleoptrata* contained mainly other genera (Table 2).

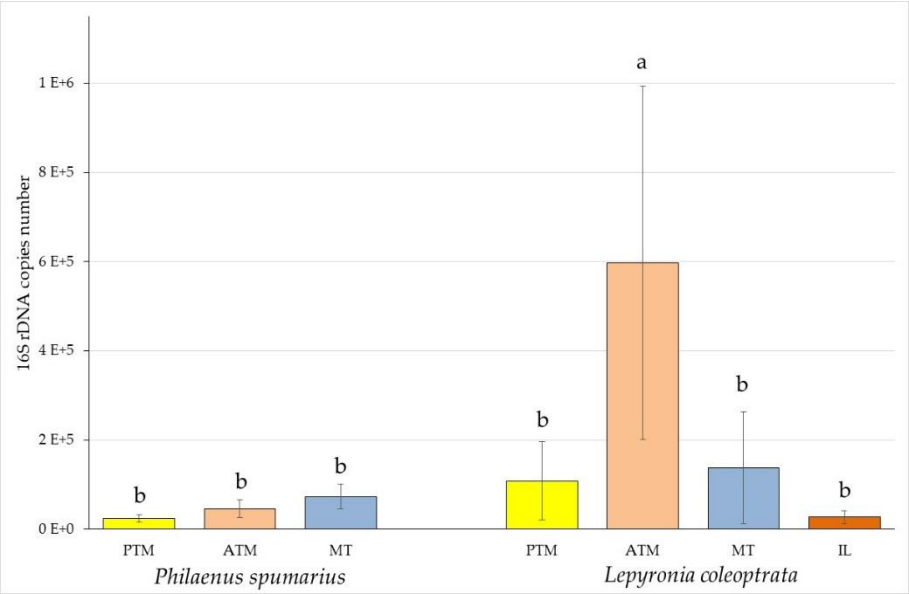

Figure 1. Mean number of 16S rDNA copies (bacterial biomass) measured for the PTM (posterior tubular midgut), ATM (filter chamber linked to conical segment), MT (Malpighian tubules) and IL (ileum) of *P. spumarius* and *L. coleoptrata*. Bars indicate standard error. Different letters over the histograms indicate significant differences among means ($p < 0.05$).

The nMDS, used to evaluate the DGGE profiles obtained through the analysis of the foam samples of the two spittlebug species, showed a certain diversity in the composition of the microbiome associated to the froth. As shown in Figure 2, bacterial communities could be grouped using the host plant as parameter, meaning that foams collected from the same plant species harbored a similar microbial community. However, the structure of microbial assemblages associated with foams collected from different plant species appeared to be distinct, with the only exception of froth samples collected from *Vicia sativa* and *Euphorbia cyparissias*, that could be grouped together. Moreover, nMDS analysis displayed that the bacterial communities of the froths collected from *Vicia sativa* showed almost an identical structure, since they could be graphically overlapped. Finally, results obtained with ANOSIM (R=0.7689; *P*=0.0001) allowed to clarify that the host plant and insect species significantly affected the bacterial community structural diversity.

Table 2. Bacterial species detected in samples of foam produced by nymphs of *Philaenus spumarius* and *Lepyronia coleoptrata*, collected from different host plants. Identification of sequenced 16S rDNA bands selected from PCR-DGGEs.

Spittlebug species	Host plant	Bacterial species	Class, Family	DGGE band
Philaenus spumarius	*Cistus monspeliensis*	*Ciceribacter selenitireducens*	α-Proteobacteria, Rhizobiaceae	F-11
	Cistus monspeliensis	*Ciceribacter selenitireducens*	α-Proteobacteria, Rhizobiaceae	F-13
	Cistus monspeliensis	*Ciceribacter azotofigens*	α-Proteobacteria, Rhizobiaceae	F-16
	Cistus monspeliensis	*Ciceribacter azotofigens*	α-Proteobacteria, Rhizobiaceae	F-17
	Cistus monspeliensis	*Brevundimonas mediterranea*	α-Proteobacteria, Caulobacteraceae	F-15
	Cistus monspeliensis	*Erwinia rhapontici*	γ-Proteobacteria, Enterobacteriaceae	F-14
	Cistus monspeliensis	*Stenotrophomonas rhizoplilia*	γ-Proteobacteria, Xanthomonadaceae	F-18
	Cirsium sp.	*Ciceribacter azotofigens*	α-Proteobacteria, Rhizobiaceae	F-31
	Cirsium sp.	*Pigmentiphaga humi*	β-Proteobacteria, Alcaligenaceae	F-10
	Vicia sativa	*Devosia oryziradicis*	α-Proteobacteria, Devosiaceae	F-12
Lepyronia coleoptrata	*Trifolium repens*	*Brevundimonas mediterranea*	α-Proteobacteria, Caulobacteraceae	F-26
	Trifolium repens	*Rhizobium skierniewicense*	α-Proteobacteria, Rhizobiaceae	F-28, F-29
	Trifolium repens	*Sinorhizobium* sp.	α-Proteobacteria, Rhizobiaceae	F-7
	Trifolium repens	*Erwinia rhapontici*	γ-Proteobacteria, Enterobacteriaceae	F-9

On the contrary, the effects of the sampling site seemed to be negligible, since foams collected in the same locality harbored different microbiome, while froths collected from *V. sativa* and *E. cyparissias,* with similar microbial structure/composition, originated from two different areas.

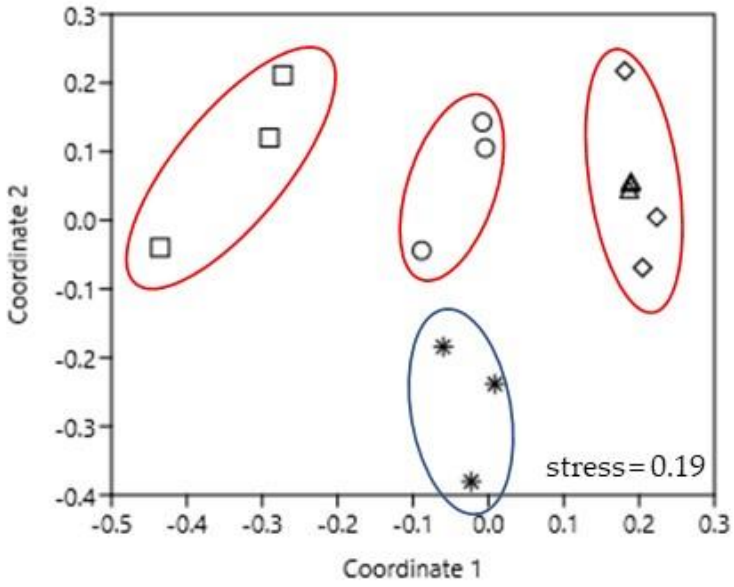

Figure 2. Non-metric multidimensional scaling (nMDS) ordination plot of bacterial communities detected in foams of *P. spumarius* and *L. coleoptrata*. Star: foam of *L. coleoptrata* collected from *Trifolium repens*; Circle: foam of *P. spumarius* collected from *Cirsum* sp.; Square: foam of *P. spumarius* collected from *Cistus monspeliensis*; Diamond: foam of *P. spumarius* collected from *Euphorbia cyparissias*; Triangle: foam of *P. spumarius* collected from *Vicia sativa.*

Discussion

The posterior part of the insect's alimentary canal is often characterized by the presence of microbial endosymbionts that are typically located in the hindgut (Ishikawa, 2003). The wide range of functions performed by these microorganisms – well summarized by Engel and Moran (Engel and Moran, 2013) – is one of the key elements that have allowed the successful evolution and spread of insect species on earth.

Although the presence of bacterial cells in adult spittlebug's gut has already been highlighted by microscopy studies (Cecil, 1930; Zhong *et al.*, 2013), and gut endosymbionts have been reported for other Auchenorrhyncha species (Wang and Wei, 2020; Gonella *et al.*, 2011), knowledge on the gut microbiota of Aphrophoridae species is still inadequate.

Results from the present study showed that *P. spumarius* and *L. coleoptrata* nymphs, harbor a large and diverse community of bacterial endosymbionts in their gut and in the Malpighian tubules, with some predominant species revealed by DGGE profiles.

A species closely related to *Sodalis glossinidius* was recurrently found in the examined *P. spumarius* gut samples, remarking the importance of this symbiont for spittlebugs. Indeed, the *Sodalis*-like bacterium inhabiting the bacteriomes of Philaenini species together with *Ca*. Sulcia muelleri can provide hosts with essential amino acids and other important biomolecules, despite its reduced genome (Koga *et al.*, 2013). Particularly, the potential capability to synthetize glutamate from glutamine (one of the main sources of amino acids in xylem sap) is remarkable, since it is complementary with the probable capacity of *Ca.* Sulcia muelleri to synthetize 2-oxoglutarate, a molecule involved in the Krebs cycle, starting from glutamate (Koga *et al.*, 2014). Moreover, the detection of *Sodalis* endosymbiont outside bacteriomes underlined the importance of its biosynthetic capabilities, marking the key role of this bacteria in the provision of essential nutrients lacking in the host diet.

Since the relationship between *Sodalis* and members of the tribe Philaenini appears to be an important aspect in the evolutionary success of these spittlebugs, further investigations are needed to clarify the precise localization of this endosymbiont and its functions in *P. spumarius.*

Our findings on *L. coleoptrata* gut microbiome, highlighted the presence of a *Rickettsia* bacterium (98.8% similarity to *Rickettsia bellii*) both in the gut and in Malpighian tubules. The *Rickettsia* group comprises intracellular bacteria well known as vertebrate pathogens, although many species belonging to this genus live in non-pathogenic association with vertebrate and invertebrate animals (Perlman *et al.*, 2006). In arthropods members of the genus *Rickettsia* usually act as vertically-transmitted facultative symbionts that play a major role as reproductive manipulators, even if the variety of their effects on the hosts is still little documented (Perlman *et al.*, 2006; Lawson *et al.*, 2001; Werren *et al.*, 1994; Hagimori *et al.*, 2006). Different *Rickettsia* species have been found in several hemipterans (Gottlieb *et al.*, 2006; Sakurai *et al.*, 2005), including Auchenorrhyncha (Davis *et al.*, 1998; Ishii *et al.*, 2013; Zheng *et al.*, 2017; Wang *et al.*, 2018; Kapantaidaki *et al.*, 2021). In these cases, *Rickettsia* spp. are involved in reproductive manipulation (Sakurai *et al.*, 2005; Himler *et al.*, 2011), increase of the host fitness (Himler *et al.*, 2011), thermotolerance (Brumin *et al.*, 2011) and protection against pathogens (Lukasik *et al.*, 2013). Since we detected *Rickettsia* sp. harbored by digestive organs of *L. coleoptrata*, we can speculate that this symbiont might be involved in digestion processes, as proposed for some cicada species (Zheng *et al.*, 2017). Otherwise, the symbiont could even be implied in the production of the froth, since a possible contribution of this bacterium was suggested in the production of the gelling saliva delivered by *Bemisia tabaci* (Gennadius, 1889) to allow stylets penetration (Brumin *et al.*, 2012).

According to our research, Enterobacteriaceae species and a member of Actinobacteria of the genus *Rhodococcus* are putative endosymbionts of both *P. spumarius* and *L. coleoptrata* nymphs.

The association between insects and Enterobacteriaceae is demonstrated to be spread and diverse (Ishikawa, 2003). Phylogenetic studies have highlighted that several insect endosymbionts are closely related with this bacteria family, indicating the presence of specific traits that allow Enterobacteriaceae to infect and establish inside insect hosts (Charles *et al.*, 2001). Beneficial effects originated by these mutualistic relationships vary from the increasing of resistance to stress and parasitism to the extension of host plant range (Moran *et al.*, 2005). The family Enterobacteriaceae comprises also diazotroph organisms that allow nitrogen (N_2)-fixation in insects (Bar-Shmuel *et al.*, 2020; Zheng *et al.*, 2017). Since spittlebugs feed on nutrient-poor and nitrogen-deficient diet, it is likely that Enterobacteriaceae inhabiting their gut are involved in N_2-fixation processes, providing the host with N usable sources (Moran *et al.*, 2005).

The Actinobacteria genus *Rhodococcus* includes several species, often found in the soil, that are able to degrade many toxic chemicals (Tsiko, 2007). Even *R. gingshengii* was first described as a fungicide degrading-bacterium, when it was isolated from carbendazim-contaminated soils and its capability to reduce this noxious compound was assessed (Xu *et al.*, 2007). Members of the genus *Rhodococcus* were also reported as symbionts of several insects, as in the case of *Rhodococcus rhodnii,* a gut symbiont of the bug *Rhodnius prolixus* Stål, 1859 that supplies its host with B vitamins (Brecher and Wigglesworth, 1944). However, this is only one of a few recognized nutritional-based associations between Actinobacteria and insects, since this group appears to be generally involved in defensive mechanisms, producing secondary metabolites with antibiotic effects (Kaltenpoth, 2009).

Regarding Auchenorrhyncha*, Rhodococcus* was previously detected in two cicada species, *Platypleura kaempferi* (Fabricius, 1794) and *Meimuna mongolica* (Distant, 1881) (Zheng *et al.*, 2017), and in the leafhopper *Homalodisca vitripennis* (Germar, 1821) (Welch *et al.*, 2015). Nevertheless, the nature of the association between *Rhodococcus* bacteria and their Auchenorrhyncha hosts is unknown. So, the role played by *Rhodococcus* sp. in spittlebugs should be further investigated.

The bacteria species discovered in the gut of *P. spumarius* and *L. coleoptrata* were identified also in the Malpighian tubules of both species. In insects, Malpighian tubules are the main excretory organs, but in Cercopoidea species they also acquire a secretory function thanks to cytological modifications that allow the production of some components of the froth, mainly mucopolysaccharides and proteins (Farina *et al.*, 2022). Trough ultrastructural studies, bacterial cells were evidenced in Malpighian tubules of *Aphrophora obliqua* (Uhler, 1896) adults, showing morphological similarity to those present in the gut of the same species (Li *et al.*, 2015). In the same research, young juveniles did not display such microorganisms leading to hypothesize an inhibitory function of the secretions of Malpighian tubules in nymphs (Li *et al.*, 2015). Our results corroborate the evidence that symbiotic

bacteria can be associated to Malpighian tubules in Cercopoidea species. Differently from what observed in *A. obliqua*, we detected bacteria in Malpighian tubules of nymphs of both species, *P. spumarius* and *L. coleoptrata* presumably because we dissected fifth instar juveniles which are quite close to the emergence and that can show dissimilar features from younger stages.

The complex composition of the froth produced by spittlebug nymphs offers a rich and diverse substrate for lots of microorganisms that could contribute to create a suitable environment for the insect and to produce direct beneficial effects for the nymph itself. So far, the microbial community of spittlebug froth has received scarce attention and information on this topic is almost absent. Our results suggest that the class of alpha-Proteobacteria is the predominant group of bacteria residing in the examined spittlebug froths, as already highlighted for the foam of the Neotropical member of Aphrophoridae *M. fimbriolata* (Tonelli *et al.*, 2020). Alpha-Proteobacteria form a heterogeneous group of microorganisms, inhabiting several terrestrial and aquatic ecosystems. Furthermore, they are involved in many associations with eukaryote organisms, including insects (Batut *et al.*, 2004).

Brevundimonas mediterranea is an alpha-Proteobacterium recently isolated and described from samples of Mediterranean Sea water (Fritz *et al.*, 2005). Members of the genus *Brevundimonas* are reported to be capable of degrading environmental contaminants (Zhang *et al.*, 2020), and mycotoxins (Peng *et al.*, 2022), but also to enhance the growth of plants and microalgae when involved in symbiotic association (Naqqash *et al.*, 2020).

In our froth samples, bacteria attributable to *Brevundimonas mediterranea* appear to occur independently from insect species, populations and localities. We can speculate that this mutualistic association could be of great importance for spittlebug nymphs.

Members referring to Rhizobiaceae family appeared to be abundant in the analyzed froth samples of *P. spumarius* and *L. coleoptrata*. The family Rhizobiaceae includes phenotypically diverse organisms, ranging from N_2-fixing legume symbionts to plant pathogens, bacterial predators and other soil bacteria. Their involvement in endosymbioses with insects has been documented for several species, including those belonging to Hemiptera order: for instance, *Ca.* Hodgkinia cicadicola is reported to be the co-resident symbiont of *Ca.* Sulcia muelleri in some cicada species (McCutheon et al., 2009a; 2009b). One of the recognized functions of Rhizobiales in insects is the fixation of gaseous N_2 into more usable forms (Bar-Shmuel *et al.*, 2020). Our analyses displayed the presence of bacteria like *C. azotofigens*, or *Rhizobium* sp. in all the froth samples, but the same species were not evidenced in gut samples of both spittlebugs. Therefore, the most likely infection source for the foam might be the soil or the host plant since members of Rhizobiaceae typically inhabit these ecological niches. Due to their abundance, probably, the spittlebugs' foam represents a suitable substrate for the survival

and growth of the bacteria of the family Rhizobiaceae, however, if any possible interaction exists with froth producing insects, it remains unclear.

The structure of the remaining part of the bacterial community associated to the spittle mass depends on both insect and host plant species, as shown by the nMDS analyses. The major source of bacteria associated to the froth might be the plant on which the nymph feeds. Differences in dietary habits of the two studied spittlebug species could explain the observed diversity.

Findings highlighted by this study suggest that the froth is a complex environment, harboring both specific mutualistic bacteria which might have defensive functions (e.g., *Brevundimonas*), and other arbitrary species which are contaminant originated from the soil or host plant as it was already highlighted for the spittlebug *M. fimbriolata* (Tonelli *et al.*, 2020) and for another animal secretions, i.e. the mucus of the garden snail *Cornu aspersum* (O.F. Muller, 1774) (Belouhova et al., 2022).

Although results obtained from DGGE and subsequently sequencing of dominant bands could be very useful for a preliminary overview of bacterial communities, the complete sequencing of extracted DNA via next-generation sequencing (NGS) analysis will be more informative in order to provide a detailed description of spittlebug facultative endosymbionts and of the bacteria hosted by the froth. Moreover, ultrastructural studies would be necessary to assess the exact localization of bacteria in the gut and Malpighian tubules of spittlebug nymphs.

More focused research on symbionts of spittlebugs could clarify specific functions played by bacteria detected in the nymphs' organs and unveil their roles in the relationship with their hosts, additional investigations could disclose the bacterial involvement in the production of the froth as well.

In conclusion, our study has allowed a preliminary exploration of the complex bacterial community associated with the gut, the Malpighian tubules and the foam of the nymphs of two Aphrophoridae species giving also the basis for further and more deepened investigations aimed at improving the knowledge on this topic and developing effective and more sustainable control strategies against spittlebug vectors of *X. fastidiosa*.

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Supplementary materials

The following supporting information can be downloaded at: www.mdpi.com/xxx/s1

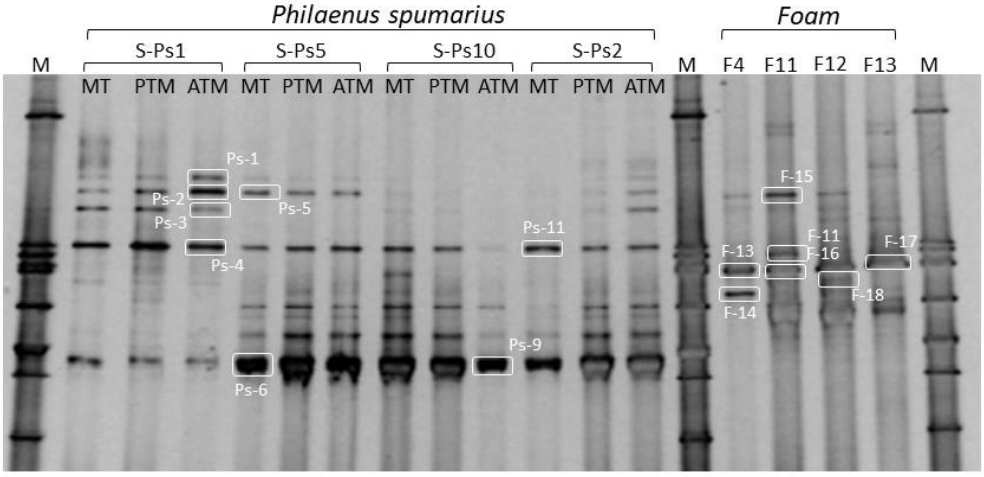

Figure S1. DGGE profiles of bacterial 16S rDNA gene fragments obtained from dissected nymphs of *Philaenus spumarius* and from foam samples collected on *Cistus monspeliensis* plants. The letter M on the gel image indicates the marker used for normalization of bands. Excided sequenced DGGE bands were pointed out. PTM, posterior tubular midgut; ATM, filter chamber linked to the conical segment; MT, Malpighian tubules; IL, ileum.

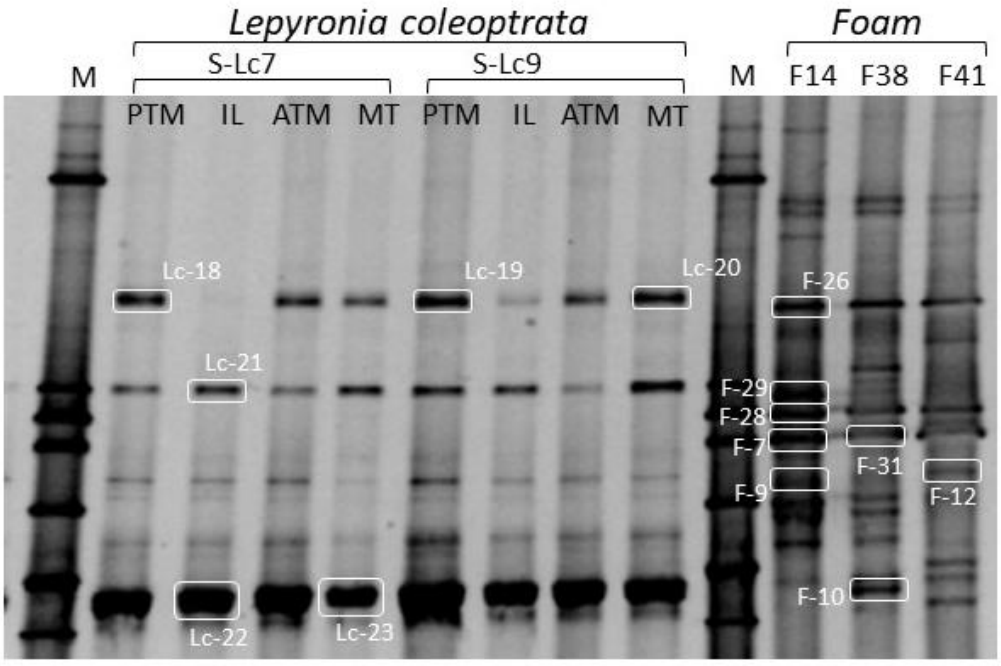

Figure S2. DGGE profiles of bacterial 16S rDNA gene fragments obtained from dissected nymphs of *Lepyronia coleoptrata* and from foam samples. Foam sample "F14" produced by *L. coleoptrata* on *Trifolium, "*F38" and "F41" produced by *Philaenus spumarius* on *Cirsum* sp. and *Vicia sativa*, respectively. The letter M on the gel image indicates the marker used for normalization of bands. Excided sequenced DGGE bands were pointed out. PTM, posterior tubular midgut; ATM, filter chamber linked to the conical segment; MT, Malpighian tubules; IL, ileum.

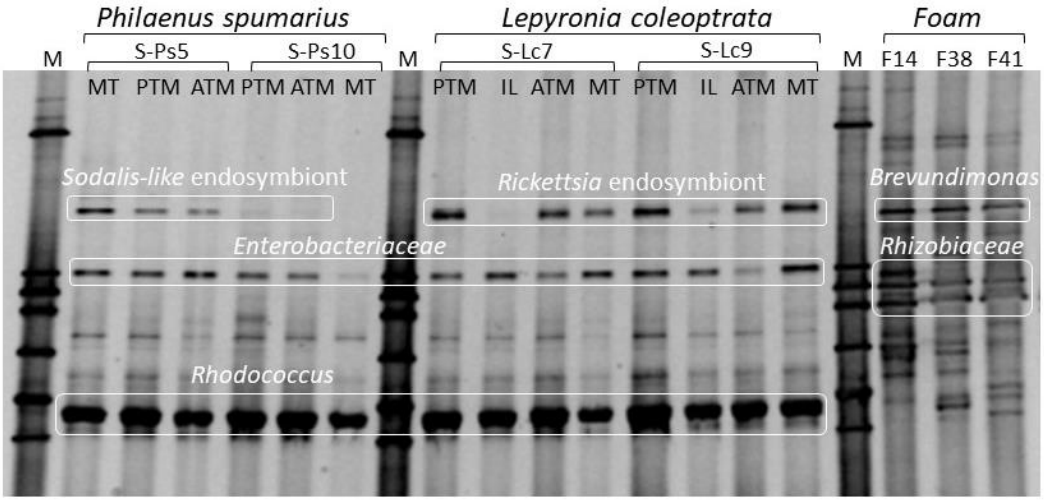

Figure S3. DGGE profiles of bacterial 16S rDNA gene fragments obtained from dissected nymphs of *Philaenus spumarius* and *Lepyronia coleoptrata* and from foam samples. Foam sample "F14" produced by *L. coleoptrata* on *Trifolium, "*F38" and "F41" produced by *P. spumarius* on *Cirsum* sp. and *Vicia sativa*, respectively. The letter M on the gel image indicates the marker used marker used for normalization of bands. Species attributions to DGGE bands were pointed out. PTM, posterior tubular midgut; ATM, filter chamber linked to conical segment; MT, Malpighian tubules; IL, ileum.

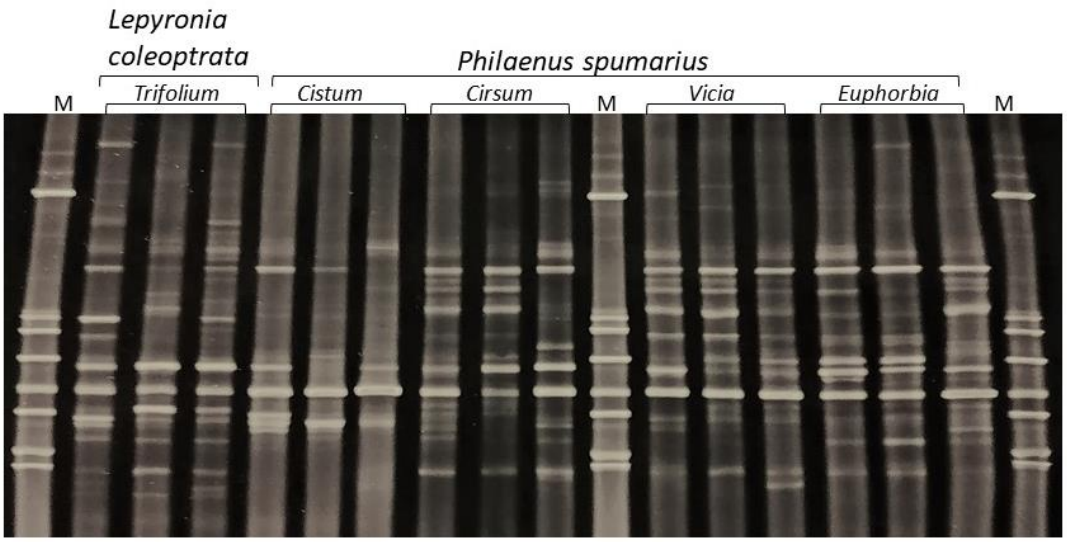

Figure S4. DGGE profiles of bacterial 16S rDNA gene fragments obtained from foam samples of *Lepyronia coleoptrata* and *Philaenus spumarius* produced on *Trifolium repens, Cistus monspeliensis*, *Cirsum* sp.*, Vicia sativa,* and *Euphorbia cyparissias*. The letter M on the gel image indicates the marker used for normalization of bands.

Table S1. Results of BLAST search on sequenced 16S rDNA bands selected from PCR-DGGEs, the accession number of the nearest known bacterial species with a sequence coverage of 100% was reported. Taxonomic identification was achieved by using different sequence similarity thresholds: a similarity ≥97%, ≥95%, ≥90%, ≥85%, ≥80% and ≥75% for assignment at the species-, genus-, family-, order-, class- and phylum-levels identification, respectively. [Webster, N.S.; Taylor, M.W.; Behnam, F.; Lucker, S.; Rattei, T.; Whalan, S.; Horn, M.; Wagner, M. Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. Environ. Microbiol. 2010, 12, 2070–2082. https://doi.org/10.1111/j.1462-2920.2009. 02065.x.].
PTM, posterior tubular midgut; ATM, filter chamber linked to the conical segment; MT, Malpighian tubules; IL, ileum

Band	**Sample**	**Nearest match (GenBank accession no.; % similarity)**	**Taxonomical identification**
Ps-1	ATM of *Philaenus spumarius*	*Sodalis praecaptivus* (AM237373; 97.4%)	*Sodalis praecaptivus*
Ps-2	ATM of *Philaenus spumarius*	*Sodalis glossinidius* (LN854557; 97.9%)	*Sodalis glossinidius*
Ps-3	ATM of *Philaenus spumarius*	*Sodalis glossinidius* (LN854557; 98.1%)	*Sodalis glossinidius*
Ps-4	ATM of *Philaenus spumarius*	*Escherichia coli* (MN083301; 100%)	*Salmonella enterica*
Ps-5	MT of *Philaenus spumarius*	*Sodalis glossinidius* (LN854557; 97.9%)	*Sodalis glossinidius*
Ps-6	MT of *Philaenus spumarius*	*Rhodococcus gingshengii* (MN826591; 100%)	*Rhodococcus gingshengii*
Ps-9	ATM of *Philaenus spumarius*	*Rhodococcus gingshengii* (MN826591; 100%)	*Rhodococcus gingshengii*
Ps-11	MT of *Philaenus spumarius*	*Escherichia coli* (MN083301; 100%)	*Salmonella enterica*
Lc-18	PTM of *Lepyronia coleoptrata*	*Rickettsia bellii* (KU586119; 99.0%)	*Rickettsia bellii*
Lc-19	PTM of *Lepyronia coleoptrata*	*Rickettsia bellii* (KU586119; 99.2%)	*Rickettsia bellii*
Lc-20	MT of *Lepyronia coleoptrata*	*Rickettsia bellii* (KU586119; 98.2%)	*Rickettsia bellii*
Lc-21	IL of *Lepyronia coleoptrata*	*Escherichia coli* (MN083301; 100%)	*Salmonella enterica*
Lc-22	IL of *Lepyronia coleoptrata*	*Rhodococcus gingshengii* (MN826591; 100%)	*Rhodococcus gingshengii*
Lc-23	MT of *Philaenus spumarius*	*Rhodococcus gingshengii* (MN826591; 100%)	*Rhodococcus gingshengii*
F-7	Foam of *Lepyronia coleoptrata*	*Sinorhizobium sp.* (CP044012; 96.5%)	*Sinorhizobium sp.*
F-9	Foam of *Lepyronia coleoptrata*	*Erwinia rhapontici* (MN826571; 99.7%)	*Erwinia rhapontici*
F-10	Foam of *Philaenus spumarius*	*Pigmentiphaga humi* (MH667611; 99.5%)	*Pigmentiphaga humi*
F-11	Foam of *Philaenus spumarius*	*Ciceribacter selenitireducens* (MH665748; 99.5%)	*Ciceribacter selenitireducens*
F-12	Foam of *Philaenus spumarius*	*Devosia oryziradicis* (CP068047; 97,2%)	*Devosia oryziradicis*
F-13	Foam of *Philaenus spumarius*	*Ciceribacter selenitireducens* (MH665748; 99.5%)	*Ciceribacter selenitireducens*
F-14	Foam of *Philaenus spumarius*	*Erwinia rhapontici* (MN826571; 99.7%)	*Erwinia rhapontici*
F-15	Foam of *Philaenus spumarius*	*Brevundimonas mediterranea* (MK250497; 99.7%)	*Brevundimonas mediterranea*
F-16	Foam of *Philaenus spumarius*	*Ciceribacter azotifigens* (KX510117; 97.6%)	*Ciceribacter azotifigens*
F-17	Foam of *Philaenus spumarius*	*Ciceribacter azotifigens* (KX510117; 97.6%)	*Ciceribacter azotifigens*

F-18	Foam of *Philaenus spumarius*	*Stenotrophomonas rhizoplilia* (MT078676; 100%)	*Stenotrophomonas rhizoplilia*
F-26	Foam of *Lepyronia coleoptrata*	*Brevundimonas mediterranea* (MK250497; 99.7%)	*Brevundimonas mediterranea*
F-28	Foam of *Lepyronia coleoptrata*	*Rhizobium skierniewicense* (MN826327; 99.7%)	*Rhizobium skierniewicense*
F-29	Foam of *Lepyronia coleoptrata*	*Rhizobium skierniewicense* (MN826327; 99.7%)	*Rhizobium skierniewicense*
F-31	Foam of *Philaenus spumarius*	*Ciceribacter azotifigens* (KX510117; 97.6%)	*Ciceribacter azotifigens*

4. Gross antennal morphology of *Philaenus spumarius* juveniles and behavioural response to olfactory plant cues

Personal contribution: Conceptualization, insect rearing, data collection, olfactometer bioassays, data analysis.

Dott. Francisco Jose Beitia Crespo[1] has collaborated on conceptualization and data collection; Dott.ssa Marzia Cristiana Rosi[2] has performed the statistical analysis. Prof.ssa Patrizia Sacchetti[2] has collaborated on conceptualization and manuscript preparation.

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Abstract

Philaenus spumarius (Hemiptera: Aphrophoridae) is the main European vector of the plant-pathogenic bacterium *Xylella fastidiosa*. Despite a large amount of literature available on this species, virtually nothing is known about host plant-finding by *P. spumarius*. The movement towards food sources has been documented both for juveniles and adults, but the mechanisms mediating the host plant location remain unknown.

In this study, preliminary investigations of the gross morphology of nymphs' antennae through scanning electron microscopy were conducted, together with Y-tube behavioural assays aimed at evaluating the response of nymphs to olfactory stimuli emitted by alfalfa plants.

Nymphs' antennae consist of scape, pedicel and flagellum. Antennal structures are distributed on the pedicel and on flagellomeres from II to V. Different sensory structures were described: basiconic sensilla, sensory cavities, campaniform sensilla and hair sensilla. Although our preliminary observations did not allow to appreciate the fine structure of the observed sensilla, an olfactory function could be hypothesized for basiconic sensilla spotted along the flagellum, since olfactory pegs also occur in adults' antennae. Results of behavioral assays in the Y-tube olfactometer suggest that nymphs could have detected olfactory cues in the odd Y-tube without orientate themselves towards the olfactometer arm bearing the triggering stimulus. Hence, host plant's volatiles may function as non-directional stimuli. Furthermore, it could be possible that nymphs move toward host plants thanks

to a combination of sensory stimuli, for example, olfactory together with visual cues, as evidenced for other Auchenorrhyncha species and suggested for *P. spumarius* adults.

Introduction

Philaenus spumarius L. (1758) (Hemiptera: Aphrophoridae) is a widespread species which occurs in most of the Holarctic realm (Yurtsever, 2000; Drosopoulos *et al*., 2010). Previously studied mainly for its polymorphism and for being a severe pest in North America (Halkka *et al*., 1973; Harper and Whittaker, 1976; Weaver and King, 1954), *P. spumarius* has received increasing attention in the last decade since it has been recognized as the main European vector of the plant-pathogenic bacterium *Xylella fastidiosa* subsp. *pauca* ST53 (Wells *et al*., 1987) (Cornara *et al*., 2016). This quarantine organism is the causal agent of the Olive Quick Decline Syndrome (OQDS), a severe vascular disease of olive trees that has led to the death of thousands of plants in South Italy (Saponari *et al*., 2013; Martelli *et al*., 2016).

Philaenus spumarius is a univoltine hemimetabolous species that overwinter as eggs, laid in clusters in herbs and stubs (Yurtsever, 2000; Cornara *et al*., 2018). Nymphs hatch from eggs early in spring and feed on numerous herbaceous plants (Weaver and King, 1954). During feeding, *P. spumarius* nymphs produce a peculiar saliva-like bubble nest consisting of hydrolyzed xylem sap, proteins and mucopolysaccharides. The self-produced foam protects the nymph against dehydration, temperature fluctuations and natural enemies (Weaver and King, 1954; del Campo *et al*., 2011; Chen *et al*., 2018; Tonelli *et al*., 2019) and represents a distinctive feature of the members of the family Aphrophoridae, which are commonly called “spittlebugs”.

The preimaginal development of *P. spumarius* passes through five instars. First-instar nymphs are tiny soft-bodied insects, approximately 1.35 mm long and light orange (Yurtsever, 2000; Cornara *et al.*, 2018). Younger juveniles occur in small spittles typically located at the bottom of plant stems (Weaver and King, 1954) and tend to aggregate inside the same foam with conspecifics or even with nymphs belonging to other spittlebug species (Halkka *et al*., 1977). A widely accepted explanation of this phenomenon is that the aggregation enables the nymphs to exploit the food source better and to save energy in producing the spittle mass (Whittaker, 1965; Biedermann, 2003, Chen and Liang, 2015; Bodino *et al*., 2019). Differently from earlier stages, later nymphs turn yellowish and greenish, usually feed on the apical part of the plant without gathering together and produce copious and easily detectable spittle (Cornara *et al*., 2018).

Both juveniles and adults of *P. spumarius* are highly polyphagous xylem sap feeders (Wiegert, 1964, Crews *et al*., 1998; Malone *et al*., 1999). Despite the wide range of host plants, nymphs show a

somewhat preference for host plants that belong to the family Asteraceae and Fabaceae, as well as for species with a high amino acid content in the xylem sap (Horsfield, 1977; Bodino *et al*., 2019). Virtually nothing is known about host plant-finding by spittlebug nymphs and adults. The ability to crawl on the soil surface and climb on plants has been observed in spittlebug nymphs, including newly hatched nymphs which move from the hatching spot to search for an appropriate host (Halkka and Halkka, 1990; Pires *et al*., 2000a; Pires *et al*., 2000b, Albre *et al*., 2021). The motility increases in later preimaginal stages, which usually change the host plants if their suitability decreases or when too many nymphs occupy the same foam (McEvoy, 1986). Although such kinetic activity towards and among food sources is known and often documented, the mechanisms that mediate the movement and the host plant location remain unknown.

Many herbivorous insects rely on Volatile Organic Compounds (VOCs) emitted by host plants for orientation and host plant recognition (Loudon, 2009; Bruce *et al*., 2005; Bruce and Picketts, 2011). Chemical cues are primarily detected by insect antennae that could be variously equipped with several kinds of sensory structures, including chemoreceptors, mechanoreceptors, thermoreceptors and hygroreceptors (Steinbrecht, 1996). The insect's ability to perceive different stimuli directly depends on the set and the arrangement of sensory structures on its antennae (Loudon, 2009).

In their study on *P. spumarius* adults' antennae, Ranieri *et al*. (2016) described a limited number of basiconic and coeloconic sensilla with a possible olfactory function. Although the olfactory sensory equipment appears to be relatively reduced in *P. spumarius* to other Auchenorrhyncha, recent studies have demonstrated the ability of both males and females to perceive several plant volatiles (Germinara *et al*., 2017; Ganassi *et al*., 2020; Anastasaki *et al*., 2021; Cascone *et al*., 2022; Rodrigues *et al*., 2022).

The study of the role of olfaction in host plant seeking in spittlebugs is still in its inception. So, research on the behavioural response of *P. spumarius* nymphs to olfactory plant stimuli could increase the knowledge of the ecology and ethology of this economically important species. Moreover, investigating the olfactory acuity of phytophagous insects could identify behaviorally active compounds that, in turn, could be successfully employed in sustainable pest management (Agelopoulos *et al*., 1999; Smart *et al*., 2014). Thus, preliminary investigations of the gross morphology of nymphs' antennae were conducted in this study, together with behavioural assays aimed at evaluating the response of *P. spumarius* nymphs to olfactory stimuli emitted by the host plant.

Materials and Methods

Insect rearing and plant material

Philaenus spumarius nymphs used for both SEM observations and behavioural assays were collected in the field in Segorbe (Comunidad Valenciana, Spain) from several host plants and reared in a screen house at the Instituto Valenciano de Investigaciones Agrarias (IVIA), on sowed alfalfa plants (*Medicago sativa* L.), without temperature and humidity control. For behavioural experiments, nymphs were divided into two age groups, according to Yurtsever (2000): first-third instar nymphs (N1-N3) and fourth-fifth instar nymphs (N4-N5) and tested separately.

Alfalfa plants, uninfested and infested by *P. spumarius* nymphs, were utilized in olfactometer bioassays as a source of olfactory stimuli. Alfalfa plants were chosen since species of the Fabaceae family are known to be preferred hosts by *P. spumarius* nymphs (Cornara *et al*., 2018).

Plants produce various volatiles in response to herbivore attacks (Dudareva *et al*., 2006). Moreover, the presence of semiochemicals in the foam created by nymphs could be hypothesized since an aggregating pheromone has been assessed for the froth of the rice spittlebug *Callitettix versicolor* Stål, 1865 (Chen and Liang, 2015). Although the volatile profile of alfalfa plants and foam of *P. spumarius* were not available at the time of the experiment, and we weren't able to discriminate between a possible effect of plant- or spittle-volatile compounds, we decided to conduct assays with infested alfalfa plants to eventually highlight some differences in nymphs' behaviour and refine our hypothesis.

To standardize the plants to be tested as much as possible several bottles filled with the nutrient solution were prepared to provide each one with four alfalfa seedlings, each equipped with its root system. These "hydroponic bottles" were arranged a week before the experiments to avoid the emission of stress-associated plant volatiles. Moreover, only actively growing plants were chosen for the experiments. Infested plants were prepared by placing a nymph on stems included in the bottle (one nymph per bottle) 24h before they were tested to allow the formation of a consistent spittle and ensure the stability of the insect on the plant. Third-instar nymphs were chosen as representatives for the N1-N3 age group to infest experimental plants, and fifth-instar nymphs were used for the N4-N5 age group.

Scanning Electron Microscopy (SEM)

Third- and fourth-instar *P. spumarius* nymphs were collected using a soft brush from alfalfa plants. Live nymphs were washed with distillate water to remove froth residuals and then cold anaesthetized at -20°C for the 60s. Subsequently, nymphs were dipped in 70% ethanol and stored until processed. Fifteen specimens (ten fourth-instar and five third-instar nymphs) were prepared for SEM observations dissecting the head from the body under a stereoscopic microscope. Antennae were left in their natural position in all the samples. Specimens were dehydrated in a series of graded ethanol concentrations, from 70% to 80%, 90%, then 95% and 99%, for 10 min per step. After dehydration, ethanol was replaced with pure HDMS (Hexamethyldisilazane) following the protocol described in Ranieri *et al*. (2016). Then, nymphs were mounted on stubs and gold-coated in a sputter coater device (S150B; BOC Edwards, Burgess Hill, U.K.). Observations were made using a Zeiss Evo 40 at the centre "Centro di Servizi di Microscopia Elettronica e Microanalisi" (MEMA) of the University of Florence.

Behavioural assays

Behavioural assays were carried out to evaluate the response of *P. spumarius* nymphs to olfactory stimuli emitted by host plants. The experiments were conducted in a glass Y-tube olfactometer (Analytical Research Systems, Gainesville, FL) under controlled temperature and humidity (21°C and 40% UR). The apparatus was set inside a dark room and was uniformly illuminated from above using neon lights.

Two types of bioassays were set up: in the first one (type 1), the nymphs' response was evaluated when volatiles emitted by an uninfested alfalfa plant was compared to the control (empty jar); in the second type of bioassay (type 2), volatiles emitted by uninfested plants were tested in comparison with those emitted by a plant infested by a spittlebug nymph. Each bioassay lasted 15 min. Each nymph's first choice was recorded at the time of its occurrence. Nymphs were considered as having made a choice when they passed the midway of one of the Y-tube arms.

Tested plants (uninfested and infested) were kept in separate jars connected to the Y-tube arms under a constant 1.2 kPa airflow. The jars were screened with a white sheet of filter paper to avoid the influence of visual stimuli. Every ten assays, plants were substituted, and their position in the Y-tube arms was swapped. The Y-tube was also cleaned using pure acetone and air-dried every five tests before beginning a new assay.

Nymphs were brought to the laboratory 48 h before the experiment and acclimatized on single-potted alfalfa plants at 21 °C and 40% RH to avoid stress due to differences between semi-field and olfactometer room conditions. Individuals were taken from their feeding plants using a thin

paintbrush and transferred into a Petri dish for 10 minutes before being tested. This step was necessary to stimulate nymphs' mobility since we observed that *P. spumarius* juveniles increased their motility after a few minutes of detaching from their host plants. After this acclimation period, a single individual was introduced into the entrance of the Y-tube and observed for 15 minutes.

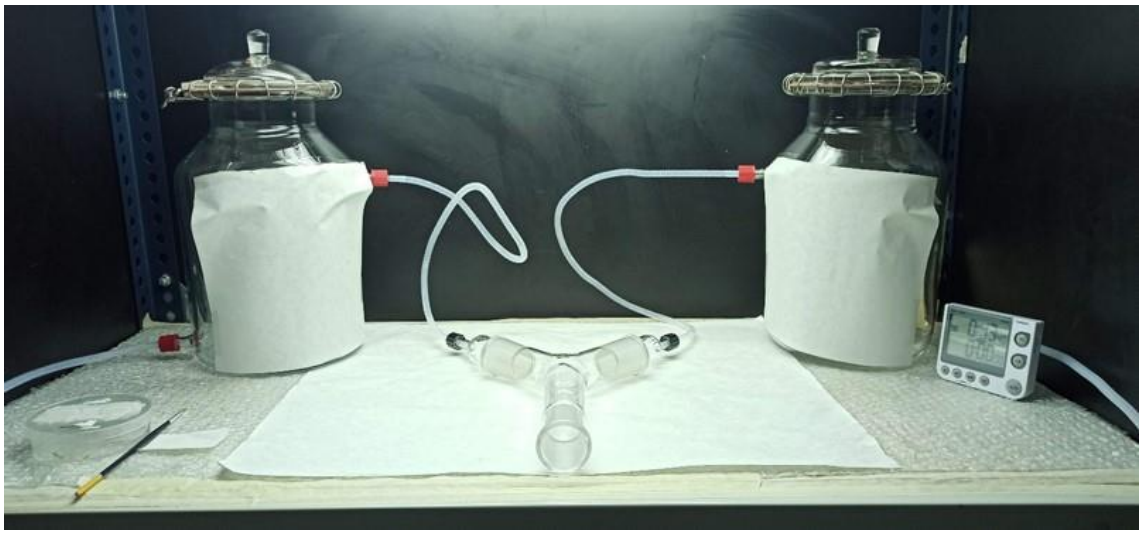

Figure 1. The Y-tube olfactometer used for behavioural assays. The jars were screened with a white sheet of filter paper to avoid the influence of visual stimuli.

Statistical analysis

Data from the two bioassay types (number of nymphs who made a first choice and time spent by nymphs to make the first choice) were analyzed separately. A Chi-square Goodness of fit test (H0 = 1:1) was performed to compare the responses obtained for each stimulus. The choices made by individuals of the two age groups were grouped for comparison. Before applying the two-way ANOVA analysis, data were assessed for normality using Shapiro-Wilk's normality test and log-transformed [$\log_{10}(x)$] for normality correction.

Finally, a two-way ANOVA test was performed to analyze the effect of nymph age and source of olfactory stimuli on time required by nymphs to make the first choice. All statistical analyses were performed using R software (R Core Team 2020, version 4.0.5).

Results

SEM observations

The antennae of fourth-instar nymphs are inserted into an antennal socket located in the transition zone between fronto-clypeus and compound eyes, below two slightly pronounced antennal ledges, similar to the arrangement observed in *P. spumarius* adults and other Cicadomorpha (Figure 2A). Antennae are inserted almost perpendicularly to the head so that they are oriented towards outside. The antenna consists ofscape, pedicel, and flagellum (Figure 2B). The scape is cylindrical and shows a smooth surface without distinct features. The pedicel (about 47 μm long) is a bolt-like segment with a concave apex articulating the flagellum. The pedicel is the most significant section of the nymphs' antenna. The flagellum (about 228 μm long) is quite thick and is composed of eight annular flagellomeres that decrease in size and length from the proximal to the distal end. The first flagellomere is multifaceted and longer than the other ones. Antennal structures appear distributed on the pedicel and the dorsolateral part of the flagellomeres from II to V. A total of 13 sensory structures were observed on nymph's antennae, which can be described based on their shape as basiconic sensilla, sensory cavities, campaniform sensilla and hair sensilla.

Seven basiconic sensilla were spotted on *P. spumarius* nymphs' antennae. The first one was located dorsolaterally on the pedicel, and four were in the dorsolateral part of the flagellomeres from II to V (Figure 2E). At the same time, the latter two were found on the flagellum apex (Figure 2C). The peg on the pedicel is conical in shape. It is inserted in a narrow, rounded, cuticular socket close to the distal margin of the antennomere and a campaniform sensillum. Dorso-lateral basiconic sensilla are somewhat elongated, pointed at the apex and inserted in round pits adjacent to the distal edge of the flagellomere, oriented nearly parallel to the flagellar extension. Finally, the two uniporous basiconic sensilla observed on the flagellum apex (on the flagellomere VIII) are inserted into two cuticular sockets, side by side, facing the outside. These pegs appear long and slender and nearly conical in shape.

Four sensory cavities appeared to be paired with the basiconic sensilla on the flagellomeres from II to V (Figure 2F). These structures consisted of a circular pit with an inner rim characterized by several cuticular processes pointing from the centre of the cavity. SEM observations couldn't allow us to detect any pegs or porous structures inside the pit.

A single button-like campaniform sensillum was observed on the dorsal surface of the pedicel, close to its distal margin. Finally, a thin hair sensillum was found on the ventrolateral surface of the pedicel, pointing to the post-clypeus (Figure 2D).

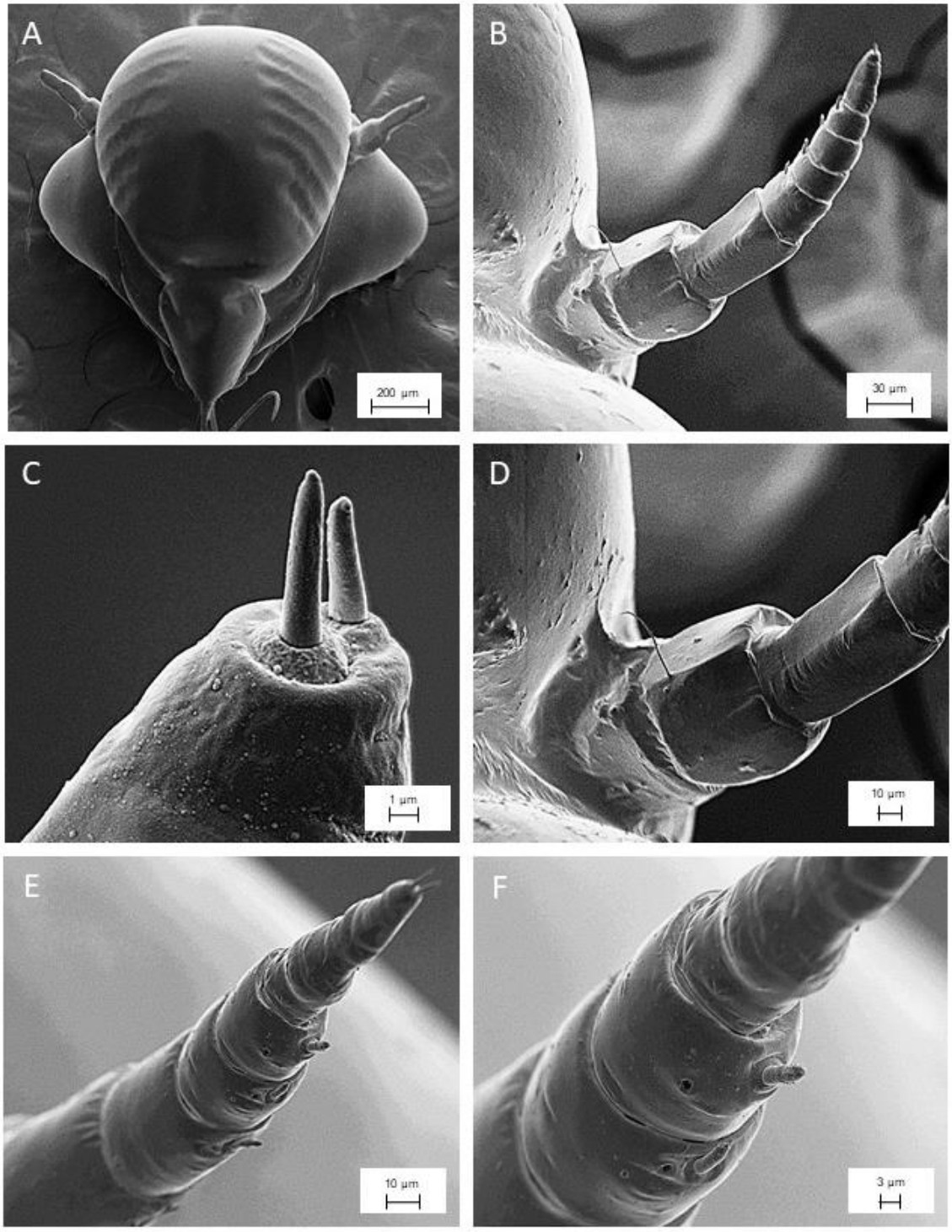

Figure 2. *Philaenus spumarius* fourth-instar nymph. A: frontal view of the head with antennae; B: ventral view of the antenna showing the antennomeres; C: flagellum tip with two uniporous basiconic sensilla; D: magnification of the antennal basis with scape and pedicel; E: flagellum consisting of eight flagellomeres; F: magnification of the central part of the flagellum showing III and IV flagellomeres with coeloconic sensilla close to basiconic ones.

Behavioural assays

There were 155 nymphs (107 for the N1-N3 age group and 48 for the N4-N5 age group) for the type 1 bioassay and 96 nymphs (49 for the N1-N3 age group and 47 for the N4-N5 age group) for the type 2 bioassay were tested in the Y- tube olfactometer. On average, the total response rate was 74.20% (70.10% for the N1-N3 age group, 83.30% for the N4-N5 age group) and 83.30% (81.63% for the N1-N3 age group, 85.1% for N4-N5 age group) for type 1 and type 2 bioassays, respectively.

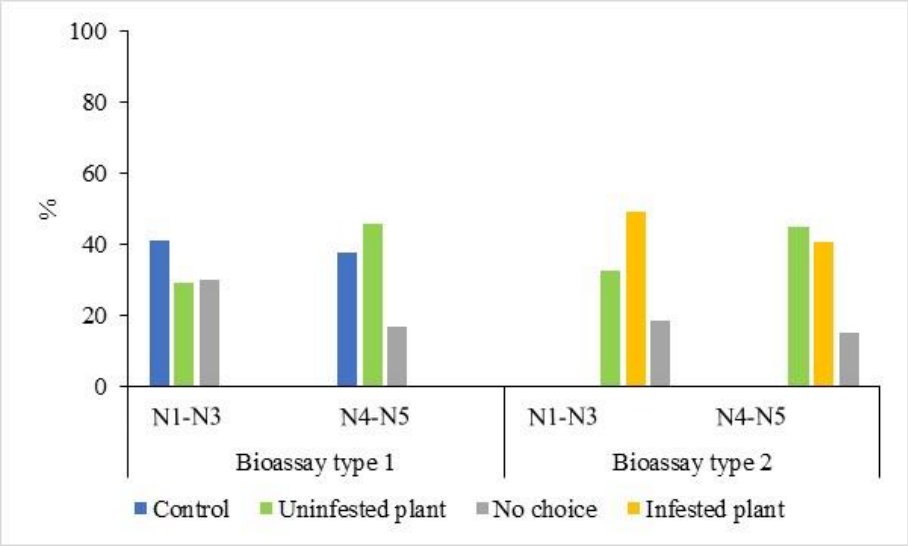

Figure 3. Response of *Philaenus spumarius* nymphs (% over the total number of tested nymphs) to possible olfactory stimuli in behavioural bioassays conducted in a Y-tube olfactometer. Bioassays type 1: Control (empty jar) *versus* Uninfested alfalfa plants (*Medicago sativa*); Bioassays type 2: Uninfested alfalfa plants *versus* Infested alfalfa plants (one *P. spumarius* nymph within its spittle per plant)

The Chi-square Goodness of fit test showed that the distribution of the number of responses among the different stimuli (Table 1) was consistent with the assumed distribution (type 1 bioassay: χ^2 = 1.96; df = 1; p = 0.16; type 2 bioassay: χ^2 = 1.26; df = 1; p = 0.26). Thus, none of the tested stimuli has elicited a significant response in *P. spumarius* nymphs.

For type 1 bioassays, the two-way ANOVA revealed no statistically significant interaction between the effects of nymphs age and the nature of the olfactory stimuli (F (1, 111) = 0.27, p = 0.60). Additionally, simple main effects analysis showed that nymph age did not have a statistically significant effect on the time spent by nymphs to make the first choice (p = 0.22). However, on average, older nymphs take less time to choose. On the contrary, the effect of the type of odour on the average time employed by nymphs for choosing is statistically significant, showing that the time needed to select the plant is significantly longer than that used to opt for the control treatment (p = 0.03).

Table 1. Response of *Philaenus spumarius* nymphs of different age groups to uninfested alfalfa and infested alfalfa plants and to control (empty jar), in Y-tube olfactometer bioassays (Chi-square Goodness of fit test).

Experiment type	Treatment	Number of nymphs who made a first choice		Total	χ^2	p
		Age group				
		N1-N3	N4-N5			
Type 1: Uninfested plant vs Control	Uninfested plant	31	22	53	1.96	> 0.05
	Control	44	18	62		
Type 2: Uninfested plant vs Infested plant	Uninfested plant	24	19	43	1.26	> 0.05
	Infested plant	16	21	37		

Table 2. The time spent by *Philaenus spumarius* nymphs of two age groups (mean ± sd) was recorded in behavioural bioassays where an uninfested alfalfa plant was tested compared to the control (empty jar). Two-way ANOVA was performed on log-transformed data, here reported as seconds (s).

Age group	**Treatment**	**n**	**mean (s)**		**sd**	
N1-N3	Control	44	328.64	±	238.77	
	Uninfested plant	31	423.87	±	276.67	
	Overall N1-N3	75	376.25	±	257.72	a
N4-N5	Control	18	246.67	±	163.20	
	Uninfested plant	22	376.36	±	247.17	
	Overall N4-N5	40	311.52	±	205.18	a
Overall control		62	287.65	±	200.99	a
Overall uninfested plant		53	400.12	±	261.92	b

Two-way ANOVA transformed data log10(x); different letters refer to significant differences between main effects.

Data from the type 2 bioassays analyzed through the two-way ANOVA showed a statistically significant interaction between the effects of nymphs age and the nature of the olfactory stimuli ($F(1, 76) = 736.70$, $p = 0.008$). Remarkably, the average time needed to make the first choice was significantly longer when younger nymphs chose uninfested plants and older nymphs selected infested plants. No statistically significant simple main effects were evidenced for both nymph age ($p = 0.49$) and the nature of the stimuli ($p = 0.30$).

Table 3. Time spent by *Philaenus spumarius* nymphs of two age groups (mean ± sd) was recorded in behavioural bioassays where an uninfested alfalfa plant was tested compared to an infested one. Two-way ANOVA was performed on log-transformed data, here reported as seconds (s).

Age group	Treatment	n	mean (s)		sd	
N1-N3	Uninfested plant	16	367.50	±	198.24	
	Infested plant	24	310.00	±	206.08	
	Overall N1-N3	40	338.75	±	202.16	a
N4-N5	Uninfested plant	21	268.57	±	183.58	
	Infested plant	19	470.53	±	234.32	
	Overall N4-N5	40	369.55	±	208.95	b
Overall control		37	318.04	±	190.91	a
Overall uninfested plant		43	390.26	±	220.20	b

Two-way ANOVA transformed data log10(x); different letters refer to significant differences between main effects.

Discussion

The gross morphology of the antenna of *P. spumarius* nymphs is somewhat different from that of the adults, although the sensory equipment appears similar. The significant difference consists in the overall shape of the antenna and particularly in the appearance of the flagellum. Nymph antennae are segmented, and formed by eight flagellomeres, while adults have a single elongated antennomere (Ranieri *et al*., 2016). Other species of the Cercopoidea superfamily show this dissimilarity between juveniles and adults (Paladini *et al.*, 2008; Dmitriev, 2010), like, for instance, the Neotropical froghopper *Notozulia entreriana* (Berg, 1879) (Hemiptera: Cercopidae) (Foieri *et al.*, 2016).

It is well known that the sensory structures on the nymph antennae of hemimetabolous insects resemble those occurring in adults (Zacharuk and Shields, 1991). However, their number and arrangement can vary during their development, resulting in differences between juveniles and adults. In *P. spumarius* adults, sensilla are gathered on the proximal part of the flagellum, while in nymphs' sensory structures are distributed along the whole segment. Basiconic and campaniform sensilla have been observed on juvenile and adult antennae (Ranieri *et al.*, 2016).

Basiconic sensilla have been described mainly in Cercopoidea species (Fennah, 1985; Liang, 2001; Liang and Fletcher, 2002; Ranieri *et al*., 2016; Zhu *et al.*, 2019) and their features (e.g., shape, size and length) are used to separate some taxa, as well as to evaluate phylogenetic relationships (Fennah *et al*., 1948; Liang, 2001, Liang and Fletcher, 2002, Paladini *et al*., 2015). The number of basiconic

sensilla observed in *P. spumarius* nymphs appear to be greater than that found in adults, where only three basiconic sensilla were spotted on the expanded base of the flagellum (Ranieri *et al*., 2016). These pegs have a porous cuticular wall and highly branched dendrites, so they were considered olfactory sensilla, as evidenced by other Auchenorrhyncha species (Rossi Stacconi and Romani, 2012). In general, sensory structures with a peg shape are chemoreceptors involved in perceiving various stimuli, including olfactory and gustatory signals (Schneider and Steinbrecht, 1968; Keil and Steinbrecht, 1984; Zacharuck and Shields, 1991). Our preliminary observations didn't allow us to appreciate the fine structure of the basiconic sensilla found on the pedicel and the flagellum of the nymphs' antennae. However, an olfactory function could be hypothesized since olfactory pegs also occur in adults' antennae. On the other hand, the two basiconic sensilla observed on the tip of nymphs' antennae appear uniporous, so that a gustatory function could be suggested.

Two types of coeloconic sensilla were observed on the antenna of *P. spumarius* adults: single-walled coeloconic sensilla and double-walled coeloconic sensilla (Ranieri *et al*., 2016). The former are aporous sensilla, probably associated with detecting environmental temperature and moisture. In contrast, double-walled coeloconic sensilla belong to the group of multiporous peg sensilla and are deemed either thermo-/chemoreceptors, thermo-/hygroreceptors and, more frequently, olfactory receptors (Altner *et al*., 1977; Keil and Steinbrecht, 1984; Pophof, 1997). The sensory cavities observed in the antenna of *P. spumarius* nymph resemble the single-walled coeloconic sensilla of the adult, but further observations are needed to clarify the internal structures contained in these pits. However, coeloconic sensilla have been observed in other Cercopoidea nymphs (Foieri *et al*., 2016a; Foieri *et al*., 2016b), and the presence of sensory structure with possible thermo-hygroreceptive function on the antenna of *P. spumarius* nymphs appear to be plausible since the specific environmental requirement of these insects (Cornara *et al.*, 2018).

Campaniform sensilla are mechanoreceptors and frequently occur near segmental junctions (Keil and Steinbrecht, 1984; Zacharuck, 1985). They are involved in proprioception since they monitor the position of the insect body parts to gravity, to each other or the environment (D'Urso and Chiarenza, 2008). Campaniform sensory structures are usually present in a few numbers in Cercopoidea, and several froghopper species exhibit a single campaniform sensillum on their pedicel (Liang, 2001; Liang and Fletcher, 2002; Liang *et al*., 2012). Differently from *P. spumarius* adults, which display the presence of mechanosensitive sensilla on the pedicel, where the Johnston's organ occurs on the flagellum, nymphs show a single campaniform sensillum on the pedicel.

Our preliminary observation of the antennae of *P. spumarius* nymphs enabled us to identify potential olfactory sensilla (mainly basiconic sensilla) and to hypothesize the ability of juveniles to perceive olfactory stimuli.

In type 1 bioassay, nymphs were exposed to volatiles emitted by uninfested alfalfa plants. In these trials, *P. spumarius* individuals of all ages spent significantly more time choosing the plant than the time employed to select the control arm, suggesting that the odours from this source were likely scanned before being chosen. Again, in the second experiment, when volatiles of infested and uninfested plants was compared, nymphs showed different behaviour according to their age and the kind of assayed stimuli. Moreover, the observed variation in the time needed for choosing the odour stimuli appears consistent with the ethology of nymphs, which tend to aggregate in the early stages of their life (Halkka *et al*., 1977). Indeed, first-third instar nymphs (age group N1-N3) seemed to require less time to choose the infested plant, suggesting a possible quicker recognition of the pest-induced plant volatiles or of the spittle volatiles that could mediate both the host-seeking and the aggregation.

On the other hand, nymphs of the fourth and fifth instar seem to be faster in choosing an uninfested plant, in agreement with their tendency to avoid aggregation with conspecifics. Differences ascertained in the behaviour of nymphs suggest the ability of nymphs to perceive and maybe discriminate olfactory stimuli. However, they didn't display a clear, attractive or repellent response to those cues.

The active movement of an insect towards a potential source (of food, oviposition sites, refuges, mates) is called searching behaviour and is mediated by both internal and external stimuli (Bell, 1990). Sensory cues can be directional or non-directional, eliciting different behaviour in the receiver insect (Bell, 1990). Non-directional sensory cues usually inform the insect that a resource is present in its surrounding environment, but they do not enable the localization of that source (Bell, 1990; Webster and Cardé, 2017). So, the insect often keeps still, scanning the sensory cues with its receptors until the sensory stimulation reaches the threshold needed to elicit the local search (Bell, 1990). Whiffs of olfactory stimuli are considered non-directional cues, as a gradient in their concentration is necessary to orientate the insect (Bell, 1990). Since we set up our olfactometer bioassays using plants as sources of olfactory stimuli and we didn't control the quality and quantity of those cues, it could be considered that nymphs were exposed to odour puffs. So, the absence of a significant response of *P. spumarius* nymphs to the tested stimuli could be interpreted as a behaviour elicited by non-directional stimuli. Indeed, nymphs could have detected olfactory cues in the odd Y-tube, but they couldn't orientate themselves and choose the olfactometer arm bearing the triggering stimulus.

Furthermore, it could be possible that the directional movement of spittlebug nymphs relied on a combination of sensory stimuli to locate their host plants, for example, on olfactory together with visual cues, as evidenced for several Auchenorrhyncha species (Todd *et al*., 1990; Bullas-Appleton *et al*., 2004; Patt and Sétamou, 2007; Dietrich, 2009) and suggested for *P. spumarius* adults (Ranieri

et al., 2016). The importance of vision in spittlebugs seems to be also confirmed by morphological observations of *P. spumarius* compound eyes of both juveniles and adults, that show features consistent with a good sensitivity level (Keskinen and Meyer-Rochow, 2004). Since, in our bioassay, the sources of the stimuli were prevented, the behavioural response of *P. spumarius* nymphs might have been affected by the lack of visual stimuli. Finally, it was proved that herbivorous insects could rely on habitat odour cues to obtain general information on where feeding or oviposition sites could occur (Webster and Cardé, 2017). Among these stimuli, ubiquitous plant volatiles (e.g. Green Leaf Volatiles) could indicate heterogeneous vegetated areas and induce a non-directional local search for a host in some phytophagous insects (Webster and Cardé, 2017). Considering that *P. spumarius* might detect only ubiquitous plant volatiles, could reinforce the hypothesis that olfactory sensory structures on *P. spumarius* adults' antennae might be little specialized. As a matter of fact, the broad host range of this spittlebug species appears to be not correlated to the small number of chemoreceptors observed on the antenna (Ranieri *et al*., 2016). Hence, olfactory sensilla in *P. spumarius* nymphs might perceive ubiquitous odour cues from the habitat, eliciting a non-directional local search. These cues could be combined with other stimuli (e.g. visual stimuli) to locate and reach a specific host plant.

Our preliminary findings represent a first step in the study of the morphology of the antenna and the host-location mechanism in *P. spumarius* juveniles and other spittlebug species.

Further investigations are needed to describe the fine structure of the antenna and clarify the function of the sensory apparatus described here. Moreover, behavioural and physiological assays are required to understand the role of olfactory and visual stimuli in the search for a host. Elucidating the host-seeking process in both nymphs and adults of *P. spumarius* could provide essential tools to develop effective and sustainable control strategies against this pest.

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5. Response of spittlebug vectors of *Xylella fastidiosa* to different colour stimuli. Preliminary results

Personal contribution: Conceptualization, insect rearing, data collection, behavioural bioassays, data analysis.

Dott.ssa Marzia Cristiana Rosi[1] has performed the statistical analysis. Dott. Claudio Cantini[2] has funded experimental activities. Prof.ssa Patrizia Sacchetti[1] has collaborated on conceptualization and manuscript preparation.

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Background

In integrated pest management, monitoring insect pests is crucial to plan efficient and sustainable control programs (Cardim Ferreira Lima *et al*., 2020; Dent and Binks, 2020). Monitoring procedures should be easy to carry out, affordable, and reliable to maximize their application's benefits.

Until now, the European vectors of *Xylella fastidiosa*, namely spittlebugs (Hemiptera: Aphrophoridae) (Cornara *et al*., 2019), are mainly monitored using the sweeping net. However, the use of this tool is time-consuming and requires skilled operators. In addition, several studies have reported the inefficacy of sweeping net in sampling adult spittlebugs from tree canopies, for example, in olive groves (Morente *et al*., 2018). Moreover, although yellow traps were found to be attractive for the adults of the spittlebug *Philaenus spumarius* (Wilson and Shade, 1967), the use of traps in monitoring *X. fastidiosa* vectors have also been considered ineffective (Morente *et al*., 2018, Cornara *et al.*, 2018; Lester *et al.*, 2019; Albre *et al*., 2021). However, many factors could bias the efficacy of coloured sticky traps, like the size, shape, colour shade, and height at which the trap is positioned (Pinto-Zevallos and Vänninen, 2013).

Although the use of trap monitoring is considered unsuccessful for *P. spumarius,* vision in Auchenorrhyncha appears to be an essential sense involved in orientation (Moore *et al*., 1993) and in the search for the host (Todd *et al*., 1990; Bullas-Appleton *et al*., 2004; Patt and Sétamou, 2007; Zhang *et al*., 2018). Although colour vision in Auchenorrhyncha has been little studied, the available information suggests that these insects possess at least three types of photoreceptors sensitive to green, blue and UV wavelengths (van der Kooi *et al*., 2021). Since the lack of information on spittlebug's visual acuity, studying their response to specific wavelengths could help increase our knowledge of their ethology. Moreover, the possible existence of wavelength-specific behaviour might be exploited to improve monitoring and trapping methods.

In this work, the visual acuity of two European vectors of *X. fastidiosa*, *P. spumarius* and *Neophilaenus campestris* (Fallén, 1805), has been investigated both in the field and in laboratory trials.

Different colour sticky traps were tested in three different olive groves in Tuscany in the fall of 2020, and behavioural experiments were conducted in the laboratory to assess the response of spittlebug adults to different light wavelengths. Here we present the results obtained in these preliminary investigations.

Materials and Methods

Field trials - Attractiveness of different coloured sticky traps

Field trials were conducted in the fall of 2020 to assess the attractiveness of different colour sticky traps to *P. spumarius* and *N. campestris* adults.

The trials were conducted in olive groves in three different localities in Tuscany (Italy): Prato (Società Agricola Fabio), Pisa (Azienda Agricola Villa Filippo Berio) and Grosseto (CNR-IBE, Azienda Sperimentale di Santa Paolina). In each olive grove, seven differently coloured sticky traps were tested: transparent, white, yellow, green, red, blue, and brown. Coloured traps consisted of plastic alveolar polypropylene "plastonda" panels, while transparent traps were made of Poliver (artificial glass polystyrene); all of them measured 20 cm × 30 cm × 2.5 mm (width, height, and thickness, respectively) and were purchased at the home improvement retailer OBI Italia (Andreani *et al*., 2021). Both sides of the panels were coated with glue applied by brush (Planatol VP 1854 PSA, Ivog biotechnical systems GmbH, Neüsaß, Germany).

Two traps were installed for each colour at two different heights: 50 cm and 100 cm. Each chromotropic trap consists of two faces: southeast-oriented and northwest oriented. The traps were positioned in a single row, spaced 15 m between them.

The spectral reflectance of the panels in the visible and UV regions, between 250 and 800 nm, was measured using a PerkinElmer Lambda 1050 spectrophotometer coupled with a specific accessory for reflectance measurements (150 mm InGaAs Integrating Sphere) (Perkin Elmer Inc., Waltham, MA, USA). Measurements (Figure 1) were performed on panels covered with glue and protected by a transparent film to obtain assessments comparable to those of traps set in the field. The transparent panel showed nearly constant reflectance over the wavelength range from 250 to 800 nm, with a mean reflectance of about 17% (Figure 1). The white trap reflected most of the light (nearly 80%) over the wavelength range from 400 to 800 nm, with a very low reflectance in the UV region. The yellow panel exhibited a maximum reflectance of about 520–550 nm (approximately 70%), while the green 500-550 nm (20%). Red and blue traps showed peaks of 620–650 nm (55% of reflectance) and 420–470 nm (about 60% of reflectance), respectively. Finally, the brown panel displayed a maximum reflectance of about 610–630 (about 25% of reflectance).

Every week captured specimens were counted and sexed, and the trap colour order was shifted. At the same time as the trap counting, adult specimens were monitored by net sweeping to assess the spittlebug population's presence and density in the olive groves.

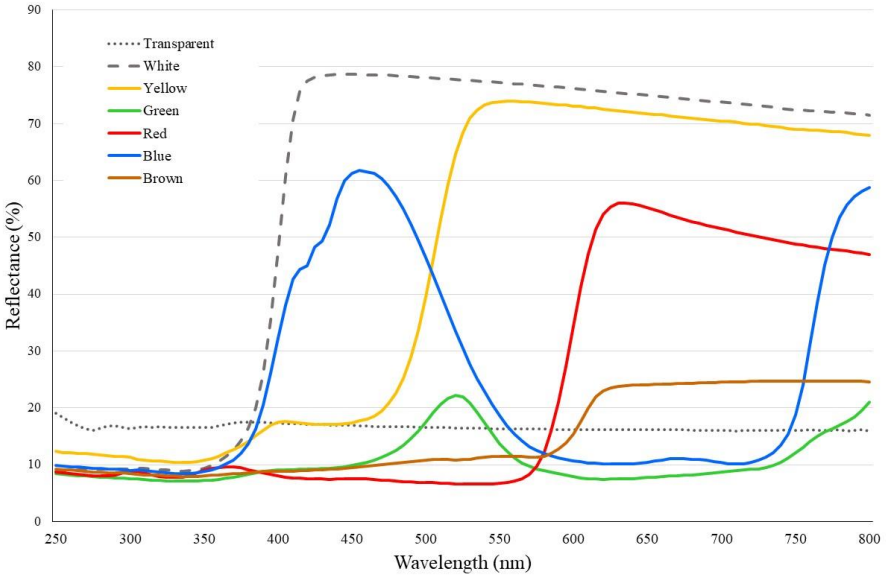

Figure 1. Reflectance spectra of the transparent (dotted line), white (dashed line), yellow (yellow line), green (green line), red (red line), blue (blue line), and brown (brown line), sticky traps used in field trials carried out in fall 2020 in three olive groves in Tuscany (Italy).

Laboratory trials - Attractiveness of different wavelengths

Experiments were conducted in late summer 2022 to study the behavioural response of *P. spumarius* and *N. campestris* adults to the light of different wavelengths. Insects were collected in the field using a sweeping net and transferred into the laboratory, where they were placed in Bugdorm© cages on *Avena sativa* plants. Males and females were kept together. Cages were maintained at room temperature (20±2°C) and under natural photoperiod. Spittlebugs were acclimated in the laboratory for at least 24 hours before being used in the assays. For the experiments, specimens were individually taken from the cage using a 1.5 mL microvial and sexed under a stereomicroscope. At the same time, taxonomical identification was confirmed using external morphological features (Holzinger *et al.*, 2003; Biedermann and Niedringhaus, 2009).

Experiments were conducted in a round choice box (30 cm in diameter) (Figure 1) inspired by Kecskeméti *et al.*, (2021). The box was divided into four identical sectors isolated from each other. The central unit of the choice box consisted of a cubic obscured chamber equipped with four vials, each entering its box sector. On the roof of the central unit, an opening allowed the entry of the tested spittlebug. The choice-box interior was entirely lined with aluminium foil. Light stimuli were provided using a mini-LED diode (internal diameter 5 mm) emitting different wavelengths: white (3300-5300 K), green (500-570 nm), blue (450-500 nm) and ultraviolet (300-395 nm). The mini-LED diodes were inserted in the box's lid, so each diode illuminated one box's sector. The box's cap could be rotated to change the position of the light stimuli.

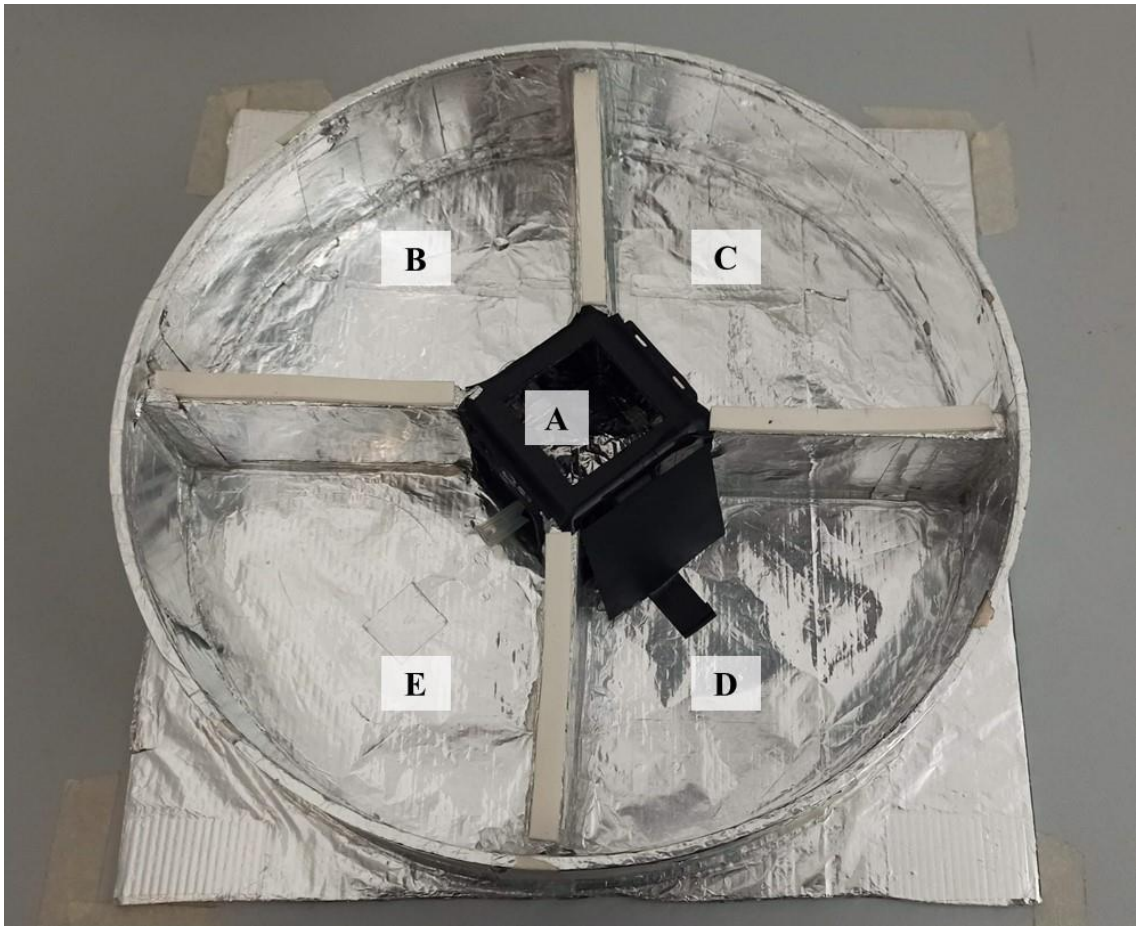

Figure 2. Choice box used for behavioural assays to evaluate the response of *P. spumarius* and *N. campestris* adults to different light wavelengths. A: central unit; B-E: choice-box's sectors. The lid of the choice box is not taken in the picture.

Trials were performed in a dark room, where a temperature of 22°C was set to equal the condition where insects were reared. Before the assays, each insect was dark-adapted for 3 minutes. The bioassay started when a single individual was gently placed in the central unit and left free to move inside the box, covered by its lid, for 7 minutes. After this period, the cap was removed, and the insect choice was annotated. Spittlebug adults were used only once and then discarded.

Three types of bioassays were conducted:

- Type 1: only one sector of the choice box was illuminated with white light;
- Type 2: green, blue and UV lights were compared. Three out of four box sectors were illuminated with the tested stimuli;
- Type 3: green and blue lights were compared. Two out of four box sectors were illuminated with the tested stimuli;

The mutual position of the tested wavelengths did not change during the trials, but the position of the stimuli in the box's sectors was rotated every ten bioassays.

Statistical analysis

A univariate ANOVA analysis compared the average numbers of male and female spittlebugs caught by different colour sticky traps.

Data from behavioural assays were analyzed by performing a two-sample test for equality of proportions with continuity correction. Only for bioassay type 1 males' and females' responses were considered together.

Statistical analyses were conducted using the R software (R Core Team 2020, version 4.0.5).

Results and Discussion

Field trials - Attractiveness of different coloured sticky traps

Only in the sampling site in Pisa, all the traps were active, even if the number of caught insects was low. Moreover, almost all the collected spittlebugs were *P. spumarius* adults. For these reasons, the statistical analysis was performed only for the Pisa olive grove, and *P. spumarius* captures.

Yellow traps captured significantly more males than the other colours and more females than red and white traps. These results are partially consistent with those reported in one previous study concerning colour attractiveness in *P. spumarius* (Wilson and Shade, 1967).

Table 1. Results of the univariate ANOVA analysis ($p < 0.05$)

Sex	Treatments	Mean squared	df	F	*p*-value
Male	Colour	0.048	6	3.82	0.00
	Height	0.000	1	0.01	0.91
	Colour x Height	0.006	6	0.27	0.95
Female	Colour	0.005	6	2.97	0.01
	Height	0.003	1	2.09	0.15
	Colour x Height	0.001	6	0.76	0.61

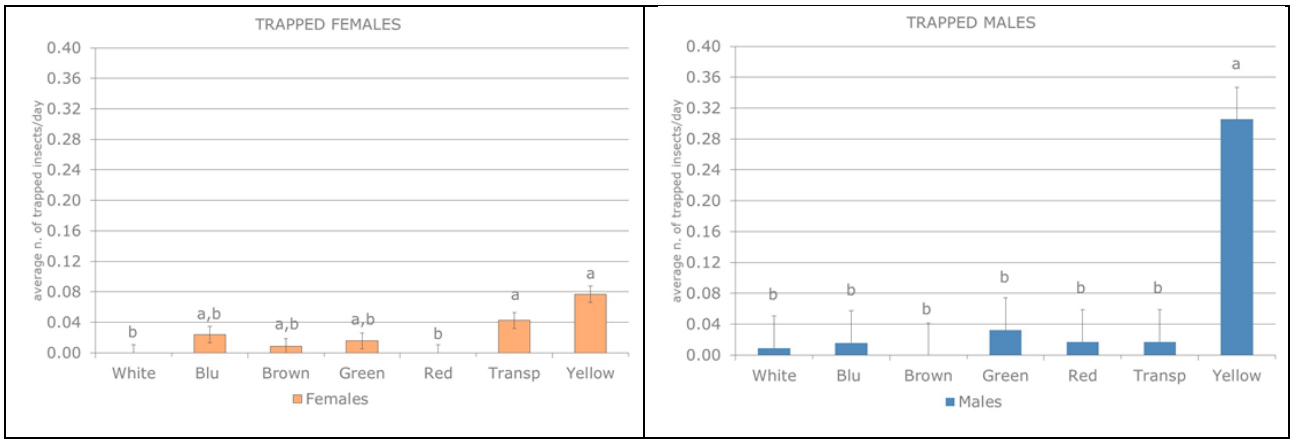

Figure 3. Average number of trapped females/males per day

The average number of *P. spumarius* adults monitored in Pisa by net sweeping and caught by yellow traps show a moderate negative correlation (correlation coefficients: -0.53). These results need further investigation to explore the efficiency of different sampling methods, the sampling time and the insect life cycle.

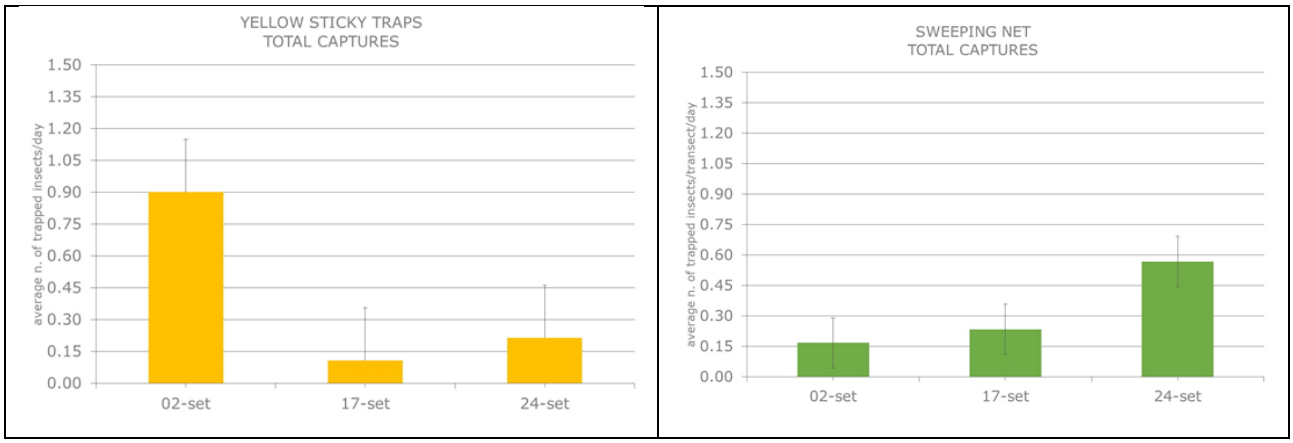

Figure 4. Comparison of the average number of trapped/swept insects daily.

Laboratory trials - Attractiveness of different wavelengths

Philaenus spumarius – In total, 40 adults of *P. spumarius* were tested for the type 1 bioassay (26 females and 14 males). The average response rate was 75%. Considering the two sexes separately, males showed a greater response rate (85.71%) than females (69.23%).

Results evidenced a clear positive phototaxy towards the white light, with no insects entering the dark sectors (Tab. 2).

Table 2 Response of *Philaenus spumarius* adults to white light in choice-box bioassays. Males and females were summed together (two-sample test for equality of proportions with continuity correction)

Chosen sector	number of adults	%	χ^2	*p*-value
White light	30	75.00	18.05	***p*<0.05**
Dark sectors	0	0		
No choice	10	25.00		

The total amount of *P. spumarius* adults tested for the type 2 bioassay was 73 (48 females and 25 males). The response rate for females was 72.92%, while for males was 76%. Results (Tab. 3) suggest that *P. spumarius* adults could perceive and discriminate the tested stimuli, like many phytophagous insects with a basic set of three photoreceptors sensitive to green, blue and UV light. Again, no insects entered into dark sectors.

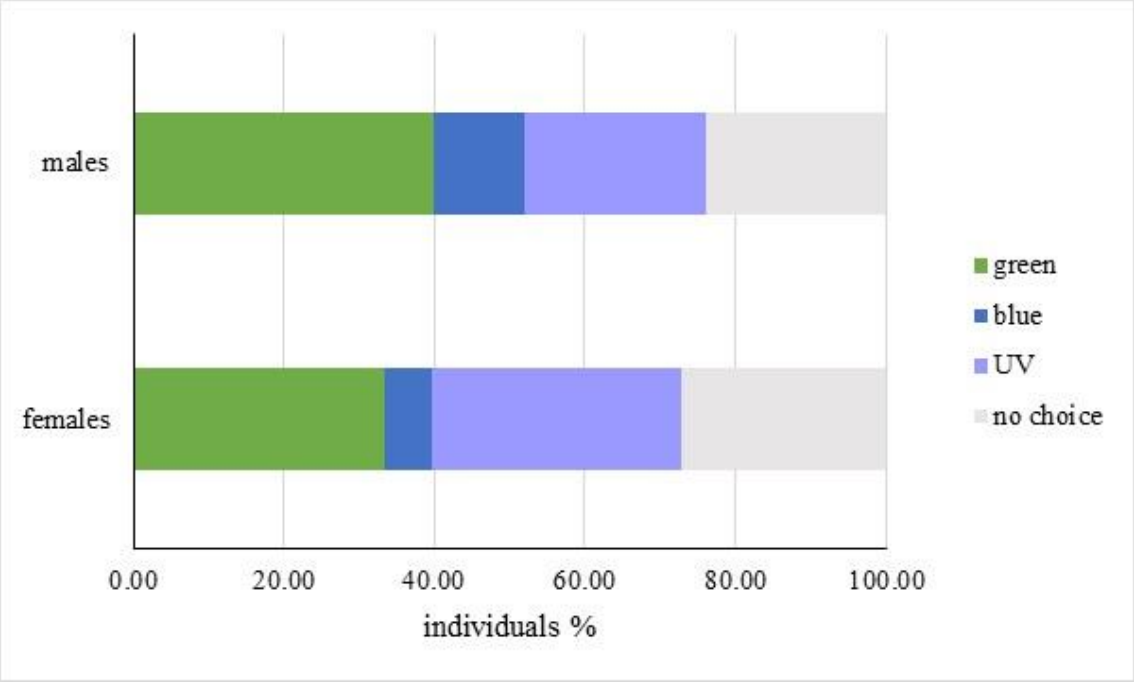

Figure 5. Response of *Philaenus spumarius* males and females (% over the total number of tested individuals per sex) to green, blue and UV light in choice-box bioassays.

Based on our results, *P. spumarius* females displayed a different behaviour to the light, suggesting the tested individuals' wavelength-specific response. Indeed, statistical analysis showed that blue light was significantly less chosen by *P. spumarius* females, while the response to UV and green lights was almost equivalent. Such differences were not evidenced in males, even if the blue light was the less chosen stimulus.

Table 3. Response of *Philaenus spumarius* females and males to green, blue and UV light in choice-box bioassays (two-sample test for equality of proportions with continuity correction)

Chosen sector	number of adults	%	χ^2	*p*-value
Females			12.667	**p<0.05**
Green light	16	33.33		
Blue light	3	6.25		
UV light	16	33.33		
Dark sector	0	0		
No choice	13	27.08		
Males			5.842	p>0.05
Green light	10	40		
Blue light	3	12		
UV light	6	24		
Dark sector	0	0		
No choice	6	24		

In the type 3 bioassay, 92 adults of *P. spumarius* (52 females and 40 males) were tested. The total response rate was 67.31% for females, while males responded at a higher percentage (77.50%).
Both males and females significantly responded to the green light, suggesting a preference for this light wavelength (Tab. 4). The absence of UV light from the set of tested stimuli affects the behaviour of *P. spumarius* adults, increasing the response to both green and blue light. This variation was particularly evident for females that responded to the blue light, increasing nearly four times their rate regarding bioassay type 2 (from 6.25% to 23.07%). In contrast, the response to green light recorded a minor increase, reaching 44.23%.

Neophilaenus campestris – In the type 1 bioassay, 63 adults of *N. campestris* (38 females and 25 males) were tested, with 53.9% of the total response rate. Considering the two sexes separately, males showed a greater response rate (60%) than females (50%).
Although more than half of the individuals made a choice, the statistical analysis did not show a clear behavioural response to white light (Tab. 5).
Results suggested that *N. campestris* adults could perceive white light since no insects entered the dark sectors, but this stimulus did not elicit a specific behavioural response.

Table 4. Response of *Philaenus spumarius* females and males to green and blue light in choice-box bioassays (two-sample test for equality of proportions with continuity correction)

Chosen sector	**numbers of adults**	**%**	χ^2	***p*-value**
Females			5.714	**p<0.05**
Green light	23	44.23		
Blue light	12	23.07		
Dark sector	0	0		
No choice	17	32.69		
Males			9.290	**p<0.05**
Green light	22	55.00		
Blue light	9	22.50		
Dark sector	0	0		
No choice	9	22.50		

Table 5. Response of *Neophilaenus campestris* adults to white light in choice-box bioassays. Males and females were considered together (two-sample test for equality of proportions with continuity correction)

Chosen sector	**Number of adults**	**%**	χ^2	***p*-value**
White light	34	53.97	0.508	$p>0.05$
Dark sectors	0	0		
No choice	29	46.03		

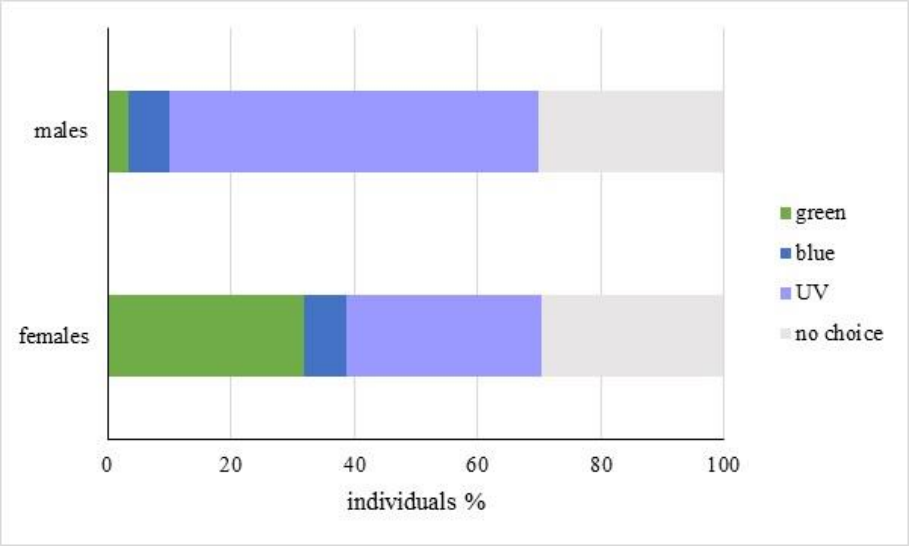

Figure 6. Response of *Neophilaenus campestris* males and females (% over the total number of tested individuals per sex) to green, blue and UV light in choice-box bioassays.

In the type 2 bioassay, 74 adults of *N. campestris* (44 females and 30 males) were tested with a total response rate of 70.45% for females and 70% for males.

Adults of *N. campestris* appeared to perceive and discriminate the tested stimuli, as observed for *P. spumarius*. Again, no insects entered the dark sectors.

N. campestris males and females significantly chose the blue light. Females did not clearly prefer UV or green light, responding equally to both stimuli. At the same time, males especially reacted positively to UV light, so it resulted as the most chosen stimulus.

Table 6 Response of *Neophilaenus campestris* females and males to green, blue and UV light in choice-box bioassays (two-sample test for equality of proportions with continuity correction)

Chosen sector	Number of adults	%	χ^2	*p*-value
Females			11,71	***p*<0.05**
Green light	14	31.82		
Blue light	3	6.82		
UV light	14	31.82		
Dark sector	0	0		
No choice	13	29.55		
Males			39	***p*<0.05**
Green light	1	3.33		
Blue light	2	6.67		
UV light	18	60		
Dark sector	0	0		
No choice	9	30		

In the type 3 bioassay, 98 adults of *N. campestris* were tested (44 females and 54 males), with a total response rate of 56.82% for females and 66.67% for males.

Both males and females significantly responded to the green light, attesting to a preference for the spittlebug species for this stimulus. However, the absence of UV light in the set of tested stimuli modified the behaviour of *N. campestris* adults since they displayed some differences in choices made in the type 2 bioassay. Firstly, the number of females that did not choose stimuli increased from 29.55% (bioassay type 2) to 43.18%. Moreover, the response to green light grew in both sexes, with

a more remarkable increase in males (from 3.33% up to 44.44%). Finally, males' response to blue light increased when UV light was absent from the tested stimuli.

Table 7. Response of *Neophilaenus campestris* females and males to green and blue light in choice-box bioassays (two-sample test for equality of proportions with continuity correction).

Chosen sector	Number of adults	%	χ^2	*p*-value
Females			20.48	***p*<0.05**
Green light	21	47.73		
Blue light	4	9.09		
Dark sector	0	0		
No choice	19	43.18		
Males			6.72	***p*<0.05**
Green light	24	44.44		
Blue light	12	22.22		
Dark sector	0	0		
No choice	18	33.33		

Conclusion

Results obtained in laboratory trials highlighted several differences in the visual behaviour of *P. spumarius* and *N. campestris*. Interesting variations occurred between the two spittlebugs and intra-specifically between males and females. Both species showed the ability to perceive and discriminate different wavelengths. Similarly, to many phytophagous insects, these spittlebugs could have at least three types of photoreceptors, sensitive to green, blue and UV light (van der Kooi *et al.*, 2021). Our results seem to confirm this assumption and also suggest wavelength-specific behaviours.

P. spumarius adults showed a more remarkable positive phototaxy than *N. campestris,* especially when white light was tested. However, phototaxy can be affected by the wavelength and intensity of light and dramatically varies among species (Shimoda and Honda, 2013). Since the tested stimuli were the same for the two species and their intensity was not modified during the trials, experiment parameters might not have been sufficient to elicit a more unequivocal response to visual stimuli in *N. campestris*.

UV light is an important stimulus in both species, as monochromatic light combines green and blue wavelengths. *N. campestris* males seem attracted by UV light, while females of both species have

interestingly shown a very similar behavioural pattern, choosing green and UV light equally. Insects seem to rely on UV light to orientate themselves, exploiting the contrast between the significant amount of UV emitted by the sky and the poor reflection of UV by the earth's surface (Wehner, 1981). Furthermore, insects could contrast green and UV light or blue and UV light to discriminate terrestrial objects from celestial ones (Möller, 2002).

The combination between UV, green and blue light appears to be essential also in spittlebugs since their behaviour has changed in trials where UV stimuli were absent.

The reflectance of leaves ranges between 500-600 nm, so phytophagous insects are generally attracted by yellow colour (Prokopy and Owens, 1983). Outcomes from this study highlighted an attractive response to the green light in both *P. spumarius* and *N. campestris*. For the former, results obtained in laboratory experiments are consistent with those obtained in the field trial, where yellow traps (maximum reflectance of 520–550 nm) captured significantly more specimens than the other colours. In particular, yellow traps caught considerably more males than all the tested colours. Similarly, in laboratory trials, *P. spumarius* males responded strongly to green light in bioassays types 2 and 3. Differently, yellow panels caught significantly more females than red and white panels but less than green and blue ones, suggesting a different wavelength-dependent response of *P. spumarius* females. A similar pattern was observed in type 2 bioassays, where *P. spumarius* females did not prefer the green light.

In conclusion, our results represent a step in studying the visual acuity of spittlebug insects. To the best of our knowledge this is the first attempt to investigate spittlebug wavelength-dependent behaviour using behavioural assays under laboratory conditions. Observations in these preliminary trials suggest an essential role of visual stimuli in the conduct of spittlebug adults, for example, in the orientation during flight or in search of host plants. Moreover, the remarkable phototaxy evidenced in laboratory trials could be exploited to develop a more effective trapping strategy for monitoring spittlebugs in integrated pest management. Further studies are needed to improve these preliminary observations and clarify whether a specific wavelength-dependent behaviour operates in spittlebug adults.

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Conclusion

The main aim of this dissertation has been to collect data on the biology and the ecology of spittlebugs vector of *Xylella fastidiosa* subsp. *multiplex*.

This work achieved the following positive results:

1 Collection of data on biology and ecology of spittlebugs vectors of *Xylella fastidiosa* subsp *multiplex* in Mediterranean environments: this work has allowed the supply of a large amount of data on spittlebugs considered as putative vectors of the quarantine bacterium *X. fastidiosa*. Our results contribute to the general knowledge of European Aphrophoridae and provide helpful information that could be successfully applied in managing the *X. fastidiosa* outbreaks in the Mediterranean basin. Outcomes from this work highlighted the necessity of further investigations to clarify the role of *P. spumarius* and *N. campestris* in transmitting *X. fastidiosa* subsp. *multiplex*. Moreover, other hypotheses have been presented and discussed. A study on the association between *S. junceum* and *R. alaternus,* and Auchenorrhyncha fauna has been planned to unveil which vectors might be responsible for transmitting *X. fastidiosa* to these two plant species.

2 Experimental tests on the antipredatory effect of the froth of *Philaenus spumarius*: we provided evidence that spittlebugs froth could play a role as multifaceted protection against generalist predators beyond protecting the nymph from thermal stress and dehydration. While its frothy state might represent a physical hindrance, hiding the nymphs from visual predators like spiders, its chemical composition could prevent detection by predators that rely most on olfactory stimuli, such as ants. Regarding the chemical effect of the froth, our results suggest that froth could contain specific odour-masking substances that hide nymphs from predators rather than acting as a proper deterrent. Further studies on the chemical composition of the froth are needed to clarify the role of this self-produced biofoam in the chemical mimicry of spittlebug nymphs.

3 Diversity of the bacterial community associated with hindgut, Malpighian tubules, and foam of nymphs of two spittlebug species (Hemiptera: Aphrophoridae): in conclusion, our study has allowed a preliminary exploration of the complex bacterial community associated with the gut, the Malpighian tubules and the foam of the nymphs of two Aphrophoridae species also giving the basis for further and more deepened investigations aimed at improving the

knowledge on this topic and developing effective and more sustainable control strategies against spittlebug vectors of *X. fastidiosa*.

A deeper investigation of the microbial community associated with the gut of spittlebug nymphs and adults and the foam has been planned to apply NGS analysis. Moreover, microscopy studies will be continued to point out the exact localization of putative symbionts and shed light on the role of these microorganisms in the spittlebugs' physiology.

4 Gross antennal morphology of Philaenus spumarius juveniles and behavioural response to olfactory plant cues: our preliminary findings represent a first step in the study of the morphology of the antenna and the host-location mechanism in *P. spumarius* and other spittlebugs species. Further investigations are needed to describe the fine structure of the antenna and clarify the function of the sensory structures observed here. Moreover, behavioural and physiological assays are required to understand the role of olfactory and visual stimuli in the search for a host. Elucidate the host-seeking process in both nymphs and adults of *P. spumarius* could provide essential tools to develop effective and sustainable control strategies against this pest.

5 Response of spittlebug vectors of *Xylella fastidiosa* to different colour stimuli. Preliminary results*:* our results provided the first accounts of the reaction of spittlebugs to visual stimuli. Such stimuli could be involved in many aspects of the behaviour of spittlebugs, nymphs and adults. Field trials and laboratory bioassays enabled promising results showing differences between *P. spumarius* and *N. campestris* and wavelength-specific responses in males and females. The knowledge of the sight behaviour of spittlebugs vectors of *X. fastidiosa* could be successfully applied to develop effective monitoring and control strategies.

List of publications

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