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# Temporal dynamics of *Xylella fastidiosa* subsp. *pauca* vector transmission to olive plants

# *Xylella* transmission by the meadow spittlebug (short title)

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# Abstract

The spittlebug *Philaenus spumarius* L. (Hemiptera: Aphrophoridae) is the predominant vector of *Xylella fastidiosa* Wells et al. (Xanthomonadales: *Xanthomonadaceae*) (*Xf*) to olive trees in the Apulia Region of Italy. Previous studies focused on assessing *Xf* transmission competence by spittlebugs and the natural infectivity of the *P. spumarius* populations. However, factors influencing *Xf* transmission by *P. spumarius* are largely unknown and these knowledge gaps hamper the comprehension of the epidemiology of *Xf*-associated emerging diseases. We performed two sets of experiments to study transmission biology of *Xf* by *P. spumarius* aimed at understanding the kinetics of the bacterial persistence, transmission efficiency and the spread rate of *Xf* among olive trees in summer and autumn. The results show that i) *P. spumarius* is a competent *Xf* vector to olive throughout its adult life, ii) bacterial load in the vector foregut increases during the first 2-3 weeks after acquisition and then becomes stable, iii) transmission rates may significantly vary during the year and under different climatic conditions, and iv) differential survival of vectors — influenced by insect age, season and climatic conditions — may affect the spread of *Xf* in olive plants. Transmission parameters of *Xf* by *P. spumarius* obtained here will improve the modelling of the pathogen spread, by explicitly incorporating the effect of insect vectors, and the designing of effective control and prevention measures against this vector-borne disease.



# Keywords

vector-borne disease, vector bacterial load, Olive Quick Decline Syndrome, OQDS, Aphrophoridae, spittlebugs, non circulative-persistent transmission.

# Introduction

Transmission biology is of major importance in outlining disease epidemiology of insect-borne plant pathogens (Jeger et al. 1998; Daugherty and Almeida 2009), and its detailed knowledge is a key to elaborate effective disease management (Almeida et al. 2005). Transmission of pathogens by insect vectors is a complex process characterized by several steps and actors, that involve different areas of knowledge, including molecular biology, physics, physiology, and ecology (Killiny et al. 2009; Sicard et al. 2018; Ranieri et al. 2020; Backus and Shih 2020). Ultimately, the most fundamental characteristic of vector transmission – especially from a disease control perspective – is efficiency or competence, i.e. how often a vector transmits a pathogen over time or per transmission opportunity (Purcell and Almeida 2005). Transmission efficiency of persistent plant pathogens by insect vectors may significantly vary over time after acquisition, and it is a process influenced by the host condition and environmental factors (Anhalt and Almeida 2008; Daugherty et al. 2009; Ghanim 2014; Daugherty and Almeida 2019). Nonetheless, epidemiological models rarely explicitly include these aspects, often because of the lack of reliable estimates of key parameters of vector transmission of pathogens. These limitation lead biases in the outcomes and predictions of disease dynamics models (Jeger 2000; Allen et al. 2019).

The meadow spittlebug, *Philaenus spumarius* L. (Hemiptera: Aphrophoridae), is the key vector of the exotic bacterium *Xylella fastidiosa* Wells et al. (Xanthomonadales: *Xanthomonadaceae*) (*Xf* hereafter) in Italy and Europe (Cornara et al. 2018, 2019). *Xf* subsp. *pauca* is associated to the most severe *Xf*-epidemic currently affecting Europe, being the causal agent of the olive quick decline syndrome (OQDS) in Apulia (South Italy) (Saponari et al. 2019). In this region, this introduced bacterium encountered large populations of a previously neglected xylem-sap feeder

insect, *P. spumarius*, and a landscape dominated by susceptible olive plants leading to a dramatic epidemic that wiped away in less than one decade the olive production in the infected area (Saponari et al. 2019; Schneider et al. 2020). Ongoing research on *Xf* in Europe has revealed that the *P. spumarius* i) is the dominant xylem-sap feeder in olive agroecosystem and Mediterranean region (Morente et al. 2018; Antonatos et al. 2019; Bodino et al. 2019), ii) adults are found naturally infected with *Xf* in olive groves during summer and autumn (Cornara et al. 2017b; Cavalieri et al. 2019), and iii) is able to transmit the bacterium from olive-to-olive and from other plants to olive in laboratory trials (Cornara et al. 2017a, 2020; Cavalieri et al. 2019).

Current knowledge on *Xf* transmission biology comes from American pathosystems – e.g. diseases of grapevine and citrus – where sharpshooters (Hemiptera: Cicadellidae: Cicadellinae) represent the main group of vectors (Redak et al. 2004; Esteves et al. 2018; Cornara et al. 2019). Once acquired through feeding in xylem vessels colonized by *Xf*, bacterial cells multiply in the foregut of the insect vector and are persistently transmitted in a noncirculative manner, with no transtadial or transovarial passage and no apparent latent period (Severin 1950; Freitag 1951; Purcell and Finlay 1979). Acquisition and inoculation efficiency of *Xf* may vary greatly according to several factors — e.g. vector species (Daugherty and Almeida 2009; Marucci et al. 2008), source or recipient plant species and tissue, conditions of the host plants (water stress, disease symptoms) (Daugherty et al. 2011; Krugner et al. 2012; Krugner and Backus 2014), season and climate conditions (Daugherty et al. 2009, 2017) — shaping vectors' feeding preferences and acquisition rates (Almeida et al. 2005; Gruber and Daugherty 2013).

Despite its importance in affecting epidemics spread and severity, several aspects of the transmission biology of *Xf* by *P. spumarius* on olive trees have not been investigated, since the previous studies on the Apulian pathosystem focused on assessing the transmission capability of

spittlebugs. In addition, some information inferred from American pathosystems may not be directly transferable to the Italian olive disease, given the differences (e.g. vector species, *Xf* genotypes, host plants) that affect transmission parameters and inoculation efficiency (e.g. Almeida et al. 2005; Esteves et al. 2018). A better knowledge on *Xf* transmission parameters of European vectors is therefore necessary, allowing epidemic models to take into account pathogen transmission dynamics, so far not explicitly incorporated in any of the proposed models of *Xf* spread in Europe (White et al. 2017, 2020; Strona et al. 2020).

The aim of this work was to study *Xf* subsp. *pauca* kinetics in *P. spumarius* over time and *Xf* transmission efficiency by *P. spumarius* to olive trees throughout the year. We investigated how acquisition, bacterial load, and transmission efficiency are affected by season, climatic conditions, and the duration of plant exposure to infectious vectors. More specifically, we carried out experiments at different times of the year to determine i) *Xf* persistence, population size in the foregut of *P. spumarius*, ii) pathogen transmission success to olive seedlings after different periods post acquisition, and iii) *Xf* spread by infected spittlebugs within an experimental population of olive seedlings under different climatic conditions.

# Materials and Methods

## Insects and plants

*P. spumarius* adults were field collected in dry meadows and olive groves located in *Xf*-free areas of Apulia (southern Italy) one to two weeks prior to the onset of each experimental assay, and maintained in mesh and plastic fabric cages (Bugdorm: 75x75x115 cm) containing potted host plants (*Medicago sativa*, *Vitis vinifera*, *Sonchus oleraceus*, *Vicia faba*, *Erigeron* spp.). The cages were placed in a climatic chamber (24.7 ± 1.0 °C, 72.1 ± 7.2% RH) in summer and at a shaded

location during autumn in the nursery “Li Foggi” of ARIF (Agenzia Regionale Attività Irrigue e Forestali) (Gallipoli, province of Lecce). The air temperature and relative humidity were recorded hourly in both conditions using data loggers (HOBO U23-002; Onset Computer, Bourne, MA, USA). Adult mortality was low (5–10% of total insects collected). To confirm the *Xf*-free status of the reared spittlebugs, before starting each acquisition assays, 20-30 individuals were collected and individually tested by real time PCR (qPCR). No positive individuals were detected.

Recipient olive plants used for all experiments consisted of certified pathogen-free olive seedlings produced and maintained in the facilities of the Premultiplication Center at CRSFA. Seedlings of about 20–30 cm in height (6–8 months old) with active growing apexes were selected and transferred at the nursery “Li Foggi” of ARIF just before starting each experiment.

## Kinetics of *Xf* colonization of *P. spumarius* and transmission efficiency

Olive-to-olive transmission efficiency, *Xf* retention and multiplication in *P. spumarius* adults were tested in four separate assays carried out in 2017-2018 in different seasons, allowing us to investigate the effects of time after pathogen acquisition access period (hereafter AAP), season (summer: late June-July; autumn: late September-November) and insect age on the transmission parameters. Acquisition of *Xf* by *P. spumarius* adults was performed by caging the insects on branches of naturally infected olive trees (4–5 years old, cv. “Cellina di Nardò”) located in olive groves in the municipalities of Gallipoli and Parabita (province of Lecce). Branches suitable for acquisition were first tested by qPCR (approx. two weeks before the AAP) to assess for the presence and abundance of *Xf* in young flushes. Source plants hosted bacterial populations ranging from a median value of $8.99 \times 10^5$ (IQR: $1.41 \times 10^5$ – $1.99 \times 10^6$) CFU/g in summer to $1.88 \times 10^5$ (IQR: $1.22 \times 10^5$ – $2.41 \times 10^5$) CFU/g in autumn. Considering separately young and mature leaves from the branches selected for acquisition, the median bacterial populations ranged

from of $2.06 \times 10^5$ (IQR: $7.69 \times 10^4 – 9.15 \times 10^5$) CFU/g of tissue in young leaves to $1.5 \times 10^6$ (IQR: $5.06 \times 10^5 – 3.98 \times 10^6$) in mature leaves. *Philaenus spumarius* adults from maintenance cages were isolated — enclosed in mesh sleeves — on a total of 10–12 *Xf*-positive branches of 6–12 field-grown olives for an AAP of three days (72h), in groups of 50–100 individuals per branch.

At the end of the AAP spittlebug cohorts were mixed and transferred to a post-AAP maintenance period in five new cages containing potted non-host plants of *Xf* subsp. *pauca*, according to EFSA (2018) (Bugdorm: 75x75x115 cm and 45x45x90; *V. vinifera*, *S. oleraceus*, *V. faba*, *Pistacia lentiscus* and *Vicia sativa*). Inoculation assays were carried out using insects randomly collected from maintenance cages at different timepoints after the AAP (Supplementary Tab. 1) and transferred onto testing plants (i.e. olive seedlings) for an inoculation access period (hereafter IAP) of three days (72 h). Each replica consisted of one potted olive seedling enclosed in a mesh sleeve, on which five *P. spumarius* individuals were confined, thus having access to the entire plant during IAP. Five replicas at each time point after AAP were carried out, for a total of 25 insects used for each inoculation assay. At some time points post-AAP insects were collected from maintenance cages and stored directly in ethanol, for qPCR estimates of bacterial load (see Supplementary Tab. 1). AAP and IAP duration (72h each) were chosen on the basis of both preliminary trials and previous studies (Cavalieri et al. 2019) in order to maximize transmission efficiency and insect survival. Both IAP assays and post-AAP rearings took place in the climatic chamber located at the above-mentioned nursery “Li Foggi” at 24.7±1.0 °C and 72.1±7.2 % RH in summer and 20.8±1.0 °C and 77.9±7.6 % RH in autumn. At the end of IAP, both live and dead insects were collected, and singly stored in ethanol 90% at −20 °C. Insect survival rate was recorded for each AAP and IAP assay. Recipient plants were treated with a systemic insecticide

158 (Imidacloprid, Confidor 200 SL) and kept in an insect-proof screenhouse. Olive test plants were
159 analysed for *Xf* both at 6 and 12 months after the experimental assays. Plants testing positive at
160 the first assay (6 months) were always positive at the second assay (12 months).

## 161 Spread of *Xf* by *P. spumarius* under microcosm conditions

162 Experimental assays in microcosms were carried out to assess influence of i) IAP duration, ii)
163 climatic conditions (semi-field vs controlled), and iii) season on the spread of *Xf*-infection within
164 an olive seedling population. The experiments were conducted in the same periods of the
165 transmission kinetic assays, i.e. summer (July) and autumn (September-October) for two
166 consecutive years (2017-2018). *Xf* acquisition by *P. spumarius* adults followed the same
167 procedure described above. At the end of 72h AAP, insects were collected from olive branches
168 and randomly transferred into mesh cages with wood frame (microcosms) (93 × 47.5 × 47.5 cm)
169 containing 16 olive seedlings each, positioned at regular spacing. In each microcosm 17
170 individuals were introduced in 2017 assays, and 32 individuals in 2018 assays. Spittlebugs were
171 released in the centre of the microcosm with a falcon tube, gently shaken until all insects left the
172 tube. To test the effect of climatic conditions, one set of cages (n = 12) was set up in controlled
173 climatic conditions (summer: 24.9 ± 1.9 °C, 74.5 ± 7.8% RH; autumn 22.4 ± 0.7 °C, 82.9 ± 6.4 %
174 RH), while another set (n = 12) was set up under semi-field climatic conditions, under a pine
175 shadowed area inside the above-mentioned nursery "Li Foggi" (summer: 26.3 ± 5.40 °C, 64.4 ±
176 22.5 % RH; autumn: 17.5 ± 3.9 °C, 63.3 ± 4.2 % RH). Climatic conditions were monitored using
177 dataloggers with probe inserted inside a randomly chosen microcosm. Insects were caged in each
178 set of microcosms for different IAP durations: 3-7-14-21 days, each with three independent
179 replicates (i.e. 3 single microcosms at each IAP per climatic condition). Olive seedlings were
180 watered during the assays every two days through individual drip irrigation, avoiding

disturbances to insects. All spittlebugs found inside the microcosms at the end of IAP, both live and dead, were collected and stored in ethanol 90% at -20 °C. Insect survival rate was recorded for each microcosm at the end of the IAP. Then, recipient olive seedlings were treated with imidacloprid and kept in an insect-proof screenhouse. Test plants were analysed for *X. fastidiosa* 6 and 12 months after the experimental assay. Plants testing positive at the first assay (6 months) were always positive at the second assay (12 months), when in average 30% more plants tested positive.

## ***Xf*** **detection in insects**

The insect head was removed using needles and a stereomicroscope. Each single head was then homogenized in CTAB buffer with a tungsten carbide bead (7mm in diameter) using the Mill300 mixer (Qiagen, Germany). Homogenized samples were incubated at 65°C for 30min prior to be treated with an equal volume of chloroform - isoamyl alcohol (24:1) followed by a 2-propanol precipitation. The pellet recovered was eluted in 30µl of sterile water. One microliter of the purified DNA was used to set up the real-time qPCR reactions (12,5 µl final volume) using the specific primers and the TaqMan probe described in Harper et al. (2010). To estimate the bacterial populations in the tested insect samples, a 10-fold serial dilution of *X. fastidiosa* cultured cells, from $10^6$ CFU/ml to $10^2$ CFU/ml, was included in all assays. The mean number of *Xf* cells in amplified samples was automatically calculated by CFX Maestro TM Software (Bio-Rad). Samples yielding doubtful qPCR results were re-amplified in direct and nested PCR assays targeting the *holC* gene according to Cruaud et al. (2018). The samples displaying the specific band on agarose gel were then considered positive.

## *Xf* detection in plants

Branches for caging the insects for field acquisition of *Xf* were selected, marked and two leaf samples collected: one including 6–8 leaves collected from the apical part of the shoots (within the last 5–8cm) and the second one including 6–8 mature leaves collected from the basal part of the shoots. Petioles and midribs were recovered from each samples and homogenized in CTAB buffer (1:10 w:v). An aliquot of 1ml of the recovered plant sap was then processed following the standard CTAB protocol (EPPO 2019). Real time PCR reactions for the detection and quantification of the bacterium in these samples were set up as previously described for the insects. For testing the recipient plants, 4–6 leaves were detached from the seedlings and processed as described above. All the recipient plants were tested twice: a first screening performed 6 months after the IAP (data not shown) and then a final assessment at 12 months after the IAP.

## Statistical analysis

The proportion of *Xf*-positive *P. spumarius* and the proportion of infected olive seedlings in the kinetic experiment were separately modelled by logistic GLMM (binomial link) with *Time post-acquisition*, *Season* and their interaction as fixed covariates and *Year* as random intercept. The *Xf* population size in infected *P. spumarius* individuals throughout the kinetic experiment was analysed separately for each assay by both linear regression and Michaelis-Menten (M-M) (1) models:

$$y = \frac{ax}{(b+x)}, \quad (1)$$

where $a$ and $b$ are model coefficients, $y$ represents number of *Xf* cells in the insect head, and $x$ represents days after AAP beginning. Models fit was compared by Akaike's Information

Criterion corrected for small sample size ($AIC_c$). In all tests, *Xf* population size was decimal log transformed to meet model assumptions. The proportion of infected olive plants was analysed by logistic GLM (binomial link) as a function of fixed covariates *Mean Xf population size in vector batch*, *Number of Xf-positive individuals in inoculation batch* and *Season*. Efficiency of transmission by *P. spumarius*, i.e. inoculation rate, was estimated using two non-linear functions derived from binomial probability model and Poisson probability model, respectively (Purcell 1981; Daugherty and Almeida 2009):

$$P_{NB} = 1 - (1 - ba)^{NB}\ , \quad (2)$$

$$P_{NB} = 1 - e^{-baNB}\ , \quad (3)$$

where $N$ is the number of vectors, $B$ is the IAP duration, $a$ is the probability that a vector is infected, and $b$ is the vector inoculation rate. $N$ and $B$ were fixed parameters in our study (5 insects, 3 days), and both $a$ and $P_{NB}$ were known for each vectors batch, given that insects and inoculated plants were individually PCR-tested for *Xf*. The constant $b$, vector inoculation rate, was the only parameter to be estimated, fitting (2) and (3) to the kinetic dataset using non-linear least-squares regression (*nls* function). We then compared the over-all fit of both models using Akaike's information criterion (AIC).

The proportion of *Xf*-positive *P. spumarius* in spread rate experiment was modelled for 2018 assays only, due to the low number of insects recaptured in 2017 assays, with *Inoculum duration*, *Climatic conditions*, *Season*, and their interactions using binomial GLM (proportion). The *Xf* population size in insects (decimal log transformed) was analysed by ANOVA using both full dataset (PCR-positive and -negative individuals) and a subset of PCR-positive insects only. The proportion of infected olive seedlings was analysed using full dataset (2017 + 2018) with binomial GLMM with *Inoculum duration*, *Climatic conditions*, *Season*, *Year* and their

interactions, with replica, i.e. single microcosm, as random intercept. Survival rate of *P. spumarius*, i.e. number of alive individuals at the end of IAP, was analyzed by binomial GLMM as a function of fixed covariates *Inoculation duration*, *Climatic condition* and *Season*, with *Year* as random intercept. All analyses were performed in R 4.0.3 (R Core Team 2020), with packages *lme4* (Bates et al. 2015), *nlme* (Pinheiro et al. 2020), and *ggplot* (Wickham 2016).

# Results

## *Xf* transmission kinetics of *P. spumarius* from olive to olive

### *Xf acquisition and retention*

The overall survival rate of spittlebugs isolated after a 3-day IAP on olive seedlings was 77.1% (343 of 445 individuals), survival of *P. spumarius* isolated on olive seedlings was similar among the assays and time periods post-AAP, with an interquartile range (IQR hereafter) between 60% and 84%. The prevalence of *Xf*-positive insects varied at different timepoints after acquisition and among assays. Overall, cumulative diagnostic results at different times post-AAP showed a proportion of 50% of *Xf*-positive insects (276 positives on 553 tested). The interaction of the fixed effects *Time post-AAP* and *Season* on the proportion of *Xf*-infected *P. spumarius* was significant (*Season* × *Time post-AAP*: $\chi^2_1 = 8.03$, $P = 0.004$) (Tab. 1). That is, the proportion of *Xf*-infected *P. spumarius* was higher in autumn (55–83%), compared to summer assays (20–60%), during the first days post-acquisition (Fig. 1). Then, the proportion of *Xf*-infected insects decreased overtime post-AAP in autumn assays (down to 40–50%), while in summer assays it remained quite stable or slightly increased (30–60%) during the whole period post-AAP, up to 78 days (Fig. 1). The two sexes had the same proportion of *Xf*-positive individuals [males = 0.455 (76/167), females = 0.541 (190/351)] ($\chi^2_1 = 0.52$, $P = 0.471$).

## *Bacterial load in the insects*

*Xf* populations in *P. spumarius* foregut varied from a few hundred to a few thousand of cells (IQR: 70–3500 cells/insect, median 836 cells/insect). 10% of positive insects hosted more than $10^4$ cells in their mouthparts, with one extreme value of $1.2 \times 10^5$. Bacterial load of infected *P. spumarius* was not constant, but increased significantly overtime post-AAP, i.e. the slope parameter *a* of M-M model was always significantly different from zero, and greater in autumn than in summer assays, indicating that *Xf* population in vectors increased faster in autumn than in summer (M-M model summer 2018: $a = 3.02 \pm 0.19$, $t = 15.61$, $P < 0.001$; M-M model autumn 2018: $a = 3.64 \pm 0.21$, $t = 17.58$, $P < 0.001$) (Fig. 2). *Xf* load during the first days after acquisition, i.e. 3–9 days after start of the AAP, consisted in a few hundreds of cells (70-600 cells per insects). *Xf* load increased overtime, reaching $10^3$–$10^4$ cells per insect approximately between 13th and 20th day post-AAP (Fig. 2). The growth of *Xf* population in insects was slightly better explained by the Michaelis-Menten model than the linear model in summer assays and in autumn 2018 assay (summer 2017: $AIC_{linear} = 79.5$, $AIC_{M\text{-}M} = 79.4$; summer 2018: $AIC_{linear} = 399.3$, $AIC_{M\text{-}M} = 393.0$; autumn 2018: $AIC_{linear} = 358.4$, $AIC_{M\text{-}M} = 356.6$), while in autumn 2017 assay the growth of *Xf* population was better described by the linear model, probably because the assay ended earlier than in 2018, and *Xf* population did not reach a plateau ($AIC_{linear} = 260.2$, $AIC_{M\text{-}M} = 262.9$). In 2018 assays the estimated asymptote of mean number of *Xf* cells in the foregut of infected *P. spumarius* was different between summer (2,455 cells, CI = 441–2,698) and autumn (4,335 cells, CI = 1,729–12,543) assay, with plateau reached around 20 days after acquisition (Fig. 2). However, in summer 2018 there were PCR-positive insects with low number of *Xf* cells, i.e. 20–50, even at 60–78 days post-AAP.

## *Xf transmission to olive*

Following acquisition from field-grown *Xf*-infected olives, *P. spumarius* adults were transferred to olive seedlings for 3-day IAP for up to 78 days post-AAP in summer 2018 assay. Overall, 29 of 82 olive seedlings were positive for *Xf* (35.4%). Percentage of *Xf*-positive olive seedlings 12 months after inoculation, i.e. a proxy of inoculation success of *Xf* by *P. spumarius*, was variable among assays and time post-AAP, the IQR was between 21% and 57% of tested olives infected (Fig. 3). The proportion of infected PCR-tested olive seedlings was not significantly affected by either *Time post-AAP* ($\chi^2_1 = 0.33$, P = 0.856), *Season* ($\chi^2_1 = 0.77$, P = 0.378) or their interaction ($\chi^2_1 = 0.69$, P = 0.403) (Tab. 1). That is, inoculation rate was constant — and randomly variable — across samples, with an average inoculation rate of 34%. The likelihood of *Xf* transmission to olives increased significantly as the average *Xf* population in insects augmented, regardless of season (*Xf population*: $\chi^2_1 = 5.72$, P = 0.017; *Season*: $\chi^2_1 = 0.50$, P = 0.479) (Supplementary Tab. 2). In other words, the larger the mean *Xf* population in *P. spumarius* individuals, the higher the probability of successful pathogen transmission (Fig. 4). Conversely, the number of *Xf*-positive insects did not significantly affect the likelihood of *Xf* transmission on olives, i.e. olive seedlings inoculated with higher number of *Xf*-positive insects (range: 1-4 positive insects/batch) were not significantly more likely to get infected than those inoculated with fewer infected insects (*Number of Xf-infected insects*: $\chi^2_1 = 1.05$, P = 0.306) (Supplementary Tab. 2). Although not statistically significant, the relationship between the number of positive insects and the successful transmission showed an apparent trend, being a recipient olive seedling exposed to four *Xf*-positive *P. spumarius* in average 2.6-fold more likely of infection compared to one exposed to a batch containing a single positive insect.

### *Inoculation rate estimates*

Poisson model provided better fit to the kinetic transmission dataset than binomial model, based on AIC score. Overall vector inoculation rate *b* (proportion/vector/day), estimated from Poisson model, was 0.062 ± 0.014 ($t_{16}$ = 4.44, P < 0.001) when pooling all the assays performed during different seasons. Considering separately the assays performed in summer and autumn provided different estimates, lower in summer (0.034 ± 0.015, $t_8$ = 2.24, P = 0.05) than in autumn (0.08 ± 0.02, $t_7$ = 4.06, P = 0.005).

## Spread of *Xf* by *P. spumarius* under microcosm conditions

Overall, recapture rate of alive *P. spumarius* from microcosms at the end of IAPs, i.e. survival rate, was 48% (1,127 alive out of 2,352 insects released). Survival of insects in microcosms was affected by both the interaction of IAP duration and season (*IAP duration* × *Season*: $\chi^2_1$ = 26.87, P < 0.001), and the interaction of season and climatic conditions (*Season* × *Climatic condition*: $\chi^2_1$ = 6.43, P = 0.011) (Tab. 2). Therefore, lower survival rate was recorded at the end of longer IAPs, especially during autumn assays, e.g. at the end of 21-days long IAPs only 3% (2017) and 4% (2018) of insects released inside microcosms were collected alive, irrespective of the climatic condition. Survival rate of caged *P. spumarius* was higher in summer, especially in controlled climatic conditions (2017: 66.7%, 2018: 85.2%) than in semi-field conditions (2017: 28.9%, 2018: 49.2%).

Effects of season, days after AAP, and climatic conditions on the proportion of infected *P. spumarius* were analyzed for 2018 assays only, given the low number of infected insects recollected during 2017 assays (n = 32, 3.9%). In 2018 assays there was a significant interaction among the three covariates — *IAP duration*, *Season*, and *Climatic condition* — on the proportion

of infected insects recaptured ($\chi^2_1 = 8.78$, P = 0.003) (Tab. 2). That is, the proportion of infected *P. spumarius* was initially lower in summer (IQR: 31-35%) than in autumn (IQR: 66-77%), then, during summer, it slightly decreased overtime both in controlled and semi-field conditions, while in autumn the proportion of infected *P. spumarius* abruptly decreased at longer IAPs in semi-field conditions, and remained quite stable in controlled climatic conditions (Fig. 5).

Given the low number of positive insects recaptured in 2017 assays (n = 32), *Xf* population in PCR-positive *P. spumarius* was analyzed only for 2018 assays (n = 444). PCR-positive individuals had in average higher *Xf* population after longer IAPs (that is to say longer post-AAP period) (*IAP duration*: $F_{1,375} = 8.75$, P = 0.003). Moreover, *P. spumarius* in the autumn assay had higher mean *Xf* loads than those tested in summer (*Season*: $F_{1,375} = 35.49$, P < 0.001) (Supplementary Tab. 3). Considering the total of recollected insects (both PCR-positive and -negative individuals) in 2018 assays (n = 957), mean *Xf* population was higher in autumn, as expected by the higher rate of positive insect (*Season*: $F_{1,696} = 10.13$, P = 0.001) (Tab. 2). However, significant interaction was observed among *Season*, *Climatic condition*, and *IAP duration* ($F_{1,696} = 8.38$, P = 0.004), that is, average *Xf* population in insects collected alive at the end of IAP in autumn was high in both conditions at shorter IAPs, then gradually declined in semi-field conditions, while in controlled conditions it remained stable; on the other hand, in summer assay mean *Xf* population gradually declined in both conditions (Fig. 6).

### *Trasmission to olive*

Transmission rate, i.e. proportion of infected olive seedlings, was affected by *Climatic condition* ($\chi^2_1 = 10$, P = 0.001), *Year* ($\chi^2_1 = 23.3$, P < 0.001), with significant interactions between *Climatic condition* × *Year* ($\chi^2_1 = 6.5$, P = 0.011) and *IAP duration* × *Season* ($\chi^2_1 = 7.17$, P = 0.007) (Tab. 2). The proportion of infected olive seedlings was higher after longer IAPs in Autumn, and higher

in controlled conditions than in semi-field conditions, especially in 2017 trials (2017: 10-fold higher likelihood of becoming infected compared to plants in controlled conditions, 2018: 1.5-fold). Infection rates were higher in 2018 than in 2017 assays (plants had a 34-fold higher likelihood of becoming infected in 2018), as partially expected given the higher number of insects released in microcosms in 2018 compared to 2017 assays (32 and 17, respectively) (Fig. 7 and Tab. 2). That is, infection spread among olives increased with inoculation time in late season only, especially in controlled climatic conditions and with higher vector density.

# Discussion

We studied aspects of *Xf* transmission biology by *P. spumarius* on olive plants in order to shed light on the epidemiology of this invasive bacterium in Europe and associate emerging diseases. Two different sets of experiments were carried out in order to gain information on the influence of post-acquisition time, seasonal, and climatic conditions on transmission efficiency, persistence, and multiplication of *Xf* in the vector, as well as on survival of the insect vector. Our results suggest that i) *P. spumarius* is a competent vector of *Xf* on olive throughout its adult life, ii) *Xf* acquisition rate by the vector under field conditions varies between seasons, iii) the bacterium load in vector's foregut increases during the first 2-3 weeks after acquisition, then becomes stable, iv) the transmission rate to olive seedlings remains constant at different post-acquisition times, but tends to increase with longer inoculation time (although differently among seasons and climatic conditions), v) insect survival is influenced by age, season and climatic conditions, affecting the transmission outcome in microcosm experiments, and possibly influencing the spreading rate of *Xf* in olive groves.

379 Unexpectedly, the higher acquisition rate of *Xf* by *P. spumarius* in autumn compared to summer
380 assays did not follow a corresponding increase in *Xf* populations in the olive source plants, while
381 the acquisition rate of *Xf* by xylem-sap feeders is usually related to the population of the
382 bacterium in host plant (Hill and Purcell 1997). In our experiments, the estimated *Xf* loads in
383 source plants were slightly higher in summer than autumn, contrary to the expectation that *Xf*
384 population in plant tends to increase during the vegetative season (Hill and Purcell 1997;
385 Giampetruzzi et al. 2020).This discrepancy may be due to the sample collection of leaf tissues,
386 which in summer included mature leaves produced on shoots of the previous year, and thus
387 possibly harboring higher number of *Xf* cells — dead or alive — than in late season samples,
388 whereas sampling in autumn included mature leaves from shoots grown during the same year and
389 thus less heavily colonized by *Xf.* Alternatively, the limited number of leaves/branch used in our
390 tests may explain the results, given the heterogeneous distribution of *Xf* in the infected plants.

391 Once acquired, *Xf* multiplied in the foregut of *P. spumarius* reaching an average of a few
392 thousand cells after 2–3 wks, although in some individuals *Xf* population was estimated
393 exceeding forty thousand cells. The increase in *Xf* population was faster, and the plateau and the
394 maximum number of cells higher in autumn than it was in summer assays. The maximum number
395 of *Xf* cells in *P. spumarius* foregut was higher than the estimates made in previous studies, which
396 tested *Xf* population in the vector only after a few days post-acquisition (Cavalieri et al. 2019), or
397 in field-collected individuals, whose acquisition conditions are unknown (Cornara et al. 2017a),
398 or exposed to different *Xf* subspecies/host plant combination (e.g. *Xf*. subsp. *fastidiosa* in
399 grapevine) (Cornara et al. 2016). Indeed, our estimates for *Xf* population in the vector after eight
400 days after the start of AAP were similar to those tested at approximately the same time after
401 beginning of acquisition by Cavalieri et al. (2019), i.e. 400-900 cells. Moreover, the population

size measured in the head of *P. spumarius* in this work is compatible with the number of *Xf* cells potentially hosted by *P. spumarius* estimated based on the cuticular surface available in foregut (≈ 67,000 cells) (Ranieri et al. 2020). Thus, our results show that *P. spumarius* can harbor in its foregut *Xf* loads similar to the ones measured in the main sharpshooter vectors in America, e.g. *G. atropunctata* and *H. vitripennis* (Hill and Purcell 1995; Almeida and Purcell 2003; Killiny and Almeida 2009).

Significant decreases of proportion of infected insects, mean *Xf* load in insects alive at the end of IAP, and survival rate were observed during autumn assays. Such trend could be explained by a higher mortality of *Xf*-infected insects compared to healthy ones, especially under the stressing conditions of the cages in semi-field conditions in autumn (colder climate and higher daily temperature variations), when insects are old and approaching the end of their life. This hypothesis would be consistent with the possible negative impact of *Xf* on fitness and survival of the spittlebug, caused by the precibarium bacterial colonization and the consequent reduction of available size of food canal. This reduction might lead to additional energy requirement to ingest the xylem sap (Ranieri et al. 2020), and variations in feeding behaviour (e.g. shorter xylem ingestion time and longer non-probing periods) (Cornara et al. 2020).

The transmission efficiency in the kinetics of *Xf* colonization experiment, i.e. the likelihood of successful inoculation of *Xf* to olive, increased with bacterium population size in vectors, while it was not significantly affected by the number of infected insects per inoculation batch, seasonal dynamics of acquisition, and time post-acquisition during kinetic experiment. The transmission efficiency by a batch composed by five *P. spumarius* was in average 34%, without significant changes or trends overtime post-acquisition or among seasons. In other words, small groups of vectors on single olive plant and controlled conditions inoculate at a constant rate, irrespective of

time post- acquisition and season. However, the increase of average *Xf* population per insect of one order of magnitude (e.g. from $10^2$ to $10^3$ cells) led to a 3.2-fold increase of the likelihood of olive infection. However, transmission efficiency at longer post-acquisition times (i.e. longer *Xf* incubation time in vector's foregut), did not increase, likely because the positive correlation between time post-acquisition and *Xf* load in *P. spumarius* was weak and there were insects with high *Xf* populations in the foregut after few days post-acquisition and others with low number of *Xf* cells after several weeks from acquisition. A positive correlation between transmission rate and bacterial load in the vector was observed for the transmission of *X. fastidiosa* subsp. *fastidiosa* by *P. spumarius* and *G. atropunctata* from grapevine to grapevine (Cornara et al. 2016; Zeilinger et al. 2018).

The overall estimated average inoculation rate was 6% of infected plants per insect per day. When considering the results obtained in different seasons, the inoculation rate per insect was higher in autumn than in summer. In the literature, concerning *X. fastidiosa* subsp. *fastidiosa*, higher inoculation rate estimates have been reported for *P. spumarius* and *G. atropunctata* on grapevine (Cornara et al. 2016; Daugherty and Almeida 2009), similar for *Draeculacephala minerva* Ball on periwinkle (Cabrera-La Rosa et al. 2008), and lower for *H. vitripennis* on grapevine and almond (Almeida and Purcell 2003; Daugherty and Almeida 2009). According to Marucci et al. (2008) and Lopes and Krugner (2016), inoculation efficiency of CVC strains of *X. fastidiosa* subsp. *pauca* on citrus of main vector species [e.g. *Dilobopterus costalimai* Young, *Acrogonia citrina* Marucci & Cavichioli, *Oncometopia facialis* (Signoret) and *Bucephalogonia xanthophis* (Berg)] was lower or similar (1–13% over two-day IAP) to that estimated for *P. spumarius* in this study. The inoculation rate of *P. spumarius* on olive calculated on data from Cavalieri et al. (2019), 6–7% over four-day IAP, obtained with the Swallow formula (Swallow

448 1985), is lower compared to our estimate, 6% per day. However, the methodological differences
449 between the different studies — e.g. AAP and IAP duration, source plants, periods of the year,
450 statistical approaches adopted — suggest caution in comparing the outcomes of the different
451 studies. Moreover, in some studies, e.g. Daugherty and Almeida (2009), estimates were obtained
452 also reviewing datasets from several studies, whose methodologies were likely not homogeneous.
453 It is worth to point out that our estimates come from an experiment not specifically intended to
454 explore the effects of inoculation time and number of insect vectors per plant, being those
455 parameters fixed (3 days and 5 insects respectively), and thus are reliable for those conditions,
456 although the inoculation rate could vary with different combinations of inoculation duration and
457 number of inoculative insects (e.g. Daugherty and Almeida 2009; Cornara et al. 2016).

458 The epidemic spreading rate of *Xf* on olive by *P. spumarius*, i.e. the increase rate of the infected
459 plants proportion with inoculation time within an olive population, was affected by different
460 factors such as inoculum period, season and climatic conditions. Indeed, the spreading rate of *Xf*
461 was higher in controlled vs semi-field climatic conditions in all the assays, possibly meaning that
462 more stable climatic conditions — e.g. lower temperatures in summer and higher in autumn
463 assays compared to semi-field conditions or lower daily variations — resulted in a faster spread
464 of *Xf* among olive seedlings, possibly due to higher inoculation rates and/or higher mobility and
465 survival of *P. spumarius* in microcosms. Furthermore, the *Xf* spreading rate on olives increased
466 with inoculation time in autumn assays only, irrespective of climatic conditions. Although the
467 transmission rate is expected to increase with longer inoculation periods (Purcell and Finlay
468 1979; Daugherty and Almeida 2009; Cornara et al. 2016), also vector behaviour — e.g. the
469 preference for low water stressed plants — may influence host plant visiting, and ultimately the
470 transmission rate (Mizell et al. 2008; Del Cid et al. 2018). In our case, the observed seasonal

variation of *Xf* spreading rate on olive seedlings could be explained by a higher mobility of the spittlebug in autumn, given that late in the season *P. spumarius* tends to move to herbaceous species for mating and oviposition (Bodino et al. 2020a). Vector movement propensity may be higher in this period, especially in a microcosm environment that does not provide good oviposition sites, thus possibly forcing the vector to visit more olive seedlings. Finally, the *Xf* spreading rate on olives was significantly higher in 2018 assays, when the vector density was about double.

In summary, this work describes the influence of seasonal and environmental factors on the transmission characteristics of *Xf* by *P. spumarius*. Such transmission dynamics could significantly contribute to determine epidemic patterns in Apulian olive agroecosystem. The highest rates of both acquisition and overall transmission of *Xf* in Apulian olive agroecosystem occur probably in early summer, being the period with highest density of vectors on olive canopies (Cornara et al. 2017b; Bodino et al. 2019, 2020a). However, because *P. spumarius* is infectious throughout its adult life, our results indicate that transmission/spreading efficiency may even increase in late season. Thus, the role of the spittlebug visiting olive canopies late in the season could be epidemiologically relevant. Moreover, inoculation events taking place late in the season could contribute to spread *Xf* at higher distances from the source plants, as the distance travelled by an insect is a function of time, being in the range of 350-650 m after 5-6 months from adult emergence in olive groves (Bodino et al. 2020b). Thus, older *P. spumarius* adults could play a role in dispersing the bacterium at longer distances to olives or to wild plants within the agroecosystem, expanding the infected area. Further studies on *Xf* spread in field or semi-field condition, as well as on seasonal variations of dispersal and mobility of vectors are needed to gain knowledge on factors driving the epidemic of *Xf* on olive. Besides transmission efficiency, *P.*

494 *spumarius* population level and preference for olive plants are major drivers of *Xf* spread in olive
495 groves. It is crucial to reduce populations of *P. spumarius* in olive groves in Apulia, since the
496 number of feeding insects is probably the most important factor determining successful
497 transmission and quick spread of the pathogen (Daugherty and Almeida 2009).

498 In conclusion, the transmission parameter estimates reported here, and their variation overtime,
499 should inform epidemic spread models of *Xf* in Italy and Europe that explicitly account for the
500 effect of vectors. *Philaenus spumarius* and its interactions with *Xf* in relation to spread are crucial
501 actors affecting the establishment, persistence, and epidemic spreading of the exotic pathogen
502 (Jeger and Bragard 2018).

503

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# Figure legends

**Figure 1:** Proportion of *Xf*-positive *Philaenus spumarius* individuals at different timepoints after acquisition from infected olive branches during Summer and Autumn 2017-2018 assays. Hollow points represent result of single insects (positive = 1, negative = 0), a small amount of variation was included to make visible the number of insects tested at each timepoint even if the multiple points had the exact same value. Red filled points and line represent proportion of positive insects (mean + SE) and the red lines the outcome of binomial GLMM model; data from insects collected immediately after AAP end were excluded from depicted GLMM model, to avoid biased estimation of infected insects.

**Figure 2:** PCR-estimated population of *Xf* in *Philaenus spumarius* foregut (decimal log-transformed no. of cells) at different timepoints after acquisition from infected olive branches during Summer and Autumn 2017-2018 assays. Hollow points represent result of single positive insects, red filled points represent proportion of positive insects (mean + SE), continuous black lines the outcome of Michaelis-Menten model and dashed grey line the outcome of linear model.

**Figure 3:** Proportion of *Xf*-positive olive seedlings inoculated by batches (n = 5) of *Philaenus spumarius* at different timepoints after acquisition from infected olive branches during Summer and Autumn 2017-2018 assays. Hollow triangles represent result of single plants (positive = 1, negative = 0), a small amount of variation was included to make visible the number of plants tested at each timepoint even if the multiple points had the exact same value. Green filled points represent the proportion of positive olive plantlets (mean + SE) and the green lines represent the outcome of binomial GLM model described in Table 1.

746

747 **Figure 4:** Proportion of *Xf*-positive olive plantlets related with mean *Xf* population size (decimal
748 log of no. of cells) in *Philaenus spumarius* inoculation batches. Hollow points represent result of
749 single plants (positive = 1, negative = 0), a small amount of variation was included to make
750 visible the number of plants tested even if the multiple points had the exact same value. Red
751 filled points represent the proportion of *Xf*-positive olive plantlets for each binned log span of
752 mean *Xf* population size in *P. spumarius* inoculation batch. Red line represents the outcome of
753 binomial GLMM model.

754

**Figure 5:** Proportion of *Xf*-positive *Philaenus spumarius* individuals after different inoculation periods in *Xf* spread experiments during Summer and Autumn 2017-2018 assays. Note that x-axis scale is equal to IAP duration + 3 days of AAP). Hollow points represent result of single insects (positive = 1, negative = 0), a small amount of variation was included to make visible the number of insects tested at each timepoint even if the multiple points had the exact same value. Filled points and triangles represent proportion of positive insects (mean + SE) recollected at different IAPs and the lines the outcome of binomial GLMM model from different climatic conditions. A small amount of horizontal variation was included for points and triangles to avoid overlap.

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**Figure 6:** PCR-estimated population of *Xf* in *Philaenus spumarius* foregut (decimal log-transformed no. of cells) of individuals recollected alive at the end of IAPs in *Xf* spread experiments during Summer and Autumn 2017-2018 assays. Note that x-axis scale is equal to IAP duration + 3 days of AAP). Hollow points represent result of single insects. Filled points and

triangles represent the mean (±SE) number of *Xf* cells in recollected *P. spumarius* at different IAPs and the lines the outcome of linear regression model from different climatic conditions.

756

**Figure 7:** Proportion of infected olive seedling after different inoculation periods in *Xf* spread experiments during Summer and Autumn 2017-2018 assays. Points and triangles represent proportion of positive insects (mean + SE) and the continuous and dashed lines the outcome of binomial GLMM model from different climatic conditions (points-continuous lines = controlled; triangles-dashed lines = natural).

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# Tables

**Table 1:** Estimated odds ratios, z-statistics significance and confidence interval (95%) from a logistic GLMM of fixed covariates *Time post-acquisition*, *Season* and its interaction on proportion of infected *Philaenus spumarius* and proportion of infected olive seedlings in kinetic experiment.

| | PCR-positive insects | Plants infected |
|---|---|---|
| Time post-AAP | 1.01<br>(1.00 – 1.02) | 0.99<br>(0.97 – 1.02) |
| Season | 4.95 ***<br>(2.84 – 8.62) | 0.93<br>(0.20 – 4.34) |
| Time post-AAP x Season | 0.95 **<br>(0.92 – 0.99) | 1.03<br>(0.96 – 1.12) |
| N | 538 | 82 |
| N (Year) | 2 | 2 |
| SD (Year) | 0.63 | 0 |
| BIC | 720.49 | 126.79 |
| R2 (fixed) | 0.08 | 0.03 |
| R2 (total) | 0.18 | 0.03 |

**Table 2:** Estimated parameters from a logistic GLMM or linear regression of fixed covariates *Inoculation duration*, *Climatic condition*, *Season* and *Year* on proportion of infected *Philaenus spumarius*, *Xylella fastidiosa* population size at the end of IAP in collected individuals and proportion of infected olive seedlings in spread rate experiment.

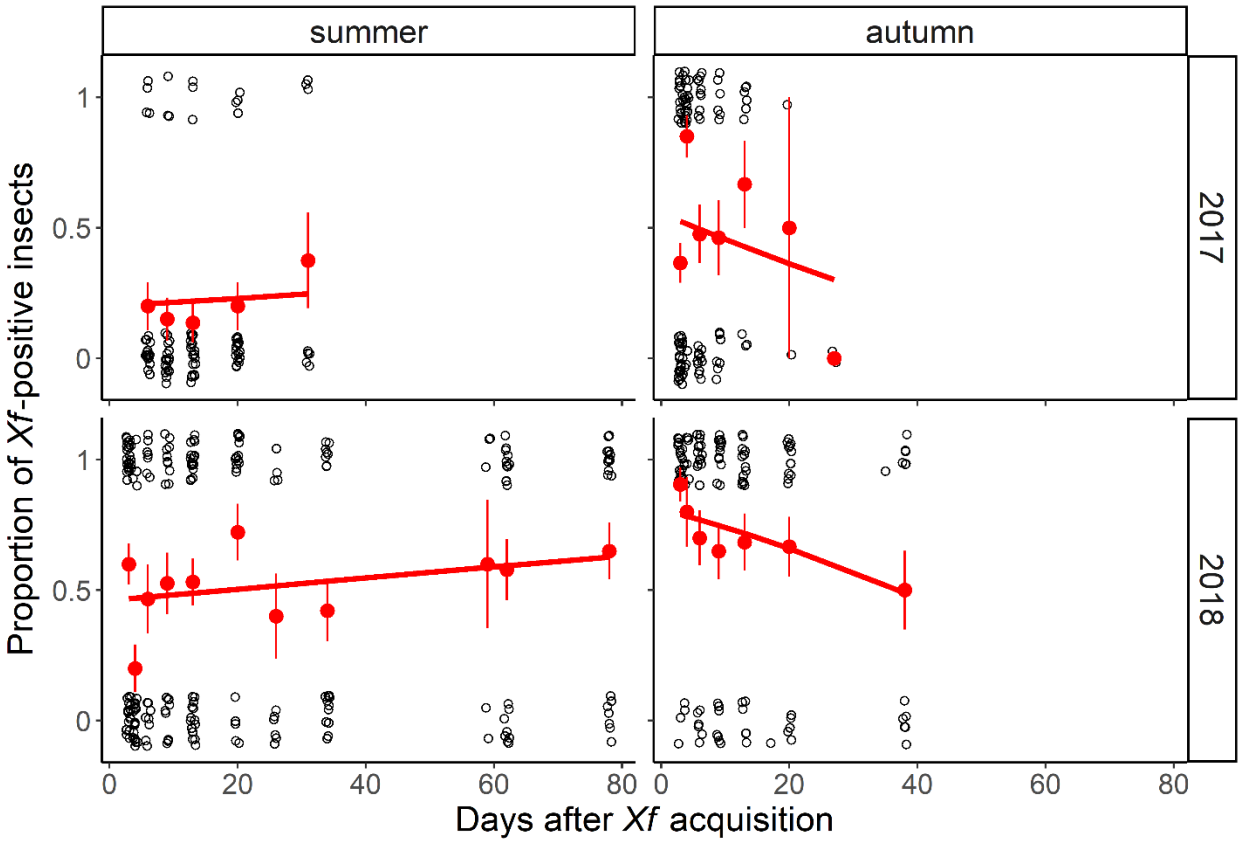
| | Survival rate | PCR-positive insects[a] | *Xf* population (pos + neg)[b] | Plants infected[a] |
|---|---|---|---|---|
| IAP duration | 0.93 *** (0.91 – 0.95) | 0.97 (0.92 – 1.01) | -0.13 *** (-0.20 – -0.06) | 1.02 (0.97 – 1.06) |
| Climatic conditions | 5.99 *** (3.74 – 9.58) | 1.38 (0.69 – 2.77) | 0.60 (-0.59 – 1.79) | 10.89 ** (2.48 – 47.80) |
| Season | 1.94 ** (1.31 – 2.89) | 8.09 *** (3.43 – 19.07) | 1.84 ** (0.70 – 2.98) | 0.57 (0.25 – 1.30) |
| Year | — | — | — | 34.54 *** (8.20 – 145.47) |
| IAP duration x Climatic conditions | 1.00 (0.97 – 1.03) | 1.01 (0.95 – 1.07) | 0 (-0.09 – 0.08) | — |
| IAP duration x Season | 0.87 *** (0.84 – 0.90) | 0.86 ** (0.77 – 0.96) | -0.08 (-0.20 – 0.03) | 1.08 ** (1.02 – 1.15) |
| Season x Climatic conditions | 0.29 *** (0.19 – 0.43) | 0.19 ** (0.06 – 0.56) | -1.47 (-2.97 – 0.04) | — |
| Climatic conditions x Year | — | — | — | 0.13 * (0.03 – 0.63) |
| IAP duration x Climatic conditions x SeasonAutmn | — | 1.21 ** (1.07 – 1.38) | 0.23 ** (0.07 – 0.38) | — |
| N | 96 | 829 | 704 | 1489 |
| N (replica) | — | 3 | — | — |
| N (year) | 2 | — | — | 96 |
| BIC | 530.96 | 1126.39 | 3545.95 | 928.86 |
| R2 (fixed) | 0.38 | 0.11 | — | 0.43 |
| R2 (total) | 0.42 | 0.11 | 0.18 | 0.45 |

[a] Logistic GLMM, estimated odds ratios, z-statistics significance and confidence interval (95%)

[b] Linear regression, estimated intercept and slope parameters, *t*-statistics significance and confidence interval (95%)

# Figures

## Figure 1



767 **Figure 2**

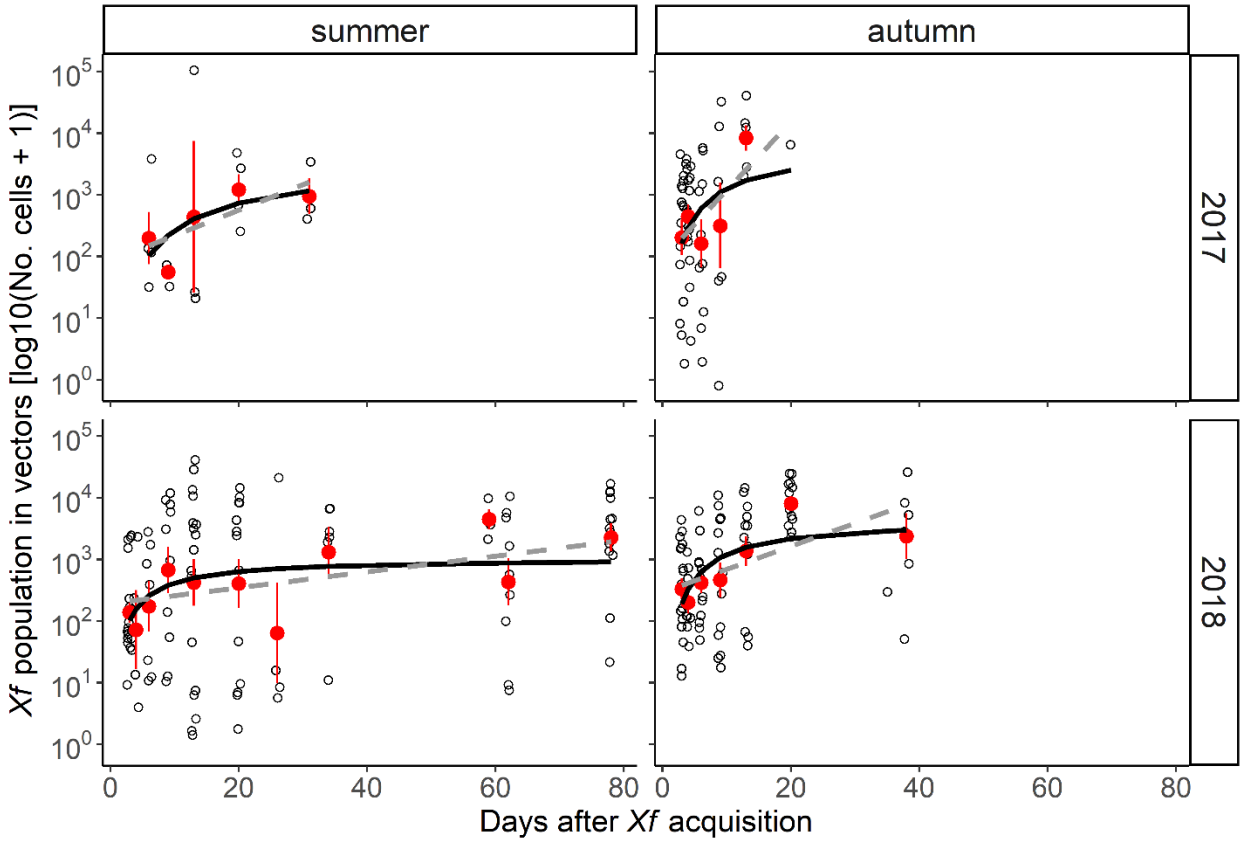



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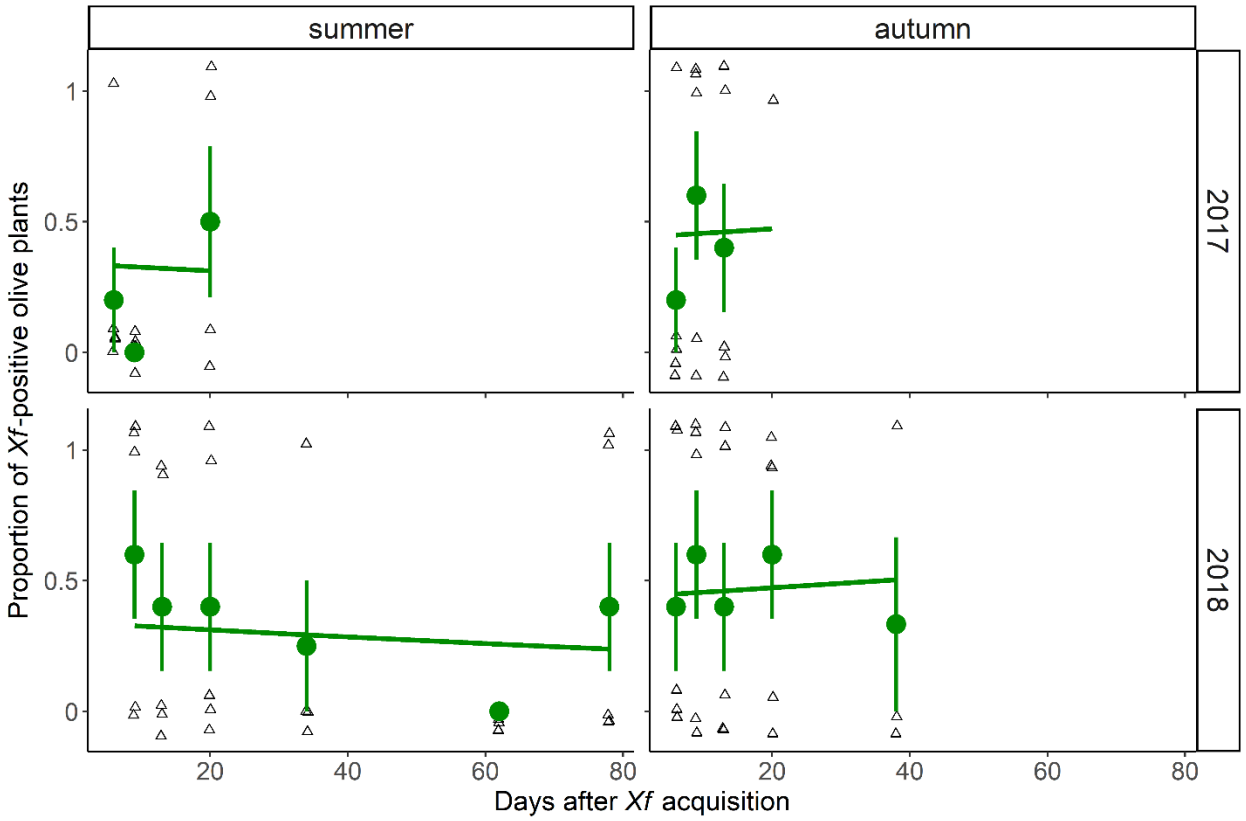
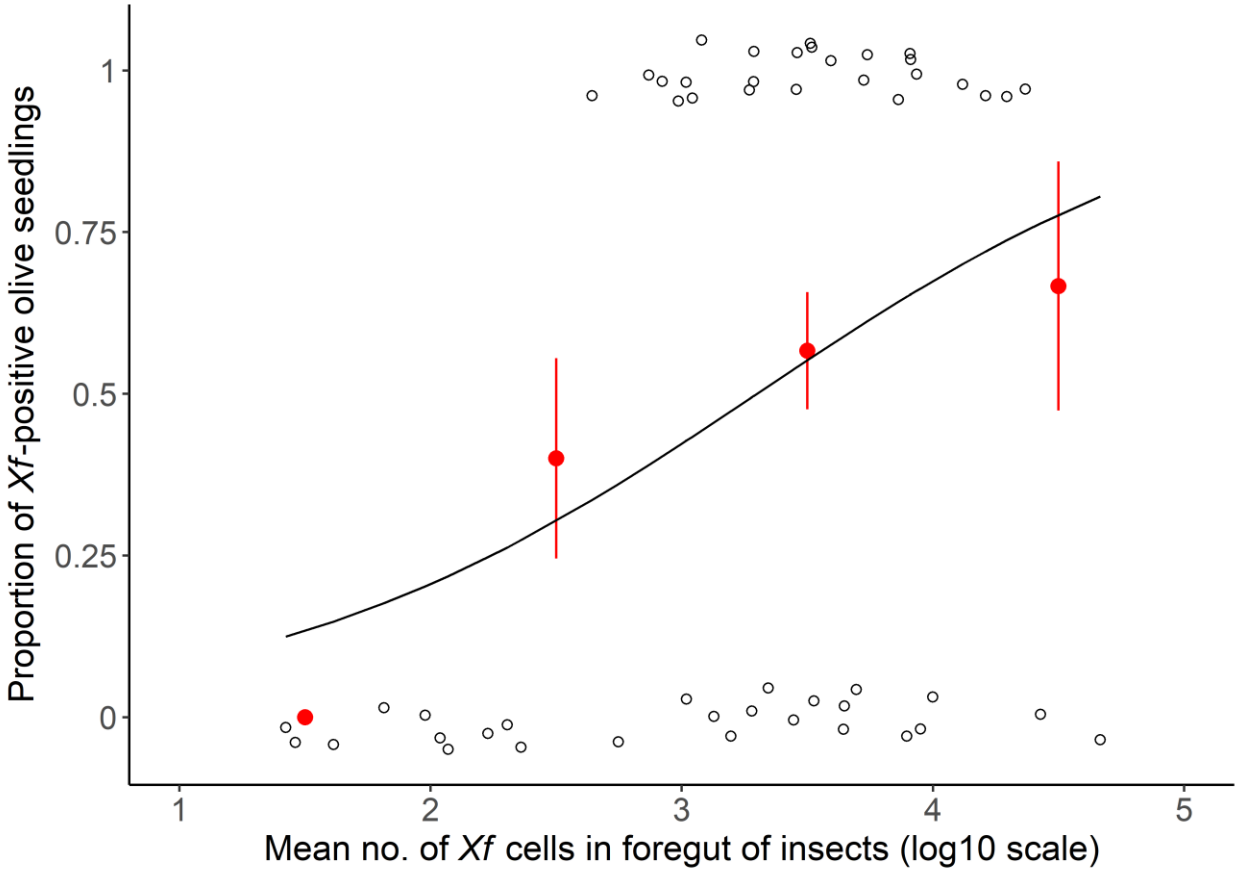
**Figure 3**


summer
autumn
2017
2018
Proportion of Xf-positive olive plants
Days after Xf acquisition
0
0.5
1
20
40
60
80

773 **Figure 4**

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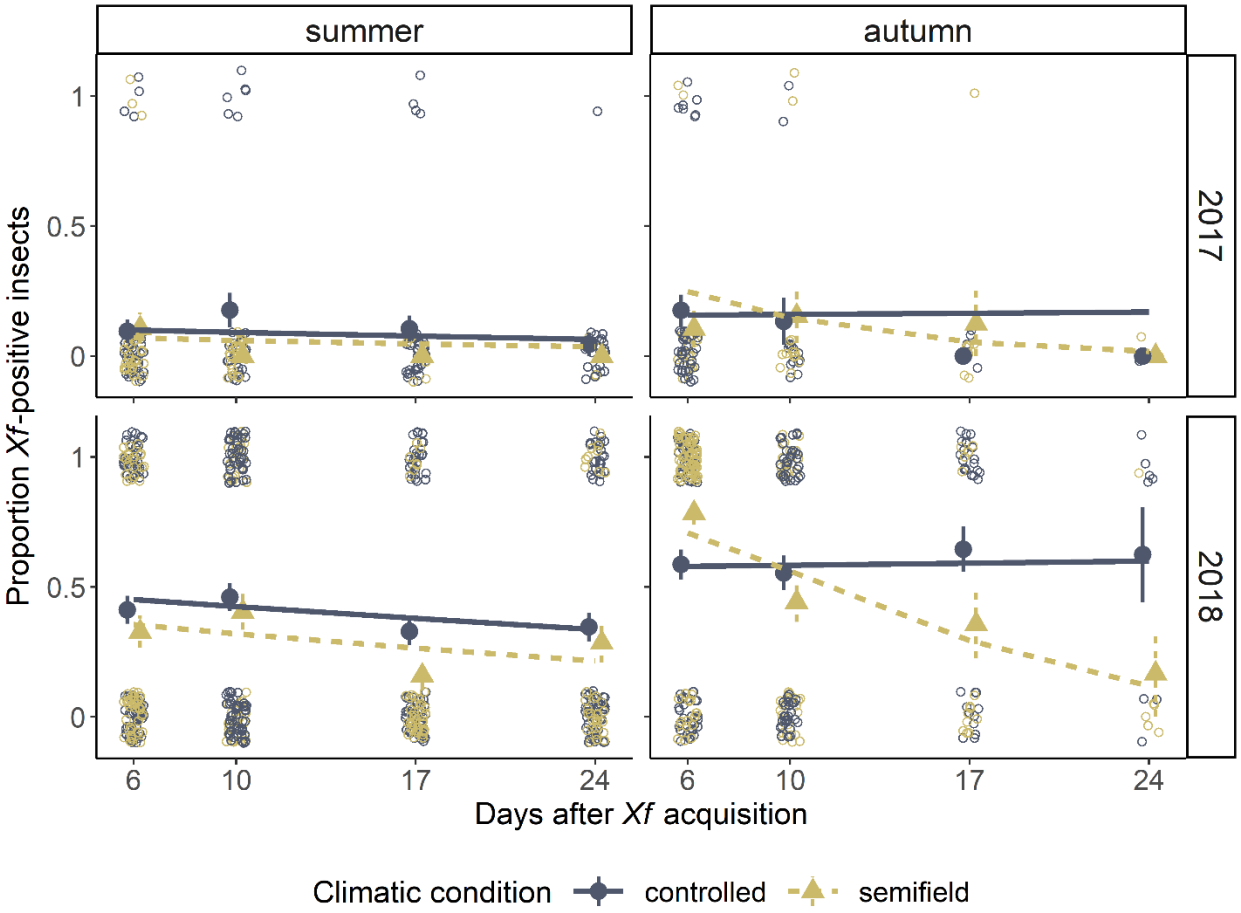
776 **Figure 5**

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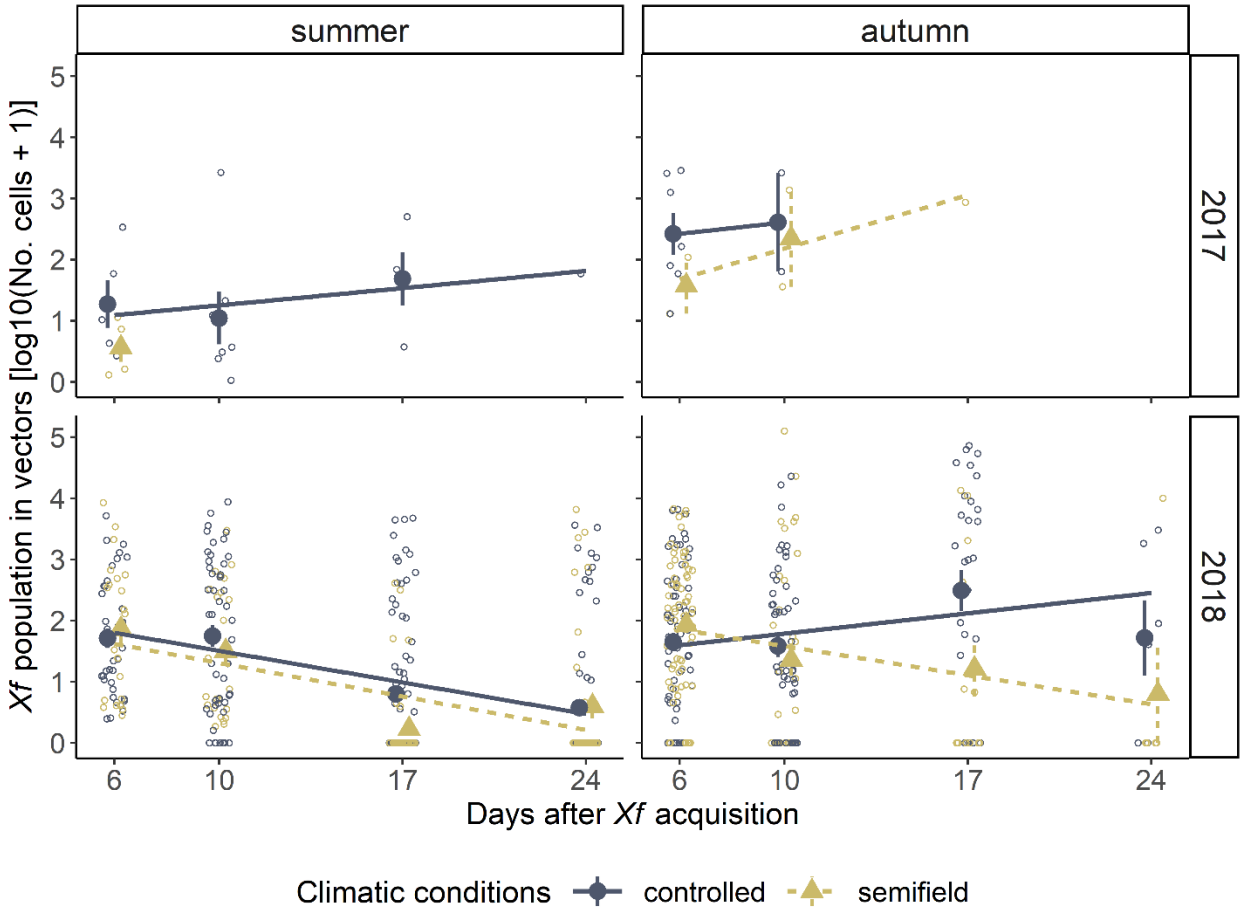
779 **Figure 6**

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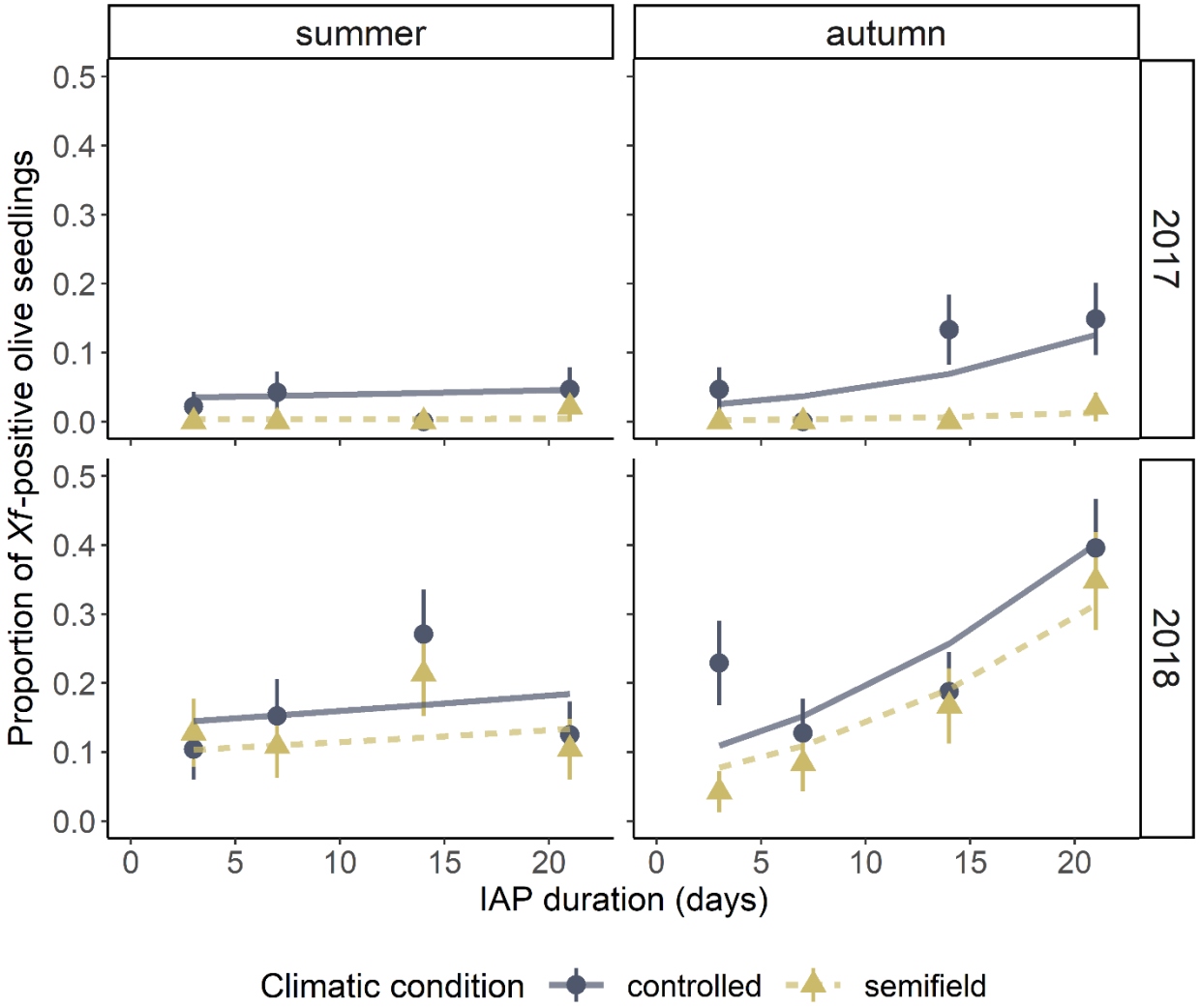
782 **Figure 7**

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