Hungarian University of Agriculture and Life Sciences

# INTERACTIONS BETWEEN CHEWING AND PIERCING-SUCKING INSECT PESTS OF CEREALS: LABORATORY AND FIELD INVESTIGATIONS

PhD thesis

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# INTRODUCTION AND OBJECTIVES

The interactions between plants and phytophagous insects form a complex system in which plants play an active mediating role in the relationships among herbivore species. These so-called plant-mediated interactions can be synergistic, antagonistic, or neutral, and they fundamentally influence population dynamics and ecosystem stability. Although such phenomena are widely documented in the literature, studies focusing on arthropod communities in cereals remain limited. Since cereals occupy a substantial proportion of the arable land in Hungary, I consider it particularly important to gain a clearer understanding of the interactions among cereal-feeding herbivores, and to explore whether any of these interactions could be exploited in integrated pest management.

Worldwide, species of the genus *Oulema* (Chrysomelidae) are among the most important pests of cereals. In Hungary, the most common species is *Oulema melanopus* (red-necked grain beetle, commonly known as cereal leaf beetle, CLB), but its frequent associates include *Oulema gallaeciana*, *Oulema cyanipennis*, *Oulema duftschmidi*, *Oulema rufocyanea*, and *Oulema septentrionis* (Pozsgai and Sáringer, 2006). These species are highly similar in their morphology, biology, and damage patterns; therefore, no distinction is made between them in practice when applying control measures. Consequently, the literature often refers to them collectively as *Oulema* species or CLB, and I will follow this approach throughout my dissertation.

CLBs are insects with chewing mouthparts. By consuming leaf tissue, they significantly reduce the photosynthetic surface of cereal plants, thereby lowering yield potential. A single larva can decrease the assimilation capacity of a plant by approximately 10%, while in cases of severe infestation, this reduction may reach up to 80%, which can correspond to yield losses of about one ton per hectare. Since its introduction and invasive spread in North America in 1962, *O. melanopus* has caused yield losses of 10–20% in infested regions, with some areas reporting up to 30%. Field observations conducted in

Washington State between 2000 and 2002 showed infestation levels ranging from 13 to 126 larvae per 100 stems (with an average of 88 larvae/100 stems), resulting in yield reductions of approximately 2–4%. (Hitchcox et al., 2002).

One of the most common piercing-sucking cereal pests in Hungary is the *Psammotettix alienus* (Dahlbom 1871) (Cicadellidae). Although its direct feeding damage is negligible, it is economically important due to its role as a plant virus vector. This species is the exclusive vector of Wheat Dwarf Virus (WDV), which can cause significant yield losses in cereal crops. Infected plants—as the virus name suggests—remain stunted, grow in a bushy form, and develop characteristic yellow chlorosis on the leaves. Root size (both length and thickness) and number are reduced compared to healthy plants. Infested plants often produce sterile spikes, and fewer spikes develop overall (Pfrieme et al., 2022). In France, the average occurrence of WDV in cereals was 13.05% in 2008, 3.94% in 2009, and 6.14% in 2010 (Abt and Jacquot, 2015).

Another significant cereal pest is the bird cherry-oat aphid (*Rhopalosiphum padi*, L.) (Aphididae), which causes both direct and indirect damage. Its direct effects are reflected in reductions in yield, thousand-kernel weight, and the number of grains per spike (Szunics et al., 2000). Data from Hungary indicate that yield losses on plants affected by *R. padi* are as follows: winter wheat 5–13%, rye 12–18%, winter barley 28–48%, and winter oat 5–12%. Its indirect damage stems from its role as a virus vector: it transmits Barley Yellow Dwarf Virus (BYDV). Infected plants exhibit symptoms similar to those caused by WDV, which are often indistinguishable by visual inspection.

Compared to chewing herbivores, the feeding behavior of piercing-sucking insects is a highly specialized process, which is detailed below. Aphids and leafhoppers insert their mouthparts into the plant and feed on the sap flowing through the vascular tissues, a process known as penetration. During the pathway phase of penetration, the progression of the stylet through the plant tissue is facilitated by the so-called salivary sheath produced by the insect. This viscous, protein-rich secretion stabilizes the stylet bundle within the leaf tissue and aids its advancement. Once the insect reaches the phloem, it secretes watery saliva, which helps overcome potential plant defense responses. At this stage, plant viruses can be acquired and transmitted. This is followed by passive ingestion from the phloem sieve elements, driven by the plant's turgor pressure, allowing the sap to flow into the insect's alimentary canal.

As this process occurs within the leaf tissue, it remains hidden from direct observation. Monitoring penetration is possible using an electrical penetration graph (EPG) device. During EPG recording, electrodes are attached to both the insect and the plant soil, completing an electrical circuit. When the insect inserts its stylet into the leaf, the circuit is closed, generating a voltage fluctuation on the monitor, known as an EPG signal. This continuous signal can be divided into segments of characteristic amplitude and frequency, corresponding to the different phases of penetration. These phase-specific signals are referred to as waveforms. Analysis of the waveforms allows precise determination of the insect's activity within the leaf tissue, such as xylem sap uptake, saliva injection, or passive phloem feeding—the stage associated with actual nutrient intake. Waveforms can vary among species, making their identification essential for newly studied insects. Accurate interpretation of these signals requires staining of the salivary sheaths (Experiment 1).

The feeding behaviors of chewing and piercing-sucking herbivorous insects differ fundamentally, which in turn elicits distinct plant responses (Denno et al., 2000; Koornneef & Pieterse, 2008). Chewing insects consume plant tissues either partially (e.g., scraping or boring) or entirely, whereas piercing-sucking insects are highly specialized for feeding on plant sap. Feeding by chewing insects typically activates jasmonic acid–dependent signaling pathways in plants, which can induce the production of digestion-inhibiting or deterrent compounds throughout the plant, depending on the species (Schweiger et al., 2014). In contrast, piercing-sucking insect feeding is more often associated with activation of salicylic acid–dependent signaling pathways and, in certain plant groups, the opening of calcium channels in phloem tissues (Tjallingii, 2006). These responses trigger the production of substances—mainly proteins—

that can coagulate within the phloem sieve tubes or the insect's alimentary canal, thereby obstructing sap ingestion (Will et al., 2007).

Understanding the dynamics of pest communities in cereals is not only of theoretical interest but also has direct practical significance. Investigating interactions between pests with different feeding modes provides opportunities to improve integrated pest management strategies. For example, if the prior presence of certain piercing-sucking species reduces the damage caused by a chewing beetle—or vice versa—this knowledge can be applied to regulate pest populations, reduce pesticide use, and enhance the effectiveness of natural enemies.

To address the research questions outlined above, a comprehensive, sequential series of experiments was designed. The aim of these investigations was to determine how the presence, feeding behavior, and colonization order of certain species influence the feeding patterns and reproduction of other species. This approach allows for a better understanding not only of direct interspecific interactions but also of ecosystem-level effects, thereby contributing to more sustainable cereal production.

The main components and objectives of the experimental series were as follows:

1. Experiment – Development of a novel salivary sheath staining method

The aim was to develop a faster, more cost-effective, and simpler histological technique. This new method enables a more precise and detailed investigation of the feeding behavior of sap-sucking insect species that produce salivary sheaths.

2. Experiment – Field studies

Investigation of the impact of natural enemies on the damage caused by cereal leaf beetles under field conditions. This experiment provided quantitative evidence of how the presence of natural enemies reduces the damage caused by *Oulema* species and its direct effect on crop yield.

3. Experiment – Effect of precolonization by *P. alienus* and *R. padi* on *Oulema* feeding

In this experiment, I used choice tests to examine how pre-colonization and feeding by the sap-feeding insects (*P. alienus* and *R. padi*) influence the behavior and feeding damage of the chewing insect *Oulema* spp.

4. Experiment – Effect of Oulema precolonization on *P. alienus* feeding and reproduction

Similar to the previous experiment, I conducted choice tests to assess how feeding by the chewing insect (*Oulema* spp.) influences the feeding behavior and offspring production of the sap-feeding insect (*P. alienus*).

5. Experiment – Electropenetrography (EPG)

This experiment complemented the previous one (Experiment 4) by providing a detailed analysis of *P. alienus* feeding behavior under different treatments: mechanical wounding, damage caused by *Oulema* species, and untreated control plants. Its significance lies in the fact that, unlike choice tests—which only show how frequently insects select a plant—EPG allows precise measurement of the time spent feeding in the leaf tissue or searching for vascular tissues. This detailed information is critical for understanding processes such as plant virus transmission, as prolonged salivation can enhance virus inoculation efficiency.

Overall, the aim of this research was to expand knowledge on plant-mediated herbivore interactions in Hungarian cereals, contributing to a deeper understanding of cereal pest communities and supporting the development of integrated pest management systems. The results are valuable not only from a scientific perspective but also provide directly applicable information for agricultural practice, thereby assisting farmers and promoting sustainable food production.

# MATERIALS AND METHODS

## 1. Objects of study

### 1.1. Plants

For the laboratory experiments, I used barley (*Hordeum vulgare*, cultivar Mv. Conchita) at the 2–4 leaf stage, each plant grown individually in small pots (200 ml capacity). The plants were propagated from certified, pathogen-free seeds and cultivated in a climate chamber at 21 °C, with 16 h of artificial light per day (L16:D8) and 40–50% relative humidity.

To maintain insect cultures, approximately 10 seeds were sown per larger pot (1.2 L). The insects were introduced onto the plants once they reached the tillering stage. Potted plants with insects were covered with isolation nets to contain and protect the experimental populations.

### 1.2. Insects

I maintained the *P. alienus* colony under the same conditions as the plants. Individuals used for the measurements were selected from this colony. To prevent long-term genetic deterioration, the colony was regularly refreshed with insects collected from the field. These field-collected individuals were kept in quarantine until one generation had developed, after which they were incorporated into the base colony. The leafhoppers were gathered from barley fields near nearby settlements (Páty, Zsámbék, Tök, Perbál), and after one generation in the laboratory, they were mixed with the established colony.

I maintained the *R. padi* colony in a similar way to the leafhopper colony, in a separate, dedicated room.

The CLBs (*Oulema* spp.) were collected from a small experimental barley plot (1.5 × 1.5 m), specifically maintained to attract *Oulema* species from the surrounding areas. The collected beetles were kept on potted barley plants under a mesh in a climate chamber at 21 °C with 16 hours of light per day (L16:D8).

A one-time species identification of a sample containing 135 individuals showed the following composition in the leaf beetle colony: *O. melanopus* 83%, *O. duftschmidi* 11%, *O. rufocyanea* 6%.

## 2. Experimental methods

### 2.1. Experiment 1 – Salivary sheath staining method

The purpose of this method is to localize and visualize the salivary sheaths of sap-sucking insects, particularly *P. alienus*, within barley leaf tissues. Using this technique, it is possible to precisely determine the position of the salivary sheath tip and, consequently, the specific part of the leaf tissue where the insect's feeding terminates.

Applied solutions: The water-based fuchsin (5%) and methyl green (1%) solutions were prepared using deionized water, while PBS (phosphate-buffered saline) was made from commercially available tablets. Leaf clearing was performed in a 20% KOH solution, and DL-lactic acid was used to clarify the thin sections. Finally, the embedding medium consisted of a 1:1 mixture of 96% ethanol and 87% glycerol.

Pre-treatment with leafhoppers: The feeding area of leafhoppers was restricted by placing micro-isolators (5 cm long, 3 cm wide, constructed from spiral binding elements covered with nylon mesh and sealed at the ends with polyurethane foam plugs) onto the first true leaf of 2-leaf-stage barley plants. Two leafhoppers were placed in each isolator and allowed to feed for 24 hours, during which they produced multiple salivary sheaths and occasionally eggs. A total of 147 replicates were performed during this pre-treatment.

Localization of salivary sheaths: A 3 cm section of the leaf was excised that had been inside the isolator and incubated it in an Eppendorf tube containing KOH solution at 40 °C for 4 minutes. The leaf was then rinsed with distilled water followed by PBS. Subsequently, it was stained in a 5% aqueous fuchsin solution on a horizontal shaker for 60 minutes at 23 °C. Under the stereomicroscope, the starting points of the salivary sheaths appeared as magenta-coloured dots.

Preparation of salivary sheath sections using hand sectioning: I made transverse sections across the leaf veins by hand with a sharp razor blade, aiming to obtain the thinnest possible slices (approximately 60 μm) that still contained intact, complete salivary sheaths. The sections were then cleared in lactic acid heated to 90 °C for 30–40 minutes, depending on the chlorophyll content.

Microscopic examination and contrast staining: On the fully cleared sections (except for the magenta-stained salivary sheath), contrast staining with methylene green solution for 0.5–1 minute was applied. The methylene green highlighted thickened cell walls, particularly the vascular bundles, in a greenish hue. Finally, the sections were mounted in an ethanol–glycerin embedding medium, allowing for precise analysis. The final staining protocol was developed after testing 34 different combinations of stains, clearing solutions, contrast dyes, and heating durations.

Storage and restaining: The stained sections can be stored for several weeks in the mounting medium or long-term in glycerin at +5 °C. Although the stained tissue may fade over time, the sections can be restained as needed.

### 2.2. Experiment 2 – Field studies

The aim of the experiment was to investigate the impact of natural enemies on leaf damage caused by *Oulema* species, as well as on the yield and quality parameters of wheat and barley. The study was conducted over two consecutive years, 2021 and 2022, on a total of four experimental plots at two different locations. In 2021, two adjacent winter wheat plots (each 0.1 ha) near Martonvásár were used, while in 2022, a 0.2 ha winter wheat plot near Martonvásár and a 0.6 ha winter barley plot near Környe were studied. No pesticide treatments were applied to the plots, and a 20 m wide untreated buffer zone was established around each plot.

The experiment was based on the exclusion of natural enemies. During the early tillering stage (Feekes F2–F4), surrounding plants within a 40 cm diameter circle around the selected plants were removed, and the soil surface was covered with a layer of sand. Three types of treatments were applied: (1) control plants without cages, (2) an exclusion treatment, in which the plants were isolated with mesh cages throughout the ripening period (35 days), and (3) an open-cage treatment, in which the plants were released during the exposure phase (21 days) following an incubation period (14 days), thereby becoming accessible to natural enemies.

Artificial infestation of CLB was carried out at the stem elongation stage (winter wheat: Feekes F5–F6; winter barley: F9). At this time, 10 adult Oulema beetles were placed on each plant. The incubation period lasted 14 days, during which the adults laid eggs and the emerging larvae began feeding. At the end of incubation, the adults were removed, eggs and larvae were counted, and initial leaf damage was recorded ("damage 1"). Subsequently, plants in the exclusion treatment were returned to the cages, while in the open treatment the cages were removed. The exposure period lasted three weeks, continuing until heading (Feekes F10).

At the end of the exposure period, at the beginning of the ripening stage, a second leaf damage assessment ("damage 2") was conducted, during which damage was recorded on the flag leaves. At the end of the experiment, the ears were manually harvested.

Natural damage gradient assessment (2022, Field 22A): At the wheat heading stage, uneven leaf damage was observed due to a naturally occurring infestation of CLB. To map this phenomenon, aerial images were taken with a drone on May 31, 2022, and a 4 × 7 virtual grid was overlaid on the field. At the grid points, leaf discoloration was visually estimated on a 0–10 scale based on drone imagery. A strong correlation was found between the leaf damage of the control plants and the estimated value of the nearest grid point ($R = 0.93$). At harvest, the grid was physically marked, and all ears were harvested from 0.25 $m^2$ quadrats at each grid point.

Determination of yield parameters: Ears were cut below the node and threshed using a Hege 16-type thresher. The number of seeds was counted with a Contador seed counter, and the total weight (yield) was measured on a precision analytical balance. Thousand-grain weight (TGW) was determined using a Marvin Seed Analyser according to MSZ EN ISO 520:2011. From ground samples (Labmill 3100), a NIR device (PerCon Inframatic 8611) was used to determine protein, gluten, starch, and moisture content, as well as water absorption capacity and Zeleny-index.

Statistical analyses: Linear mixed models (LMM) were applied, with field and cluster included as random spatial factors. The relationships between damage caused by *Oulema* species and yield parameters were examined. Special attention was given to the extent of leaf damage (Damage 1 and Damage 2) and to the effect of natural enemies on crop yield.

### 2.3. Experiment 3 – Effect of precolonization by *P. alienus* and *R. padi* on the feeding behavior of *Oulema* species

Under laboratory conditions, choice tests was conducted to examine how precolonization by piercing-sucking insects (*P. alienus* and *R. padi*) affects the feeding behavior of CLB. Inverted Petri dishes were used as arenas. Two leaves (which weren't separate from plants) were placed in each arena: an untreated control leaf and a precolonized leaf by the sap-feeding insects. Micro-isolators were applied for precolonization, which were positioned on the first true leaf of 2–4 leaf-stage barley plants, one per plant. The insects were allowed to feed on the leaves for 48 hours. Two types of pre-colonization treatments were applied:

1. In the leafhopper treatments, two *P. alienus* individuals were placed per micro-isolator (N=20),
2. In the aphid treatments, five *R. padi* individuals were placed per micro-isolator (N=20).

For the control leaves, the micro-isolators were left empty (N = 40). After the precolonization period, treated and control leaves were paired in the arenas, and a single CLB adult was placed under each arenas to feed for 24 hours. At the end of the experiment, the beetles were removed, the damaged leaf sections were excised, and highresolution photographs were taken. Leaf damage was quantified using ImageJ software, and the data were analyzed in R 4.5.0.

### 2.4. Experiment 4 - The effect of *Oulema* species precolonization on feeding behavior and offspring of *P. alienus*

The effect of feeding by *Oulema* species on the feeding behavior and offspring production of *P. alienus* was investigated under laboratory conditions using a choice arena assay. The first true leaves of 2–4 leaf-stage barley plants were used, onto which micro-isolators were placed. A precolonization period of 24 hours was applied.

Three types of treatments were applied within the micro-isolators:

1. Control (C), in which the leaves were left untreated and only the micro-isolator was placed on them,
2. Artificial treatment (AT), in which transverse cuts were made on the leaves, taking care not to damage the main vein,
3. Infested with *Oulema* species (O), in which a CLB was allowed to feed on the leaf for 24 hours .

Before the start of the choice experiment, the CLB were removed, and the leaves pretreated in different ways were placed in pairs under inverted Petri dishes, which served as arenas, similar to the previous experiment. The precolonized leaves were paired as follows:

1. Artificial treatment – *Oulema*-infested (N = 40)
2. Control – Control (N = 40)
3. Control – Artificial treatment (N = 40)
4. Control – *Oulema*-infested (N = 40).

In each arena, a 3 cm section of leaf tissue was made accessible using a paper strip. Then, a female *P. alienus* individual was put in into each arena, where it was allowed to feed for 24 hours.

At the end of the experiment, the leafhoppers were removed from the arenas, and the sections of leaf tissue exposed to feeding were excised and stored in PBS solution. Salivary sheaths were stained with 5% acid fuchsin, cleared in lactic acid, and stored in glycerol. The number of salivary sheaths and deposited eggs was determined using a stereomicroscope.

The statistical analysis was performed using R version 4.5.0. To evaluate the data, a model describing the feeding behavior and oviposition preferences of females (N = 99) was applied. Generalized linear mixed models with a Gaussian error distribution were used for the data, while a zero-inflated model was incorporated for the egg counts to account for the excess zeros.

### 2.5. Experiment 5 - Electropenetrography (EPG)

In the EPG experiment, the pre-treatments used for the experimental setup were identical to those applied in the previous (Experiment 4) pre-treatments:

1. control (C) (N = 60),
2. artificial treatment (AT) (N = 60), and
3. *Oulema*-infested (O) (N = 120) leaves were used.

The precolonization lasted for 24 hours. For the measurements, Giga-4 and Giga-2 EPG devices (EPG Systems Ltd.) were used. For EPG preparation, the leafhoppers were collected from the stock population and anesthetized with $CO_2$ gas, then attached to an electrode using a thin gold wire and silver glue. The duration of the experiment was six hours. For evaluation, the *Oulema*-infested leaves were photographed from below, and the damaged area was quantified using ImageJ software.

The EPG waveforms were evaluated based on the definitions of Tholt et al. (2015). The following waveforms were distinguished:

Ps1: penetration into deeper leaf tissues, Ps2: feeding from xylem, Ps4a: salivation into the phloem, Ps4b: actual passive phloem ingestion, Ps3 and Psmix: their exact meanings are not yet fully clarified.

The statistical analyses were performed in R 4.5.0, based on separate datasets:

Differences between leaf damage types (N = 176) were analyzed: the C (control), AT (artificial treatment), and OT (*Oulema* treatment) groups were compared. The effects of experimental duration, sex, treatment type, and their interactions on the number of transitions between penetration phases, the proportion of time spent in different feeding phases (non-penetration, xylem, phloem), the time until first xylem and phloem penetration, the Ps4a/Ps3 ratio and in females, the total Ps4/Ps4a ratio were evaluated. Mixed-effects Cox models and generalized linear mixed models (GLMMs) were applied for the analysis.

Effect of damage intensity (N = 112) was analyzed: only data from the OT group were considered. The intensity of damage was treated as a quantitative variable, and its effects, together with experimental duration, sex, and their interactions, on the main feeding parameters were evaluated. Cox models and GLMMs were applied for the analysis.

# RESULTS AND DISCUSSION

## 1. Experiment – Salivary sheath staining method

Using the new and simplified method I developed, 122 out of 147 targeted salivary sheaths (83%) were successfully stained for clear evaluation. A total of 113 leafhoppers (30 males and 83 females) produced these sheaths. During the refinement of the method, 35 different combinations, consisting of variations of stains, clearing agents, and contrast dyes, were tested. The entire procedure was completed in an average of 1.5 hours, which is significantly shorter than previously reported methods, where 1–2 days were typically required.

The localization of the salivary sheath was performed by staining the leaves with an aqueous fuchsin solution after clearing them in KOH. The starting points of the sheaths appeared as magenta dots, which made it possible to prepare a targeted single section. The selected salivary sheath was exposed by manual sectioning. A simple, microtome- and embedding-free procedure was applied, producing sections approximately 60 µm thick. With proper cutting accuracy, the entire salivary sheath structure was clearly visible in a single section. For microscopic examination, the fuchsin-stained sheaths were contrast-stained with methyl green, which provided a greenish hue to the thickened cell walls (e.g., vascular bundles), allowing them to be clearly distinguished from the salivary sheath. The sections were mounted in an ethanol–glycerol (1:1) solution, which facilitated microscopic examination.

The new procedure represents a significant advance in the study of feeding patterns of piercing-sucking insects that feed with salivary sheaths, as it is designed to be simple, rapid, cost-effective. Whereas previously used methods often employed toxic and volatile solvents such as diethyl ether, the technique I developed doesn't use these harmful reagents. Prior filtration before fuchsin staining was required, as residual pigment particles could have caused misleading results. The application of alkaline KOH solution in the first clearing step proved more effective: it rendered the leaf more transparent and rigid, thereby facilitating sectioning. The lactic acid treatment helped preserve the integrity and flexibility of the cell walls during post-sectioning clearing. The critical optimization step was chosen to be the direction of sectioning. Since leafhoppers are positioned parallel to the leaf veins while feeding, and their mouthparts move most easily laterally, the salivary sheath branches are generally located perpendicular to the vein plane. This allowed the entire salivary sheath structure to be revealed in a single targeted section, eliminating the need for a series of sections. The manual sectioning method was found particularly suitable for herbaceous monocots such as barley, compared with earlier methods that were based mainly on sclerotized leaves. The methyl green contrast staining provided a clear distinction between the fuchsin-stained salivary sheaths and the greenish vascular bundles. The method can likely be applied to other piercing-sucking, salivary-sheath-feeding insect species, such as *R. padi*. The use of ethanol–glycerol solution allowed the samples to avoid fixation and traditional slide embedding, which would have significantly slowed down the workflow. Moreover, it enabled long-term storage and restaining of the samples.

This technique made it possible to routinely examine the complete structure of salivary sheaths, where it could be determined whether feeding had occurred in the xylem or phloem, and an approximate estimate could be given of the number of attempts required to locate the appropriate feeding site. This can significantly contribute to more accurate interpretation of EPG waveforms, as well as to a better understanding of the feeding behavior and pathogen-vectoring role of piercing-sucking insects.

## 2. Experiment – Field studies

When the effect of natural enemy access was examined, it was observed that leaf damage was 30% lower by the end of the exposure period in the treatment exposed to natural enemies than in the exclusion treatments. Chewing damage was found to be strongly correlated with the number of larvae counted at the beginning of the exposure period. On naturally infected control plants, damage was initially lower, but by the end, the damage on plants in the open-cage treatment did not differ significantly from that on the controls.

### 2.1. Effect of CLB Damage on Yield

The thousand-grain weight (TGW) was significantly reduced by leaf damage, being identified as the most sensitive parameter: a 22–29% decrease in thousand-grain weight was observed depending on the severity of the damage. Total yield was found to be 50% lower at the grid points showing the most severe damage in the natural damage-gradient study. Protein content was found to increase with higher leaf damage, while starch content was slightly decreased, and moisture content showed a positive relationship with leaf damage. Gluten content and the Zeleny index were not affected.

## 2.2. The direct effect of natural enemies

Leaf damage was reduced by the presence of natural enemies, but no significant direct effect was found between the presence of natural enemies and yield parameters. However, larval density negatively affected both yield and thousand grain weight. In the natural gradient study, the total yield decreased by 15.7%, and the thousand grain weight by 11.7% compared to what would have been obtained if the zero-damage yield had been extrapolated across the entire field..

Our results confirm that natural enemies effectively reduce leaf damage caused by *Oulema* species. Both the exclusion experiments and the natural damage gradient showed that higher yields were observed in cases of lower damage, but the relatively weak correlations reported here can be attributed to the patchy and variable occurrence of the beetle of the beetles. Natural infestations—excluding the most severe patches—caused only moderate yield reductions.

The *Oulema* species are regulated by numerous natural enemies, such as the egg parasitoid *Anaphes flavipes* (Förster, 1841) (with an average egg parasitism rate of 40% (Meindl et al., 2001)), and various predators (e.g., Nabidae, Coccinellidae, Carabidae, spiders) (Kheirodin et al., 2020, Meindl et al., 2001, Szabolcs and Horvath, 1991). In the exclusion treatments, 30% more leaf damage was observed.

In cases of severe leaf damage, substantial yield loss can occur: in our study, the most heavily damaged zones showed a 51% reduction in yield, while the field-wide average decreased by 16%. Other studies have generally reported more moderate effects. (e.g., in Swiss 5,4%(Jossi and Bigler, 1996)). The starch content decreased significantly, while the protein content increased, especially in cases of early damage. The thousand-grain weight consistently declined, negatively affecting the market value of the yield.

The damage caused by the CLB is spatially and temporally variable, influenced by their population dynamics, weather conditions, natural enemies, and landscape structure. Natural enemies are generally effective, whereas preventive insecticide applications are not always successful. Chemical control is justified only in cases of severe infestation and should be targeted. Early monitoring is indispensable.

### 3. Experiment - The effect of precolonization by *P. alienus* and *R. padi* on the feeding behaviour of *Oulema* species

Based on statistical analysis, no significant difference was found in the feeding preference of *Oulema* species between control plants and plants precolonized by the sucking insects (*P. alienus*: $p = 0.2717$; *R. padi*: $p = 0.2550$). However, observed trends suggested that beetles fed less frequently on leafhopper-precolonized plants and more frequently on aphid-precolonized plants compared to controls. This indicates that, although both sap-feeding pests feed similarly, aphid-precolonized plants may provide better digestibility for the CLB. It is possible that differences in the salivary composition of the two sap-feeders play a role: aphids might suppress plant defenses more effectively, making these plants more attractive to *Oulema* species than those precolonized by leafhoppers. These hypotheses require further investigation, as no data were found in the literature addressing this phenomenon.

The results indicate that the interactions are highly species-specific, and the effect of a previously feeding sap-sucking insect can be positive, negative, or neutral. Positive examples from the literature include *Brevicoryne brassicae* (cabbage aphid) (L., 1758) (Aphididae), which enhanced the development of *Pieris brassicae* (cabbage butterfly) (Linnaeus, 1758) (Pieridae) larvae on cabbage (Soler et al., 2011), , and *Aphis nerii* (oleander aphid), which accelerated the development of *Danaus plexippus* (monarch butterfly) (Linnaeus, 1758) (Nymphalidae) on common milkweed (Ali et al., 2014). In these cases, it is likely that the aphids suppressed the plant's jasmonic acid-based defenses, thereby benefiting the chewing larvae.

A negative effect has been observed between *Empoasca fabae* (Harris, 1841) (Cicadellidae) leafhoppers and *Leptinotarsa decemlineata* (Colorado potato beetle) (Say, 1824) (Chrysomelidae), where the leafhopper reduced the beetle's egg laying and larval survival. (Lynch et al., 2006). This is consistent with my results, where *Oulema* species chose plants precolonized with *P. alienus* less frequently. Similar negative interactions have been reported between *Brevicoryne brassicae* (Linnaeus, 1758) (Aphididae) and *Plutella xylostella* (diamondback moth) (Linnaeus, 1767) (Plutellidae) on oilseed rape. (Nouri-Ganbalani et al., 2018), as well as from the competition between *Tuta absoluta* (Meyrick, 1917) (Gelechiidae) and *Macrosiphum euphorbiae* (Thomas, 1878) (Aphididae) on tomato (Dong et al., 2020).

Neutral effects have also been reported in the literature: for example, *Rhopalosiphum padi* did not influence the behavior of *Oulema melanopus* (Linnaeus, 1758) (Chrysomelidae) on winter wheat, and on *Brachypodium distachyon* L. the larvae of the cotton bollworm (*Helicoverpa armigera*) (Hübner, 1827) (Noctuidae) showed no effect on development, even though plant salicylic acid levels increased.

Although my experiment did not directly examine plant phytochemical changes, the observed patterns suggest that the cell damage caused by leafhoppers triggered a stronger defense response than that caused by aphids. The antagonism between the salicylic acid (SA) and jasmonic acid (JA) pathways—particularly the SA-mediated inhibition of JA signaling—likely played a key role in the differential responses of *Oulema* species to plants precolonized by the two phloem-feeding species.

### 4. Experiment - The effect of *Oulema* species precolonization on the feeding behaviour and reproduction of *P. alienus*

When examining the feeding preferences (penetration frequency) and oviposition behavior of female *P. alienus* induviduals, no significant differences were found in the number of salivary sheaths produced on treated versus control leaves in any treatment group; in all cases, the distribution conformed to the null hypothesis, indicating that the females did not discriminate between leaves ($\chi^2 = 3.5$; df = 3; p = 0.9494). Similarly, egg laying on the two leaves did not differ among the treatment combinations, although in this case the number of replicates was very low. Based on these results, neither prior feeding by *Oulema* species nor mechanical damage significantly influenced the choice of female cereal leafhoppers.

This result reinforces that plant-mediated interactions are highly species-specific and not necessarily predictable. For example, in other studies, feeding by *Leptinotarsa decemlineata* (Say, 1824) (Chrysomelidae) reduced *Myzus persicae* (Sulzer, 1776) (Aphididae) populations on potato by up to 48%, regardless of whether the feeding occurred prior to or simultaneously with aphid colonization. Interestingly, despite this negative effect, aphids still preferred the previously damaged plants based on their volatiles, which may represent a defensive strategy to avoid natural enemies. (Davidson-Lowe et al., 2018). A similar negative interaction was observed on broccoli, where feeding by *Pieris rapae* (Linnaeus, 1758) (Pieridae) larvae suppressed populations of *Brevicoryne brassicae* (Linnaeus, 1758) (Aphididae). (Blubaugh et al., 2018).

There is also example of a positive effect: the presence of *Sitona lineatus* (pea weevil) (Linnaeus, 1758) (Curculionidae) increased the mobility and survival of *Acyrthosiphon pisum* (pea aphid) (Harris, 1776) (Aphididae), thereby facilitating the spread of plant viruses (Chisholm et al., 2019).

My study did not reveal a similar effect, but this doesn't rule out the possibility that plant response pathways—such as those mediated by salicylic acid and jasmonic acid signaling—were operating in the background. It is important to note, however, that the number of penetrations alone does not necessarily provide sufficient information about the precise processes, which is why I also investigated this phenomenon using EPG.

## 5. Experiment – Electropenetrography (EPG)

Based on the results of the statistical analyses, the type of damage had a significant effect on the phloem-feeding behavior of female leafhoppers: the sumPs4/Ps4a ratio was significantly lower in the artificially wounded (AT) group compared to the control (C) ($\chi^2_2 = 7.92$; $P = 0.019$; $p = 0.038$). In contrast, no significant difference was found between natural chewing damage caused by *Oulema* species

(OT) and the control. When comparing the sexes, I found that males reached the phloem more quickly than females ($\chi^2_1 = 4.78$; $P = 0.029$), which is consistent with previous observations made on the American grapevine leafhopper (*Scaphoideus titanus*) (Ball, 1932) (Cicadellidae) (Chuche et al., 2017). Although the number of transitions between penetration phases and the increase in the proportion of the xylem phase showed a significant correlation with the duration of the experiment, neither sex, treatment type, nor their interaction had a detectable effect on these variables.

When examining the extent of feeding damage caused by *Oulema*, it was revealed that the interaction between leafhopper sex and damage intensity significantly influenced the number of transitions between penetration phases ($\chi^2_1 = 7.47$; $P = 0.006$). Female behavior remained stable in this respect, whereas in males, more severe damage resulted in a significant reduction in the number of switches between feeding phases.

The results suggest that different types of treatments affect the behavioral and physiological responses of leafhoppers in distinct ways. The phloem-feeding activity of females responded sensitively to the type of treatment: while mechanically induced damage (AT) significantly reduced the proportion of time spent in the phloem phase, natural feeding damage caused by CLB (OT) did not elicit such an effect. This indicates that leafhoppers may be able to distinguish between plant injuries of different origins and selectively adjust their behavior accordingly—presumably in response to physical or chemical cues emitted by the plants. These findings are consistent with previous reports showing that mechanical wounding and herbivore-induced chewing trigger distinct protein profiles and molecular responses in plants.

Sex-specific differences were also pronounced: males reached the phloem tissue more rapidly than females, which may indicate divergent foraging behavior or physiological requirements. Feeding damage intensity was found to be decisive only in males, where stronger injury resulted in a reduced frequency of feeding phase transitions. This suggests that male behavior is more sensitive to environmental stressors and that they are likely to develop adaptive responses distinct from those of females. In contrast, females exhibited no treatment-related differences either in feeding activity or in oviposition behavior.

Given the limited body of literature on sex-related differences in behavioral and feeding patterns, the findings of the present study may highlight new and promising avenues for research, particularly regarding divergent response patterns between sexes and their ecological significance.

# CONCLUSIONS AND RECOMMENDATIONS

The study of cereal-based food webs encompasses numerous intriguing and complex aspects. The resulting findings not only enrich theoretical ecological knowledge but may also hold practical relevance for plant protection, agroecology, and integrated pest management. Taken together, the experiments highlighted that the biotic factors affecting cereals—particularly the activity of insect species with different feeding strategies—generate complex, species-specific interactions that can influence both pest behavior and the physiological responses of host plants.
The main conclusions and related recommendations are summarized below:

## 1. Salivary sheath staining

The newly developed method represents a significant advancement in the study of feeding patterns of piercing-sucking insects, as it is faster, simpler, and more cost-effective.

Recommendation: It would be advisable to broaden the scope of this method and adapt it to other host–plant systems—for instance, to dicotyledonous species.

## 2. Field studies

Field experiments clearly demonstrated that the presence of natural enemies provides an important ecosystem service by reducing the damage caused by *Oulema* species.

Recommendation: The adoption of management practices that support the conservation of natural enemies is recommended, such as the maintenance of diverse habitats, the establishment of tree belts, and the implementation of targeted, rational (non-routine) spraying that takes into account the spatial distribution of pests, allowing the survival and long-term persistence of biological agents.

3. The effect of *R. padi* and *P. alienus* precolonization on the feeding behavior of *Oulema* species

The results confirmed that plant-mediated interactions between different insect species are strictly species-specific, and their outcome largely depends on the host plant's responses. The observed changes in CLB behavior in response to leafhopper and aphid infestation are particularly noteworthy and warrant further investigation.

Recommendation: It would be advisable to complement the experiments with phytohormonal analyses to obtain a more detailed understanding of the mechanisms underlying the plant responses.

4. The effect of *Oulema* species precolonization on P. alienus feeding and reproduction

The experiments did not reveal significant differences in leafhopper behavior among the different treatments, suggesting that chewing damage, in this case, does not substantially affect the preference of the piercing-sucking species. This supports the statement in the literature that such interactions are system-specific.

Recommendation: Extending the observations to other species pairs and host plants, as well as examining the combined effects of different biotic and abiotic stress factors, could open up new research directions.

5. Electropenetrography (EPG)

The data indicate that *P. alienus* is capable of detecting the type of plant damage, and its feeding behavior – particularly in males – is adaptively modified accordingly. The differing effects of natural and artificial damage highlight that the origin of injury is a key factor in regulating feeding behavior.

Recommendation: Further research is needed to determine which tissue-level or molecular processes are responsible for the observed behavioral differences, and which signaling pathways are activated in response to different types of plant damage.

# NEW SCIENTIFIC RESULTS

1. A novel histological method was developed, which is faster, simpler, and more cost-effective than previous approaches, allowing the feeding behavior of piercing-sucking insects – primarily leafhoppers – to be more thoroughly characterized.
2. The results quantitatively demonstrated that the presence of natural enemies significantly reduced the leaf damage caused by *Oulema* species.
3. An increase in feeding damage by the *Oulema* species led to a reduction in thousand grain wheight and overall yield.
4. Precolonization by *P. alienus* and *R. padi* did not have a significant effect on the feeding of *Oulema* species; however, an interesting trend was observed in that plants pre-treated with aphids were preferred compared to control plants, whereas plants pre-colonized by leafhoppers were less frequently chosen than controls.
5. Pre-colonization by *Oulema* species had no effect on the feeding behavior or oviposition of *P. alienus* in choice tests.
6. EPG analyses revealed that mechanical damage significantly influenced the duration of salivation within the phloem phase, suggesting that the penetration behavior of the cereal leafhopper depends on the biotic or abiotic origin of the plant injury.
7. Significant sex-related differences were observed in the time required to reach the first phloem penetration in EPG measurements.
8. Feeding damage caused by *Oulema* species significantly affected the total number of transitions between penetration phases in EPG measurements.
9. In males, damage caused by *Oulema* species significantly reduced the number of transitions between feeding phases in EPG measurements.

# PUBLICATIONS

## 1. Journal articles

**Gerstenbrand, R.** ; Fülöp, D. ; Samu, F. ; Tholt, G. Simplified and rapid staining of leafhopper salivary sheaths in plant tissues for electrical penetration graph waveform correlations Micron 140 Paper: 102959 (2021)

Samu, Ferenc ; Szita, Éva ; Simon, Janka ; Cséplő, Mónika ; Botos, Erika ; Pertics, Botond ; Růžičková, Jana ; **Gerstenbrand, Regina** ; Rakszegi, Marianna ; Elek, Zoltán ; Vida, Gyula ; Tholt, Gergely Cereal leaf beetle (*Oulema* spp.) damage reduces yield and is more severe when natural enemy action is prevented Crop protection 185 Paper: 106893 , 9 p. (2024)

## 2. Conferences

Samu, F ; **Gerstenbrand, R.** ; Simon, J. ; Fülöp, D. ; Szita, É. ; Botos, E. ; Tóth, F. ; Molnár, B. P. ; Vida, Gy. ; Jordán, F. ; Tholt, G. Gabona táplálékhálózatok vizsgálata – fajok közti interakciók feltárása komplex megközelítéssel In: Haltrich, Attila; Varga, Ákos (szerk.) 68. Növényvédelmi Tudományos Napok Budapest, Magyarország: Magyar Növényvédelmi Társaság (2022) 74 p. pp. 17-17. , 1 p.

Samu, F ; **Gerstenbrand, R.** ; Simon, D. ; Szita, É. ; Tóth, F. ; Molnár, B. P. ; Vida, Gy. ; Jordán, F. ; Tholt, G. Természetes ellenségek szerepe gabona táplálékhálózatokban: van-e tényleges hatás a terméseredményre? 7. Kvantitatív Ökológiai Szimpózium Vácrátót (2022) pp. 25

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## 3. Additional Scientific Works

Fulop, David; Szita, Eva ; **Gerstenbrand, Regina** ; Tholt, Gergely ; Samu, Ferenc Consuming alternative prey does not influence the DNA detectability half-life of pest prey in spider gut contents PEERJ 7 Paper: e7680 , 14 p. (2019) doi: 10.7717/peerj.7680

Pertics, Botond Zsombor; Tholt, Gergely; Kis, András; Szita, Éva; Gerő, Kornél; **Gerstenbrand, Regina**; Simon, Janka; Samu, Ferenc Widely-based full-genome analyses enable development of universal and strain-specific PCR toolkit for wheat dwarf virus detection, revealing new alternative hosts and challenging strain-host specificity Plant Methods 21 Paper: 100 (2025) doi: 10.1186/s13007-025-01420-6

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