FINAL REPORT

Cascading effects of direct and indirect natural enemy – pest interactions in a spider – leafhopper– plant virus – plant system

NKFI OTKA K116062

Arthropod natural enemies play an important role in regulating insect prey populations not only by consuming them, but also, through non-consumptive effects, by their shear presence causing stress to prey. Prey behaviour may dramatically change in response to predator stress (PS), including reduced plant consumption in herbivores. By utilising a 'spider – leafhopper – plant virus – plant' model system, the goal in the OTKA project was to understand how predation and PS influences virus vector sap feeding insects, and if there is any cascading effect of PS on virus transmission. The model system included the spider *Tibellus oblongus* (Walckenaer, 1802), the leafhopper *Psammotettix alienus* (Dahlbom, 1851), the wheat dwarf virus (WDV) and cereals as host plants. The model predator and prey are among the dominant herbivorous and predatory arthropods in cereal fields and margins in Europe. *P. alienus* is the only known vector of WDV which causes severe symptoms and losses on cereals. *T. oblongus* is an abundant and important natural enemy of the leafhopper (Samu et al., 2013).

In the grant proposal we asked how predators interact with leafhopper feeding through predation and PS – with emphasis on the latter –, and investigated the cascading consequences of this interaction on virus transmission. Our objectives were to study a) How spider PS influences leafhopper penetration, sap feeding and fitness and how this relates to virus transmission capability; b) What are the sensory modalities of predator perception by the leafhopper; c) Whether PS affects virus spread indirectly in a mesocosm setup; d) What is the real-life significance of *T. oblongus* and *P. alienus* interactions? Are field abundances and virus incidences correlated? e) Can we work out molecular methods to routinely detect WDV and leafhopper predation by spiders?

Effect of predator stress on leafhopper feeding

One central question to our proposal was how does PS influence insect herbivores' feeding behaviour? In the work package that addressed this question we included several experiments, some executed to the detail to result in high impact publication. Part of the study was planned and conducted as a co-operation between our lab and the Harwood Laboratory at the Department of Entomology, University of Kentucky, Lexington, USA, another study was done in co-operation with the Agricultural Biotechnology Institute, Gödöllő, Hungary.

Predator stress effect on salivary sheath formation **-** In this experiment we observed PS effect of spider (restricted, i.e. cannot reach prey) on the penetration and ingestion stages of the leafhopper by microscopically detecting shape and penetration depth of salivary sheath. By this method we could separate penetrations that could not reach the final feeding destination and ended in mesophyllum from penetrations that reached the phloem vessels in the leaf.

Salivary sheaths (ss) produced during penetration permanently remain in the plant tissues after the stylets were removed. The presence of ss clearly indicates penetration, while the end of each ss in plant tissues (in mesphyllum or in vascular bundle) indicates how far the penetration proceeded (Ammar & Hall, 2012). We conducted laboratory experiments to show the effect of predator presence on the feeding behaviour of leafhoppers, quantified by the number of salivary sheaths.

Trials were made in divided micro isolators (n = 80), where a dividing mesh prevented actual predation by spiders, but made possible for the animals to sense the presence of each other. We applied the isolator to the plant, driving one of the leaves through the device. Cuttings in the sponges and on the separator mesh tightly

fitted to the surface of the leaf (see also Fig. 7). Experiments were performed in climate chambers (L/D: 16/8 h, 25°C). The spider-treated trials were separated from controls, in order to prevent olfactoric and visual disturbance that might be caused by the spiders to control leafhoppers. Trials were run for 24 hours. After 24 hours plant leaf areas from the leafhopper side of the microisolators were cut out, cleared by lactic acid, then ss were stained by McBride solution. Clarified and stained leaf samples were examined under microscope.

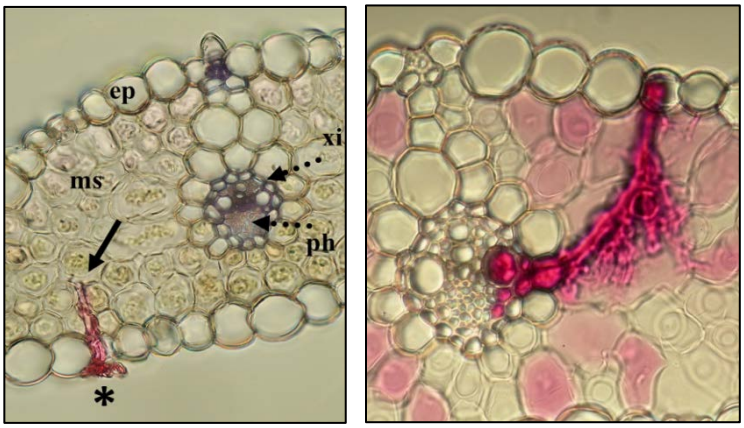

Figure 1. Stained salivary sheaths ending in mesophyll tissue (left) and in vascular tissues (right) of barley leaf.

We have categorised ss as follows: ss originates from: upper/lower side of a leaf; ended up in mesophyll (incomplete penetration) / vascular bundle (complete penetration) (Fig. 1). In microcosm tests salivary sheaths, as indicators of penetrations were observed. The total number of produced ss did not differ significantly under predation stress treatment (t_{78} = -0.54, P = 0.59).

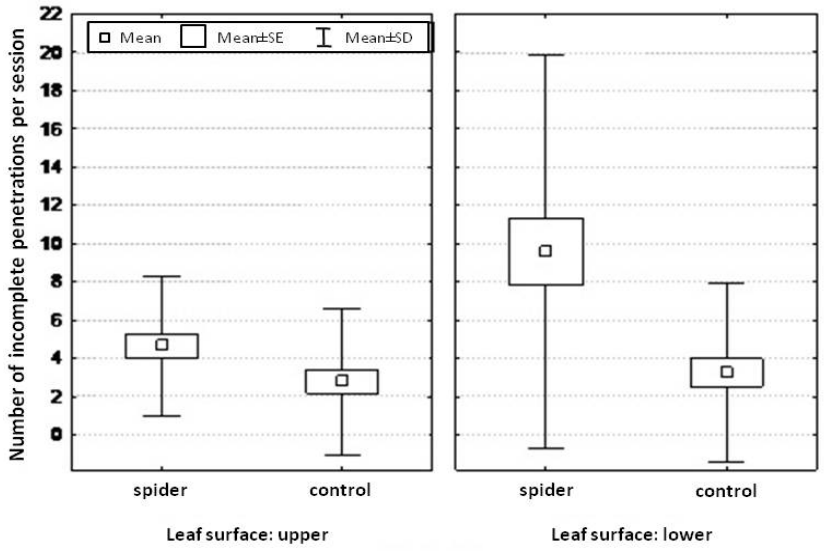

Figure 2. The number of incomplete salivary sheaths produced with the absence or presence of a spider predator on the upper vs. the lower surface of the leaf.

Leafhopper's in the control group produced more ss on the upper leaf surface and with better success, nearly 90% of ss were complete on the upper side, while on the lower side of the leaf the ratio of complete penetrations was 65%. However, in the presence of the spider several differences in the depth of the penetration and the leaf surface where it originated from was shown. The preference for upper vs. lower surface of the leaf, which in the presence of spider shifted to the lower surface. In the presence of the spiders leafhoppers also produced significantly more incomplete ss (t=-3.69 p= 0.0003). In the case of complete penetrations the interaction between leaf surface preference and spider treatment is notable.

Effect of PS on leafhopper feeding stages, detected by EPG - In this experiment we could detect in detail how hidden stages of the penetration process, also in relation to virus transmission, change in the presence of a predator (Tholt et al., 2018).

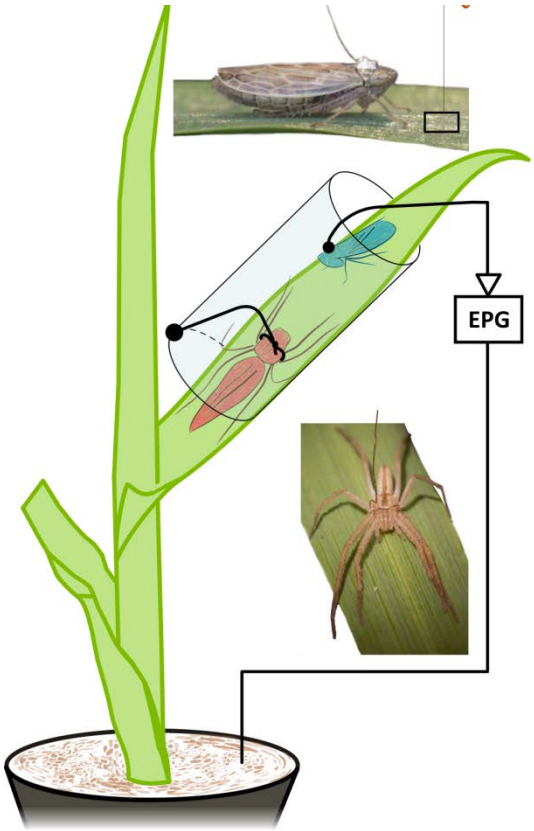

Figure 3. Schematic representation of EPG measurement with a leashed spider present.

Penetration events can be studied by electrical penetration graph (EPG), which technique consists of incorporating an insect and a plant in a common electrical circuit. When a sap-feeding insect penetrates its stylet into plant tissues, the circuit closes, and a fluctuating voltage, called 'EPG signal' generates. Different EPG signals (EPG waveforms) indicate different insect activities and stylet positions, so ingestion from phloem (and other phases of the feeding) can be separated. EPG waveforms of *P. alienus* penetration and correlation of stylet tips position was previously described (Tholt et al., 2015).

EPG recording sessions were five hours long; there were 10 sessions in total 31 treatment and 32 control cases. The penetration process was monitored using the EPG technique within a microcosm enclosed in an open ended glass tube. The microcosm contained (i) a section of the barley leaf; (ii) the leafhopper attached to the EPG electrode wire; and in the case of predator treatment (iii) a spider, also leashed on a wire fixed at the other end to the tube, ensuring that it could not reach the leafhopper (Fig. 3). Microcosm setups were made for each of every EPG channels and were placed inside the Faraday cage of the EPG device.

The leafhopper altered its feeding behaviour in response to predation risk. Considering the total duration of the behaviour, leafhoppers spent significantly less time engaged in penetration activities in the predator treatment than in the control (Fig. 4A). Overall, 16.2% of all penetrations reached phloem tissues (successful recordings of a phloem preparation phase during the event); while in 83.8% of the penetrations the stylets were withdrawn before phloem contact (Fig. 2). In the predator treatment fewer penetrations reached the phloem tissue (12.8%) in comparison to the control treatment (18.6%) ($\chi^2 = 4.29$, d.f. = 1, P = 0.038; Fig. 4C).

To detect possible non-consumptive effects on the different penetration phases we considered two main parameters, the total duration of a penetration phase and the delay until first occurrence of a phase. Starting time (delay) and total duration of the phases were calculated from the raw waveform data from the EPG measurements. Overall, leafhoppers spent significantly less time in the travel phase of the penetration process, when predators were present (Fig. 4D). The spider's presence also resulted in an increased delay until the occurrence of the first travel phase event (which is equal to the delay until first penetration) i.e. in the predator treatment there was a lower probability of starting a penetration than in the control (Fig. 4B).

In the case of drinking, predator presence did not influence either aspect of this phase but the time delay for leafhoppers to start their first drinking phase was longer in the predator treatment (Fig. 4F). We tested predator effect on the duration of the phloem preparation phase (Fig. 4G). When this phase occurred it was significantly shorter in the predator treatment. However, neither occurrence probability nor the time until the first occurrence of the phloem preparation phase (Fig. 4H) differed between the treatments. Phloem ingestion was significantly influenced by predator presence (Fig. 4I), yet the zero-added model suggests a response that differs from that in the preparation phase. The probability of the occurrence of phloem ingestion significantly depended on predator presence. In the predator treatment eight of 31 leafhoppers initiated phloem ingestion, while in the control group this phase occurred in 17 out of 32 leafhoppers. The time delay to initiate phloem

ingestion was significantly longer in the predator treatment (Fig. 4J), but the total duration of this phase did not differ significantly between the treatments.

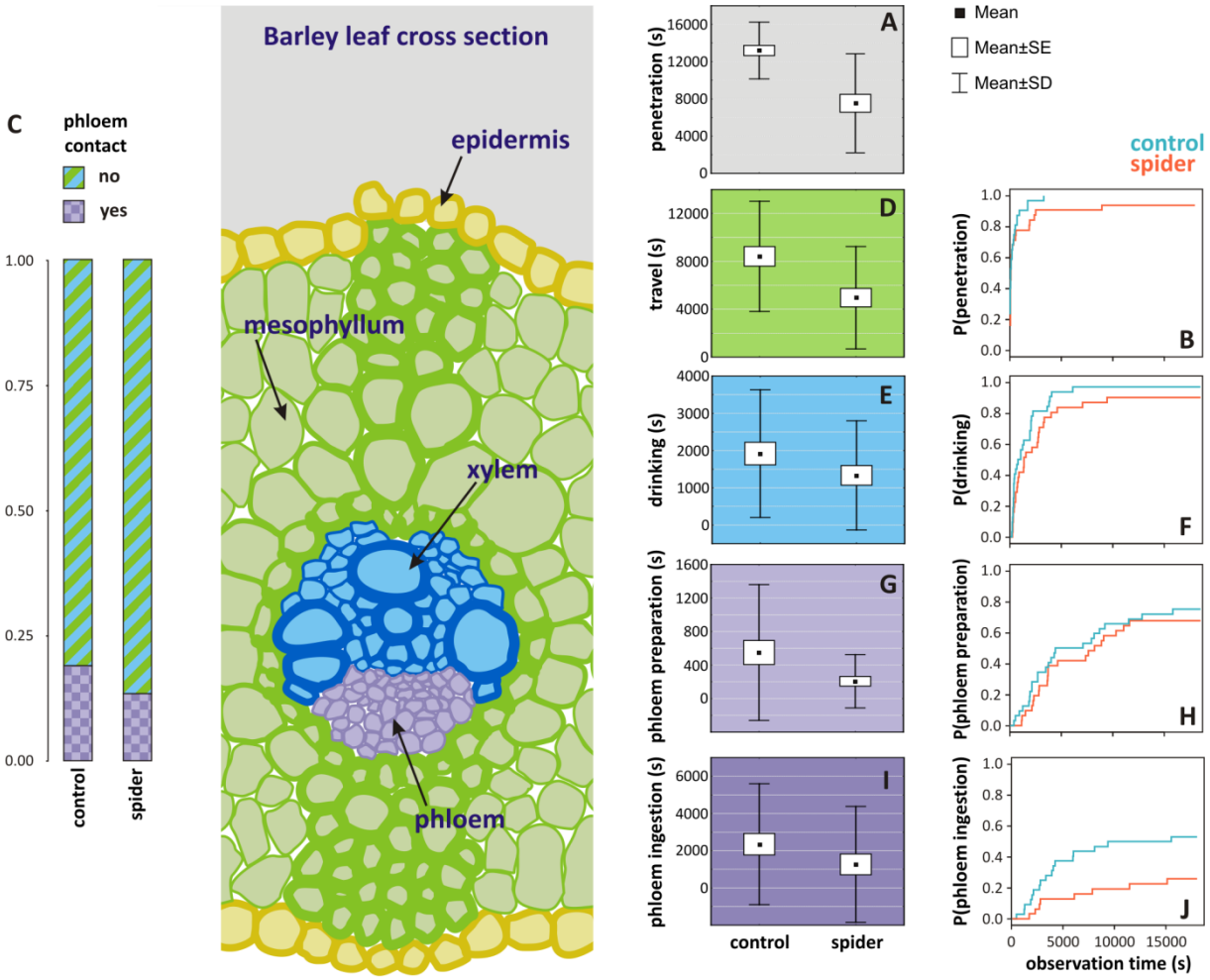

Figure 4. The effects of predator presence on penetration. PS effect on penetration phases are referenced against (colour coded) a leaf cross section where the phases take place. Between treatment comparison of total penetration duration and the probability of occurrence, respectively, for the following phases: travel (**D**,**B**), drinking (**E**,**F**), phloem preparation (**G**,**H**), phloem ingestion (**I**,**J**) and for complete penetrations (**A**,**B**). Boxplots depict mean, S.E. and S.D. derived from the raw data. Histogram under (**C**) gives the proportion of penetration events that either reached or did not reach phloem in the treatments.

In summary, phloem ingestion, the feeding phase when virus acquisition occurs, was delayed and was less frequent. The phase when pathogen inoculation takes place, via the secretion of virus infected vector saliva, was shorter when predator was present. Our study thus provided experimental evidence that predators can potentially limit the spread of plant pathogens solely through influencing the feeding behaviour of vector organisms.

Linking leafhopper feeding to WDV transmission **-** In co-operation with the Agricultural Biotechnology Institute we identified the localization of systemized WDV infection in the plant tissues to determine which penetration stage can be responsible for virus uptake and inoculation (Tholt et al., 2018).

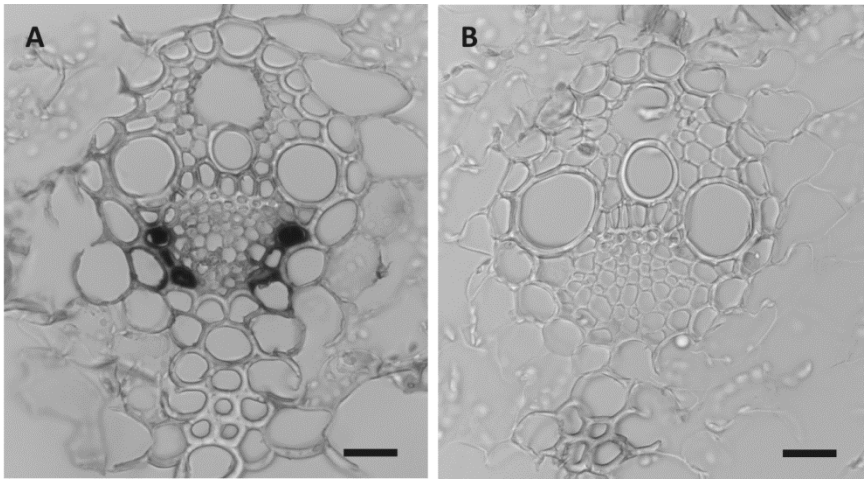

Figure 5. Barley leaf cross sections. WDV mRNA hybridization on virus infected (**A**) and control (**B**) leaves. (Scale bars = 20 μm.)

We transferred WDV from infected stock plants to barley plants using *P. alienus.* Infection was subsequently confirmed by PCR. Samples for in situ hybridization were collected four weeks after inoculation and then tested by our previously described protocol (Medzihradszky et al., 2014).

To assess how altered feeding activity might affect virus transmission, the previously unknown localization of WDV had to be confirmed. We examined the virus DNA localization in WDV infected leaf tissues, as well as mock treated controls, by in situ hybridization. This revealed that four weeks after virus infection in the treated samples (n = 12) WDV was clearly detectable only in the phloem tissues (Fig. 5A). No signals were detected in the control samples (n = 10) (Fig. 5B).

Although WDV has been considered as a phloem restricted virus, this is the actual first evidence which supports this claim. To prove this phenomenon was crucial because the most notable effect of PS on leafhoppers' penetration behaviour was the significant delay and reduction of salivation and ingestion activities in phloem tissues. Thus, in case of *P. alienus* predators can reduce leafhoppers possible vector capabilities by limiting the time is spent in those plant tissues where the virus is localized.

Effect of PS on leaf consumption as a function of temperature **-** In a collaborative experiment with University of Kentucky we investigated whether environmental factors, such increased temperature originating from growing conditions or climate change may change PS effect in a tritrophic system (Beleznai et al., 2017). We analysed how two spider species, with differential responses to warming, interacted with spotted cucumber beetles at different temperatures, and how these interactions finally affected cucumber plant damage. Our initial prediction was that spiders would increase beetle mortality, change beetle activity and decrease damage to plants.

We performed laboratory experiments exposing an important pest species to two spider predator species at different temperatures. Heat tolerance was characterised by the critical thermal maxima parameter (CTM50) of the cucumber beetle (*Diabrotica undecimpunctata*), wolf spider (*Tigrosa helluo*), and nursery web spider (*Pisaurina mira*). Cucumber beetles and wolf spiders were equally heat tolerant (CTM50 > 40 °C), but nursery web spiders had limited heat tolerance (CTM50 = 34 °C).

Inside mesocosms, beetle feeding increased with temperature, wolf spiders were always effective predators, nursery web spiders were less lethal at high temperature (38°C). Neither spider species reduced herbivory at ambient temperature (22°C), however, at warm temperature both species reduced herbivory with evidence of a dominant non-consumptive effect (Fig. 6). Our experiments highlight the contingent nature of predator-prey interactions and suggest that non-consumptive effects should not be ignored when assessing the impact of temperature change.

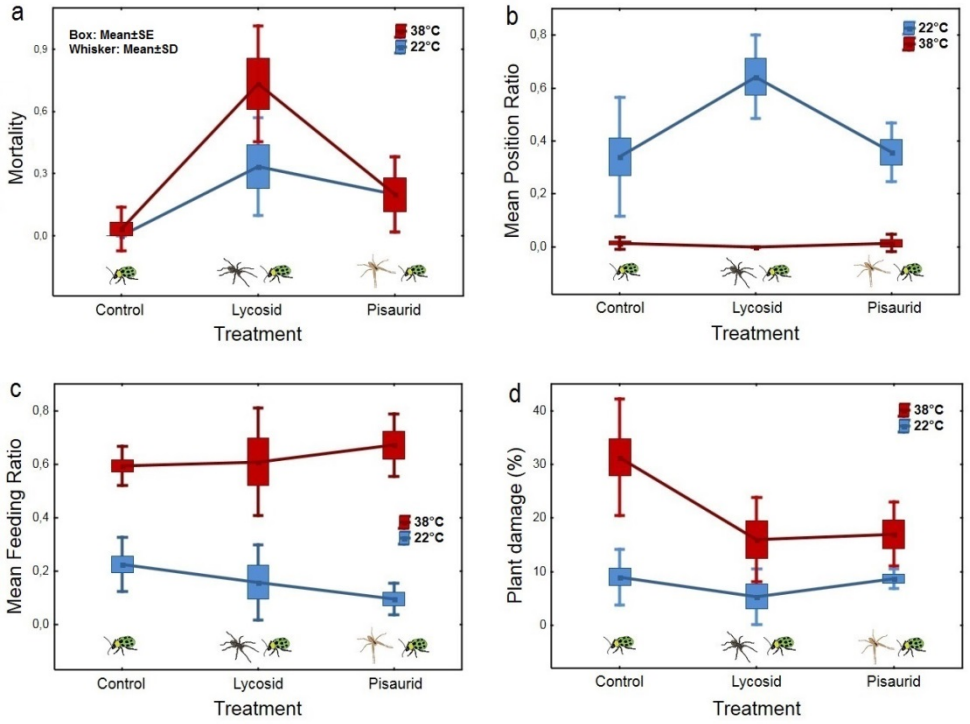

Figure 6. Cucumber beetle responses. Mean cucumber beetle responses in mortality (**a**), Mean Position on plant (**b**), Mean Feeding Ratio (**c**) and Plant Damage (**d**) to the three predator treatments (control, lycosid, pisaurid) under the two different temperatures (22 °C, 38 °C).

If warming is limited (for which management options exist) the complementarity of predators' heat tolerances may offer biological control over a wider temperature range, and the mode of predator effect (consumptive vs. non-consumptive) may also complement each other. The present results underline the effectiveness of generalist arthropod predators in such systems. The interaction between the thermal environment and the modes of predator effect found here, shows the necessity to study this potential interaction in other studies that asses the effect of global warming on ecosystem functioning.

Effect of PS on leafhopper fitness **-** The indirect effect of predator presence might not only affect actual feeding behaviour, but on the long run may decrease the fitness of the herbivore. To test this effect we exposed *P. alienus* individuals to spider presence in divided microisolators.

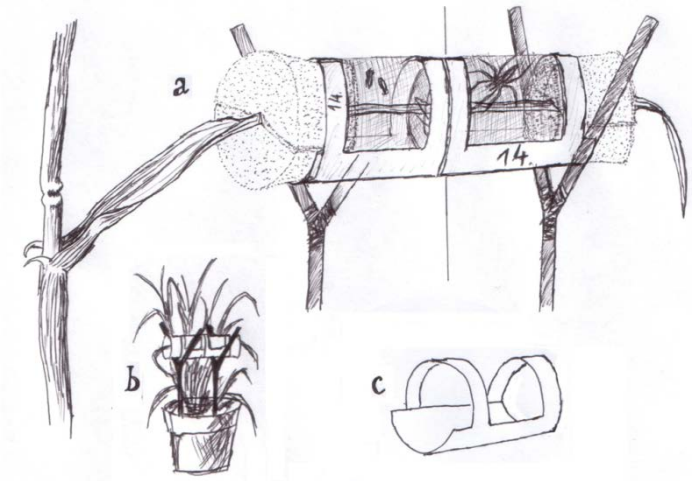

Figure 7. Schematic representation of leafhopper fitness micro-isolator experimental setup.

Experimental design (plants, microisolators, spiders) was the same described in salivary sheath morphology experiments. Two leafhoppers, a male and a female were inserted into the isolator's cell which was adjacent to the stem. Into the opposite cell a spider were inserted (treated), or left empty (control) (Fig. 7.).

Experiments (exposure to spider) lasted for 4 days. We ran two sets of experiment, all together 50-50 replicates for both the control and the spider treatment. After the treatment isolated leaf sections were carefully removed and stored in a solution of distilled water, 99% glycerine, and 85% lactic acid (1:1:1 vol) in thermostat (37.5°C) for overnight.

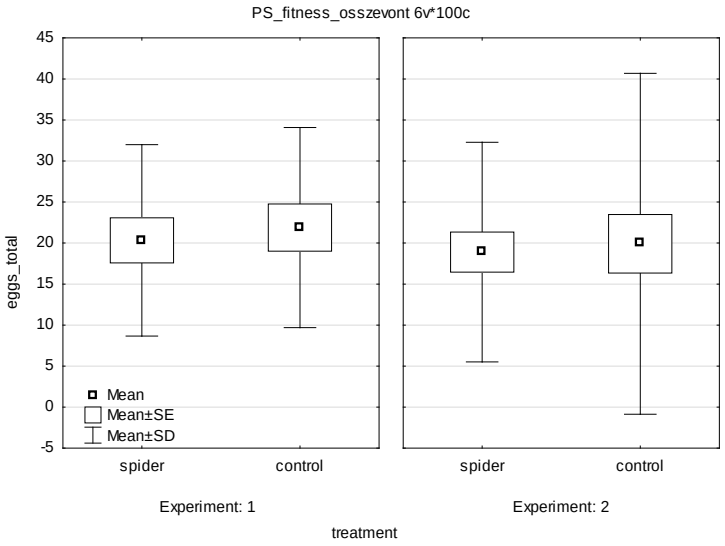

Figure 8. Total number of leafhopper eggs laid (on leaves + on artificial surfaces) in two experimental sessions under PS and control treatments.

After leaves were soaked in with McBride's stain for 6 hours (room temperature), then washed in PBS-solution, and put back into the lactic acid solution filled vials overnight.

The leaves were examined under light microscope where we counted the stained leafhopper eggs injected in the plant tissue. Besides we had to count the eggs layed on the isolator's mesh, on the plastic spine, and on the sponge-plug.

The total number of eggs produced by the leafhoppers did not differ statistically between the spider and the control treatment in either experiment, even though on both occasions eggs laid in the spider treatment were numerically slightly less (effect of treatment: $t_{98} = 0.38$, NS, Fig. 8).

The effect of leafhopper body condition on how feeding behaviour changes as a response to PS - Effect of PS on leafhopper foraging and feeding behaviour is likely to be modulated by leafhopper body condition. Leafhopper body condition can be experimentally varied by different starvation periods. Behavioural observations were conducted in mesocosm arenas of potted plants. Potted plants were enclosed by a plexiglass tube meshed on top, where we observed a) the unrestricted foraging and feeding behaviour of leafhoppers in control situation and in the presence of unrestricted spider. b) we observed the effect of previous starvation on leafhopper feeding behaviour in EPG observations.

a) We ran pilot experiments to study starvation status on the feeding behaviour in predator presence. Leafhoppers were starved for 30 min, 1hr, 2hrs, 3hrs, 4hrs. During 30 min observations there was no difference in feeding frequency among the no-predator observations. However, when a spider was present leafhoppers suffered 100% predation over the 30 min observation period. We conducted further trials to see the effect of extended starvation period on leafhopper survival, without predator presence. According to these observations after 12 h starvation there was a 20% mortality, but after 24 h starvation it raised to 100%.

b) More detailed observations on the effect of starvation on leafhopper feeding behaviour were done with EPG recordings. We have compared the effect of four starvation treatments [n=29 (control, 0), n=36 (1h), n=31 (2h), n=28 (4h)] on the start of first penetration which was determined from EPG curve analysis. The median time to start penetration was marginally different between treatments according to Wilcoxon test of survival ($\chi^2 = 4.29$, d.f. = 3, P = 0.042), and there was a significant difference between the start of the first penetration in unstarved (median =287 sec) and that of the leafhoppers in the starvation treatments (medians 136, 119, 114 sec, respectively; proportional hazards test: $\chi^2 = 4.76$, d.f. = 1, P = 0.029) (Fig. 9).

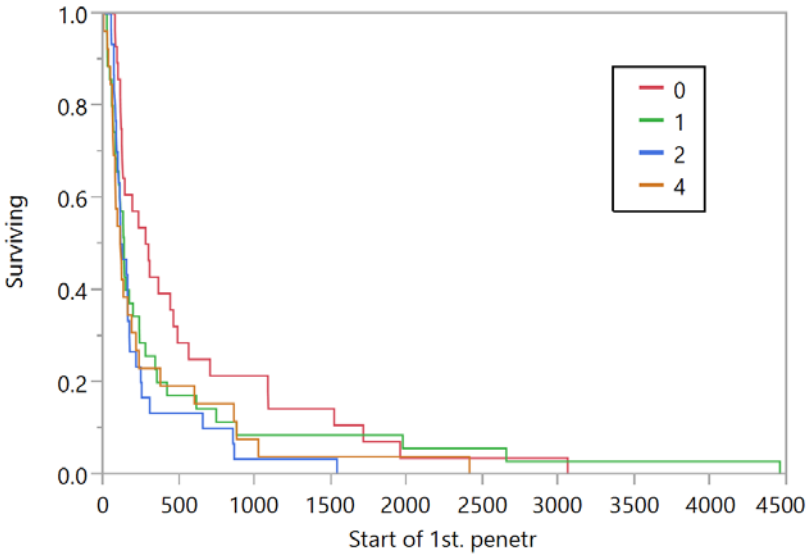

Figure 9. Survival curve of leafhoppers of different hunger status for starting first penetration. Survival is defined as the proportion of leafhoppers has not started penetration to the time point. Start of first penetration in sec from beginning of experiment. Different colours signify hunger status of starving for 0, 1, 2 or 4 hours right before the experiment.

Modalities of perception by the leafhopper that may play role in host plant and predator detection

In these studies, we collected headspace volatiles from various subjects, tried to test leafhopper olfactory responses to these stimuli with various methods and attempted to find out why our attempts were eventually much less successful than in the case of usual insect subjects (e.g. moths) in chemical ecological studies.

Feeding behaviour of herbivorous insects is greatly affected by environmental olfactory cues which might originate from the host or non-host plants as well as from animal sources, such as predators or conspecific individuals. A starting point to investigate such sensory processes is to collect volatiles from subjects of interest and store and use these in trials testing the olfactory responses of the insect. We collected headspace volatiles with open type collecting device for 4 and 24 h time intervals, after absorbing the volatile component on charcoal filter eluting of odorants was performed with *n*-hexane organic solvent. The solutions were stored at -40°C. Volatiles were collected from the following subjects: (a) *Psammotettix alienus*, (b) barley (different growth stages), (c) leafhoppers during feeding, (d) barley infected with WDV, (e) leafhoppers feeding on WDV infected barley, (f) non-host plant (*Carex sp*.), (g) spider: *Tibellus oblongus*, (h) spider silk; plus control collection to all subjects.

The volatiles of spider and spider silk were tested using a gas chromatograph coupled with mass spectrometer (GC-MS). The volatile composition of spider silk was similar to spider volatiles; however, the amount of the components were lower in spider silk. We also analysed the volatile profile of the plant (barley) using GC-MS. We found that the plant produces the typical green leaf volatiles, which have been used to stimulate the antennae.

We intended to test the effect of the collected volatiles on leafhopper antennae with Electroantennography (EAG) device. Since initial measurements were more noisy than in other insect subjects, we set out to develop a protocol that maximized the resulting signal-to-noise ratio. The most appropriate preparation method was to fix the head of the leafhopper in a glass capillary filled with Ringer solution. We cut the bristle (arista) of the antenna and fixed it into an approximately same diameter glass capillary. We found that the leafhopper antenna is extremely sensitive to ambient vibrations, therefore we used an anti-vibration table, on which we finally achieved a suitable low noise baseline. In the next step, the antenna was stimulated with volatile collection extract collected from barley samples and synthetic odours using a stimulus controller. The synthetic odours or the volatile collection extracts were applied onto a small filter paper (1x1 cm). This filter paper was placed into a Pasteur pipette serving as a stimulus. However, most likely because of responses from large mechanoreceptors, we were unable to separate olfactory responses from blank stimulation.

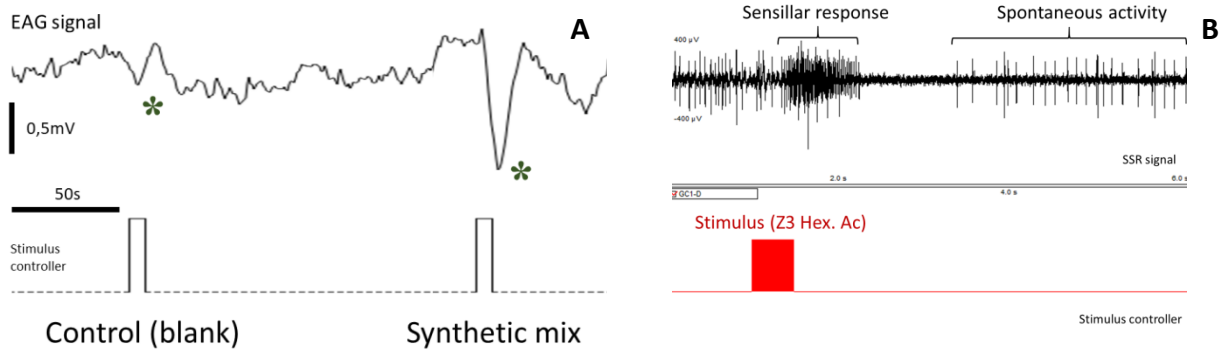

Figure 10. (A) Antennal responses (marked by asterix) measured by EAG and (B) single cell responses on labium measured by SSR and of *P. alienus* female to Synthetic volatile compounds mix.

A total of 30-30 male and female leafhopper antennae were tested with different odours and stimulation methods. To eliminate mechanical responses, GC-EAD measurements were performed using the volatile collection extracts. Although this method succeeded in eliminating noise, however, olfactory responses were only recorded in a few cases and in a rather unpredictable manner: in different replications, we had a response to different odours. As a next step, based on the GC-MS results, we wanted to stimulate the antenna with high odour concentration, therefore we prepared an odour mixture with high concentration of synthetic barley volatile compounds (100-100 µg of Z-3-hexenyl acetate, Z-3-hexanol acetate, octanal and decanal). GC-EAD recordings were executed with this synthetic mixture (Fig. 10) in 12 replicates. The evaluated results still showed that antennal responses are sporadic.

To investigate why we were so unsuccessful with studying the olfactory detection of leafhopper, we decided to examine the sensory hairs (sensilla) on the antennae. We produced light microscopic, SEM, and layer photomicroscopy (Keyence microscope) images of the antennae using various staining and native techniques. Based on the SEM images, we identified one mechano- or hygroreceptor on the first, second and third segment of the *P. aliens* antenna. We found only 1 or 2 potential olfactory chemoreceptors per segment which were difficult to identify, because they were covered with a leafhopper produced substance, so-called brochosomes (Fig. 11 A-C). These proteins are produced exclusively by leafhoppers, are extremely water repellent, and their role presumably lies in reducing the chance of honeydew adhering to the individual. By trying several different preparation methods (native, dehydrated and rinsed in alcohol, purified with acetone; fixed on a carbon tape and charred), the brochosome cover could not be reduced. We concluded that there are no chemoreceptors on the other segments of the antenna, and overall *P. alienus* antenna is poor in chemoreceptors to an extreme degree.

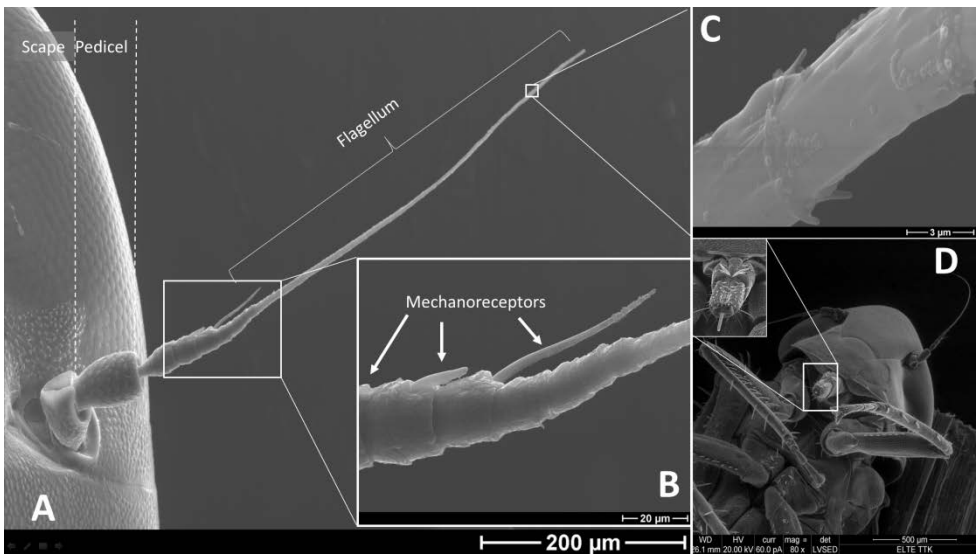

Figure 11. *P. alienus* antennae anatomy. Overview of a female antenna (A); Detailed view of the mechanoreceptors of the first 3 segments (B); Detailed view of the flagellum covered by brochosomes (C); Lateral view of the head of *P. alienus*, with enlarged pictures of the labium with sensilla (D).

Preparing the SEM images, we observed that, unlike on the antenna, on the labium there are a number of sensilla that are presumably non-contact chemoreceptors (Fig. 11 D), which due to their position, can be responsible for the detection of olfactory stimuli. Therefore we also tried the EAG setup together with stimulus controller to examine the response of labial sensilla to olfactory stimuli. Unfortunately, we could not properly stimulate the labium using this method. As a final step in our efforts, we used the Single Sensillum Setup (SSR), by which we were able to prove that these labial sensilla can detect olfactory stimuli (Fig. 10B), but these studies, as of the end date of the project, did not get further from that point.

Mesocosm studies into the cascading effect of predator presence

In this study we observed how spider presence affects virus spread as a mass effect in mesocosm enclosures. The basic idea was that virus carrying leafhoppers would infect plants with lower frequency if predators are present on / in the vicinity of the plant. In a mesocosm we intended to separate plants with predators and predator free ones with a "semi permeable" net, through which leafhoppers can move freely, but spiders cannot.

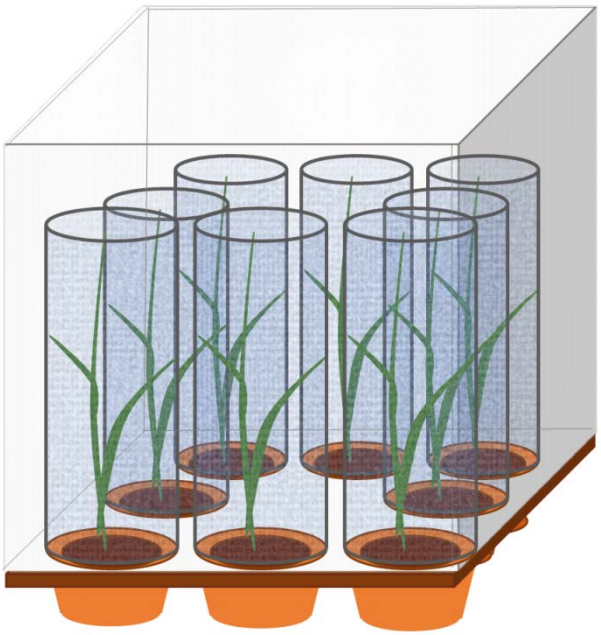

Figure 12. Schematic plan of the mesocosm design. One spider was placed in 4 randomly selected enclosure.

We had several preliminary trials to finalize mesocosm design, experimental procedures and testing. Our first design, which most resembled the initial plan in the project proposal, consisted of a large cage (38x52x52 cm) with a dividing mesh in the middle, where spiders could not get through but leafhoppers could. Due to difficulties of homogeneously growing in the right density and growth stage of experimental plants, after several variations and trials, we decided on a setup, where barley plants were pre-grown in small rectangular pots, and for one mesocosm 8 plants reaching the predetermined growth stage (4 leaves) were assembled together gap-free in a cardboard template into a 32x32x40 cm arena enclosed in a fine mesh cage (Fig. 12). Each plant was enclosed with a cylinder of "semi-permeable" mesh, through which leafhoppers could move, but spiders could not. Four randomly assigned plants had a spider, four were predator-free. Leafhoppers were selected from our WDV infected stock populations and placed on WDV infected barley plants (checked with PCR testing) for 4 days. According to preliminary experiments that guaranteed that the vector animal becomes a virus carrier. During one experimental trial we made parallel observations on 5 mesocosm arenas (out of these one served as procedural control, did not contain spiders at all). We had two such trials, during Trial 1. we entered 12 leafhoppers / mesocosm arena for 24 h; in Trial 2. we entered 9 leafhoppers / mesocosm arena for 6 h exposure. All plants after the trial were sprayed with the insecticide Karate Zeon and this treatment was repeated on two more occasions plus the plants were kept separately from our leafhopper stock populations and under mesh enclosure. These procedures were meant to ensure that during the incubation period there are no nymphs emerging from viable eggs, and there are no remaining or vagrant animals that would transfer virus infection to plants that remained uninfected during the trial. After 5 weeks of incubation samples were taken from the plants and virus presence was tested with PCR. In Trial 1 we had 100% virus infection both with spider presence and without spiders (also 100% in control). In Trial 2 we could show WDV in plants with spiders in 87.5% of the cases, exactly the same percentage was found in the control mesocosm, whereas 75% of the predator free plants in the treatment mesocosms became virus infected (χ^2 = 0.24, d.f. = 1, P = 0.62).

In the two final mesocosm trials statistically the predator treatment effect was not significant. These results contradict with previous results from EPG monitored behavioural reaction of leafhoppers to spider presence. Interpretation of these results is not straightforward. One possibility is that a certain early penetration stage (stage 3) might already involve phloem contact. Correlating penetration stages in EPG recording with concrete physiological events is methodically difficult, since an independent assessment of is needed with independent method(s). Alternatively, we may have the correct interpretation of penetration stages, but the mass effect is contrary to our null hypothesis, and predator presence results in shorter feedings that through more frequent relocations of the vector animals results in not fewer, but rather in even higher transmission events. In the extension year we could investigate the first option in greater detail, for which we also needed to work out a fast and reliable histological method to determine stylet tip positon in the plant tissues.

Field surveys to study virus, vector and predator incidence and their possible correlations

In the field survey we intended to a) provide object for laboratory methodological and systematic study that originate from natural, field conditions; b) to obtain data about the field occurrence of the main components of the studied model system: natural enemy spiders, virus vector leafhopper and the WDV virus.

We conduced systematic field surveys in 2016 and 2017 in the area of the following settlements: Páty, Vértesboglár, Tabajd, Zsámbék, Velence and Martonvásár, altogether at 12 locations in each year with 3 sampling session (temporal replicates) per year. One location consisted of a cereal field and a neighbouring grassy field margin. Arthropod samples were taken by motorised suction sampler (5 samples / sampling session/ habitat), leaf samples (3 x 10) were hand collected. A nation-wide more extensive sampling was conducted to establish the incidence of WDV. At every sampling point 10 leaf samples from 10 plants were collected. In 2017 we had 26 sampling points, in 2018 39 and in 2019 140 sampling points. In separate studies, using previously collected data, we analysed the composition of the spider natural enemy complex (Samu et al., 2018), which gave us a figure for natural enemy abundance and diversity in typical agricultural landscape settings. In another study we could show the beneficial influence of natural enemies on pest damage in wheat (Lajos et al., 2020).

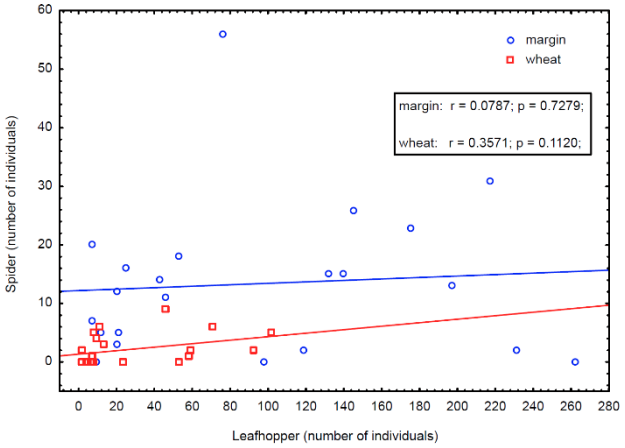

Figure 13. Correlation between leafhopper and spider abundance in wheat fields and margins

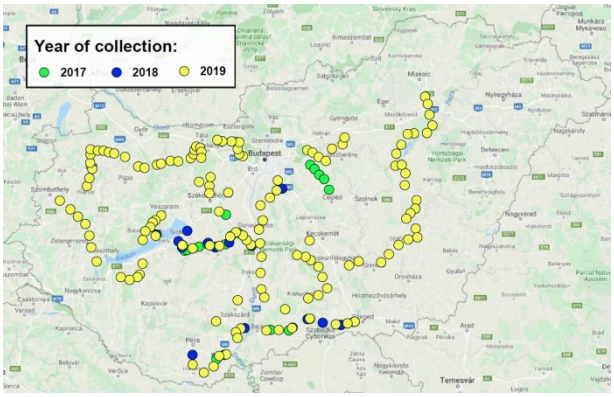

Figure 14. Sampling points of the nation-wide WDV survey.

During the systematic survey we could show the presence of 33 spider species (1127 individuals) and those of 5 *Psammotettix* species (2773 individuals). The abundance of spiders and leafhoppers showed weak, non-significant positive association both in the margin and in the wheat habitats (Fig. 13).

We also analysed the congruence between WDV infection in the plant and leafhopper samples considering both the crop field and margin habitats. Positive association was found between leafhopper and wheat WDV infection considering all samples (χ^2_1 = 7.11; P = 0.007) and in the wheat field samples (χ^2_1 = 4.07; P = 0.04), but no significant association if only margin samples were considered. If we considered the association of WDV infection between the crop field and its margin, then we also found a significant association in the plant

samples (χ^2_1 = 9.31; P = 0.002), but no significant relationship between the WDV carrying status of the leafhoppers collected from the field and its margin.

In the nation-wide survey (Fig. 14) we found a very low incidence of WDV infection in the wheat samples. In 2017 0/26, in 2018 1/39, in 2019 3/140. In some years WDV can cause serious damage for growers, however its occurrence shows huge yearly variations (Pribék et al., 2006; Pocsai & Murányi, 2010). On the other hand, the difference between higher incidence in systematic samples (with temporal and spatial replicates) versus one-time 10 sample survey suggests that even if the virus is present in a field, it might infect only a small(er) proportion of plants.

Molecular ecological studies to follow cascading events in our model system

Within the framework of the project we aimed to integrate DNA-based molecular studies into our methodology in order to check for the presence of WDV in various lab studies, as well as in field samples, and also to establish, whether and to what extent our model natural enemy spider feeds on the leafhopper prey under field conditions. The latter involved the development of molecular gut-content analysis for the first time in Hungary.

Infrastructure development, foundation of molecular ecology lab **-** With the support of the grant we established a brand new basic PCR laboratory. The PCR cabinet allows us to work in sterile environment by minimising the possibility of contamination while the consumed equipment (PCR machine, centrifuge, electrophoresis unit) makes us able to carry out elementary molecular ecological investigations form DNA extraction to amplicon clean-up for sequencing. To make the molecular lab operational we needed to develop and implement reliable protocols to detect WDV and *Psammotettix* DNA from various samples. This task was at the beginning assigned to Ms. Orsolya Beleznai, who tried to implement protocols she had learnt during her visit to University of Kentucky, USA. After two years she left for abroad, and we could employ Dr. Dávid Fülöp with a much broader molecular experience. From 2017 on Dr. Fülöp basically restarted protocol development and his work resulted in stable protocols that are now routinely used in the lab.

PCR protocol to test for WDV **-** We tested and adopted published protocols for virus DNA extractions from plant and insect materials and PCR reactions and designed, tested and compared primers and alternative methods. We adopted the WDVrepDetF and WDVrepDetR primer pairs amplifying 450 bp of the rep region of the WDV genom (Kis et al., 2016). We also tested the effect of sample pooling on DNA isolation. Our result suggests that our detection method is strongly reliable even in the case of one infected sample out of twenty. Based on this methodological framework we were able to develop pipeline for monitoring WDV presence in our *Psammotettix alienus* cultures, and forecast WDV infections in plants even before presence of any clinical signs. This protocol is in continuous use for testing for WDV in lab grown plants and leafhopper stock populations, virtually in all experiments (e.g. mesocosm study) and in testing field samples (e.g nation-wide random survey for WDV).

PCR protocol to test for Psammotettix DNA **–** Ten taxon specific primer pairs, amplifying 200-300 bp long segment of mitochondrial cytochrome-oxidase I (COI) barcoding region was designed using NCBI Primer-blast home page (www.ncbi.nlm.nih.gov/tools/primer-blast/) following the recommendations of King et al. (2008) and tested in silico for *Psammotettix* specificity against GenBank nucleotid database of Arthropoda taxa with the same application. After optimisation we chose the Psam268F and Psam483R primer pair for further application.

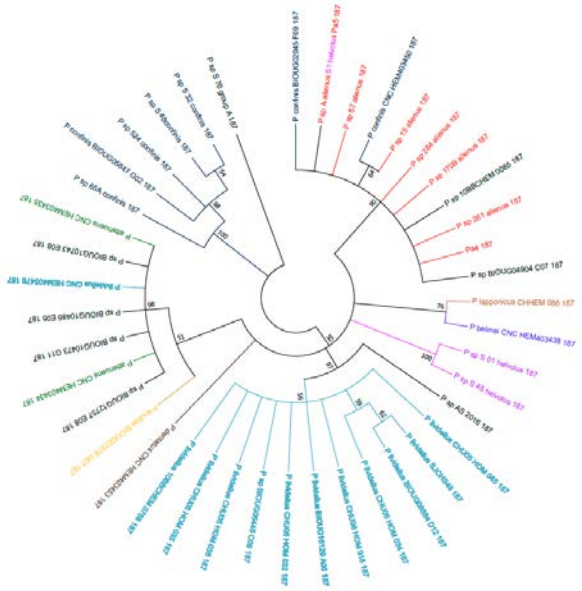

Figure 15. 50% consensus Neighbor Joining tree of the overlapping 187 bp length region of COI sequences (1000 Bootstrap replicates). *P. alienus* is marked with red.

Psammotettix alienus is the only known vector of WDV, virus carrying could not be shown from other congeneric species, some of which also occurs in agricultural fields, although they can be mostly found in grassy field margins and meadows. Therefore, we examined if the development of species specific primers was possible.

We used the available COI sequences identified at species level in Genbank and the COI sequences from Abt et al. (2018) to construct phylogenetic trees and networks to identifying stable monophyletic groups corresponding with morphospecies (Fig. 15). This tree clearly indicates the unresolved position of *P. alienus*. This is due to the typical short sequence lengths used in gut content analysis, the high similarity in sequences, the low number of sequenced species (9 from 121) and probably misidentified specimens. According our recent knowledge 100% species-specific primers could not be designed. Sequenced PCR products could be identified to species level following the species concept in Abt et al. (2018).

Molecular gut content analysis calibration **–** Having developed the PCR protocol for the detection of *Psammotettix* DNA, we intended to use it to identify whether predators consumed this prey. First we tested in silico for applicability using the primer blast resource of the NCBI genbank website. For laboratory testing *Psammotettix*, *Drosophila* (stable food for spiders in our laboratory) and *Tibellus* DNA were used. With the above confirmation of applicability, in order to interpret future field data, the rate of prey DNA decay in the predators' gut, described by DNA detectability half-life ($t_{1/2}$), was needed to be established. In a paper in PeerJ (Fülöp et al., 2019) we described *Psammotettix* DNA detectability in our model predator, *T. oblongus*. Since DNA decay may depend on the presence of alternative prey in the gut of generalist predators, and this issue has not been investigated in spiders, this effect was also the objective of our study. In a laboratory feeding experiment we determined $t_{1/2}$ of *P. alienus* in the digestive tracts of the spider with or without the additional food of fruit flies, *Drosophila melanogaster*. After feeding treatments spiders were starved for variable time intervals prior to testing for leafhopper DNA in order to establish $t_{1/2}$. Detectability limit was reached at 14 days, where c. 10% of the animals tested positive. The calculated $t_{1/2}$ = 5 days value of *P. alienus* DNA did not differ statistically between the treatment groups which received only the leafhopper prey or which also received fruit fly (Fig. 16).

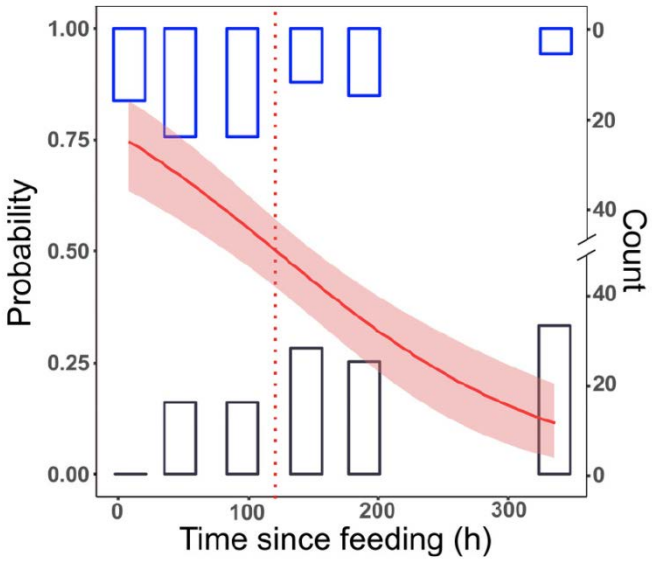

Figure 16. Detection of prey DNA in *T. oblongus* gut content with diagnostic PCR. Red line: fitted logit model with SE.; dotted line: $t_{1/2}$, bars: cumulative number of positive (blue) and negative (black) PCR.

F***ield applicability trial of molecular gut content analysis*** **-** To test whether our PCR protocol can be used to determine if field collected spiders contain *P. alienus* DNA in their gut contetnt, a small field survey was conducted on 10.10.2019. in self-sown wheat, near Perbál. With hand collection we collected N = 40 *T. oblongus* individuals. These post-harvest fields with self-sown cereal had a modest *P. alienus* density. Leafhopper species identity present in mass in the field was checked from sweep net samples. Each collected spider individual was immediately killed on dry ice, transferred and stored in a freezer. These individuals were tested for the presence of *Psammotettix* DNA the same way as individuals from the laboratory feeding trials. Five out of 40 field collected spider were tested positive to leafhopper DNA. For certainty amplicons from all positive samples were sequenced by Sanger method, quality checked and they were all identified as *P. alienus* using nucleotid search (nt-blast) on Genbank and Boldsystems database and other molecular species identification/delimitation methods.

Methodological results

We were able to detect significant changes in leafhoppers penetration behaviour as a consequence of PS effect in our EPG experiments, parts of them occurred during mesophyll phase. The mesocosm experiments also raised the possibility the observed waveforms during feeding might have a different role in WDV transmission than we previously thought. Therefore we have decided to revise our previously published waveform correlation (correlating EPG waveforms with their biological meanings) study (Tholt et al., 2015). To ascertain the biological meaning of a recorded waveform, independent information is needed about stylets tip position within plant tissues and other ongoing biological processes.

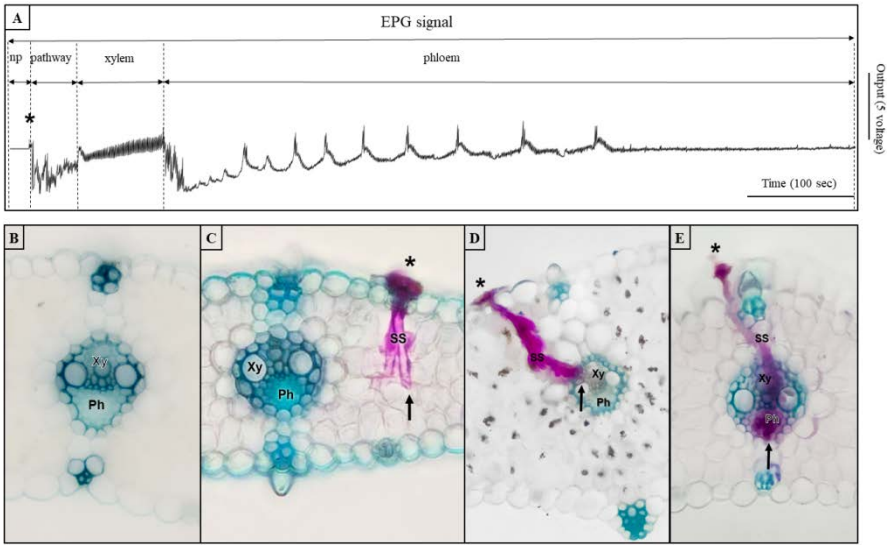

Figure 17. An examplar EPG waveform of *Psammotettix alienus* with stained salivary sheaths. The salivary seath developments (where they end) can be connected to certain waveforms representing different stages of the penetration process.

Stylet tip position in plant tissues can be observed by making histological images of interrupted feeding, where the salivary sheath developed up to a certain feeding stage can be visualised (Fig. 17). To produce such histological slides in large quantities we developed a simplified histological procedure where each step was optimized to offer a rapid process that does not require special equipment, can be applied to many samples, has good success rate and a low cost of errors in terms of time and materials. We described the procedure, using the *P. alienus* – barley model system, in three steps. (i) Clarification of entire plant parts and pre-staining salivary sheaths with aqueous fuchsin. This step allows to identify salivary sheath starting points on the surface. (ii) Knowing salivary sheath location, using hand sectioning, produce a single c. 60 µm section that contains the entire salivary sheath. (iii) Counterstain the section with methylene green and, after further clarification, study under light microscope in a glycerol - ethanol embedding solution, without fixed mounting. The resulting manuscript was published in the journal Micron (Gerstenbrand et al., 2021), while systematic EPG correlation studies based on this method have been finished and they are in the analysis phase.

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(references marked bold were part of the OTKA project, which was acknowledged therein)

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