Evaluating biological control of the soybean gall midge (*Resseliella maxima* Gagné) in Minnesota

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Gloria Melotto

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Robert L. Koch and Amelia R. I. Lindsey (Advisors)

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Literature Review

Soybean (*Glycine max* (L.) Merr.) is a vital source of vegetable oil and animal protein feed worldwide (Sugiyama et al. 2015, Pagano and Miransari 2016), with the highest protein content (40-42%) of all food crops and the second-highest oil content (18-22%) among food legumes (Robert 1986, Pagano and Miransari 2016). This makes soybean one of the four key crops, along with maize, rice, and wheat, that satisfy two-thirds of the total agricultural calorie demand (Ray et al. 2013). Additionally, soybean is among the 16 primary crops cultivated worldwide (Foley et al. 2011, Pagano and Miransari 2016), highlighting the necessity to enhance soybean research to meet the growing global crop demand as the human population increases (Masuda and Goldsmith 2009, Tilman et al. 2011). To achieve this goal, research should focus on improving the potential yield of soybean while considering maximum cultivar production under certain environmental conditions, providing adequate nutrients and water, and controlling insect pests and diseases (Salvagiotti et al. 2008, Pagano and Miransari 2016).

In 2022, the United States ranked as the second largest producer of soybean globally, contributing to 31% of the world's production (USDA 2022). Minnesota alone contributes 9% to the total US soybean production (USDA, 2022). However, soybean fields in MN face multiple insect pests that can impact its yield, including *Aphis glycines* Matsumura (Hemiptera: Aphididae), *Tetranychus urticae* Koch (Acari: Tetranychidae), *Cerotoma trifurcate* (Forster) (Coleoptera: Chrysomelidae), caterpillars (Lepidoptera), grasshoppers (Orthoptera: Acrididae), and stinkbugs (Hemiptera: Pentatomidae) (Bennett et al. 1999, Kandel 2010, Ragsdale et al. 2011, Koch and Pahs 2014). In addition to these common pests, a new insect pest, the soybean gall midge (*Resseliella maxima* Gagné (Diptera: Cecidomyiidae)), has been detected in Minnesotan soybean fields since 2018 (Gagné et al. 2019). In addition to Minnesota, *R. maxima* infestation is also found in other states in the Midwest US (McMechan et al. 2021b).

The Cecidomyiidae family is commonly known as the gall midge family. This family is one of the largest and most diverse families within the order Diptera, with approximately 6600 described species worldwide and potentially thousands of undescribed species (Dorchin et al. 2019). A curious fact is that despite the name "gall midges", not all species in this family form galls (Dorchin et al., 2019). The subfamily Cecidomyiinae is composed of herbivorous and predatory species (Dorchin et al., 2019).

Within Cecidomyiinae, 75% of species are herbivorous, and most of them are host-specific and develop in one or a few closely related host plants (Dorchin et al., 2019). Many genera within this subfamily have specialized and diversified on specific plant families (Gagné 1989, Gagné and Jaschhof 2017, Dorchin et al. 2019).

The cosmopolitan genus *Resseliella* is in the subfamily Cecidomyiinae, and includes 56 described species (Gagné et al., 2019). Some species in this genus are important pest species, such as *Resseliella soya* (Monzen) that attacks leaf veins and petioles of soybean in Japan, and *Resseliella yagoi* Yukawa and Sato that feeds in pears (Gagné et al., 2019). Another important species is *Resseliella theobaldi* Barnes, which attacks raspberry plants in Europe (Pitcher 1952). *Resseliella theobaldi* is particularly important because it exhibits a similar biology to *R. maxima*. Specifically, it lays eggs on plant fissures, the larvae feed on the outer tissues of host stem before reaching maturity, and finally, they drop to the soil to pupate (Pitcher, 1952). Although the *R. maxima* genome was recently sequenced (Melotto et al. 2023c), the biology of *R. maxima* largely remains unknown, making *R. theobaldi* a valuable comparison for studying the biology and ecology of *R. maxima*.

The origin of *R. maxima* remains unknown, as it could either be exotic or native to the US. If it is exotic, it is possible that it occurred somewhere else in the world but was not documented before arriving here. On the other hand, the fact that *R. maxima* feeds on other legumes (i.e., sweet clover (*Melitotus alba* and *M. officinalis*), alfalfa (*Medicago sativa*), and beans (*Phaseolus vulgaris* and *P. lunetus*)) (McMechan et al. 2021a, Sever 2021, Potter et al. 2022), suggests that it might be a native oligophagous insect that underwent a host range expansion to include soybean (Kogan 1981).

Cecidomyiidae adults are delicate and tiny flies, typically with a wingspan of less than 3 mm. Their larvae are legless, elongated maggots with modified mouthparts for consuming liquids (Gagné 1994). A detailed description of *R. maxima* is provided by Gagné et al. (2019). Briefly, *R. maxima* adults have a light to dark brown body and measure about 6 mm in length. Their antennae have a unique pattern of dark and light bands, which varies between males and females. Their wings are mottled with yellow and black scales, and their legs are marked by distinctive alternating bands of dark and light colors (Gagné et al., 2019). Another species of gall midge found in Minnesota soybean fields is *Karshomyia caulicola* (Coquillett), which may be mistakenly identified as *R. maxima* (Koch et al. 2020). However, these two

species can be easily differentiated. *Karshomyia caulicola* infests soybean plants later in the season and is always associated with white mold (*Sclerotinia sclerotiorum* (Lib.) de Bary) infected tissues (i.e., inside, and outside of *Sclerotinia*-infected soybean stems, pods and other tissues). *Karshomyia caulicola* late instar larvae have less intense orange coloration than similarly sized *R. maxima* larvae, with distinct morphological characteristics described in Koch et al. (2020). Adult *K. caulicola* differs from *R. maxima* due to its uniform grayish antennae, wings, and legs, creating a striking contrast between the two species.

Due to the lack of success in laboratory rearing (Koch and McMechan personal communications), there is limited knowledge about the biology of *R. maxima*. As a result, much of the knowledge specific to this pest's biology has been made from observations in the field. It is believed that *R. maxima* overwinters as larvae in silken cocoons in the soil. In spring, they pupate, and adults emerge around mid-June to establish the next generation in nearby soybean fields (McMechan et al. 2021b). Early vegetative growth stages of soybean plants, typically the V2 or V3 growth stages (i.e., two or three trifoliate leaves expanded), are most susceptible to *R. maxima* infestation because natural fissures develop below the cotyledonary node, where *R. maxima* females are suspected to lay their eggs (McMechan et al. 2021b). Once hatched, *R. maxima* larvae feed on the plant phloem at the base of the plant, eventually reaching the pith before dropping to the ground to pupate or overwinter (McMechan et al. 2021b). Instead of forming galls, this feeding leads to necrotic lesions at the base of the plant, which can be recognized as a symptom of *R. maxima* infestation (McMechan et al. 2021b). Depending on the extent of the infestation, soybean plants can wilt and die in just a few days, resulting in yield reductions of up to 31% in field interiors and 100% in field edges (Helton et al., 2022; McMechan et al., 2021a).

Management options for this pest are limited. Research has shown that chemical control may not be sufficient for management of soybean gall midge infestations in soybean fields (Hodgson and Helton 2021, McMechan 2021, Castro 2022) and soybean gall midge-resistant soybean varieties are not available. Recently, hilling emerged as a potential cultural control method (McMechan et al. 2023). Nevertheless, its application remains uncommon in soybean systems due to concerns about soil disturbance, which may lead to root damage and increased vulnerability to soil erosion (McMechan et al.

2023). Therefore, alternative management options, such as biological control, need to be evaluated for the development of effective integrated pest management (IPM) of *R. maxima*.

Biological control involves the use of living organisms, known as biological control agents, to manage insect pest populations (DeBach 1964). As defined by Heimpel and Mills (2017) this method provides indirect benefits to humans through the biological control agent's direct or indirect negative impact on one or more target species. However, in the case of *R. maxima* infestation in soybean fields, limited information is available regarding potential biological control agents that could contribute to managing this pest.

For other cecidomyiid species, several biological control agents have been documented, particularly parasitoids and predators (Austin 1984, Floate et al. 1990, Holland and Thomas 2000, Olfert et al. 2003, Smith et al. 2004, Sampson et al. 2006, Corlay et al. 2007, Buhl and Duso 2008, He and Wang 2011, Abram et al. 2012b, Johnson et al. 2013, Roubos and Liburd 2013, Matsuo et al. 2016, Thompson and Reddy 2016). Hawkins and Gagné (1989) investigated parasitoid communities for 191 species of gall midges, including 8 species of the genus *Resseliella* from around the world. These *Resseliella* species were reported to be attacked by 0 to 2 species of parasitoids (Hawkins and Gagné, 1989). An example is the raspberry cane midge, *R. theobaldi*, that is parasitized by *Aprostocetus epicharmus* Walker (Hymenoptera: Eulophidae) in Europe (Vétek et al. 2006). Besides Eulophidae (Sampson et al. 2006, Roubos and Liburd 2013), other Hymenopteran families have been documented parasitizing cecidomyiids, such as Platygastridae (Austin 1984, Olfert et al. 2003, Buhl and Duso 2008, He and Wang 2011, Abram et al. 2012a, Johnson et al. 2013, Roubos and Liburd 2013), Pteromalidae (Smith et al. 2004, Abram et al. 2012b, Thompson and Reddy 2016), and Braconidae (Matsuo et al. 2016). Recently, we documented a new species of parasitoid from the genus *Synopeas* (Hymenoptera: Platygastridae) associated with *R. maxima* infestation in Minnesotan soybean fields (Melotto et al. 2023b).

In addition to parasitoids, several predatory groups, such as Aranaeidae (Xia et al. 2021), Carabidae (Floate et al. 1990), Coccinellidae (Corlay et al. 2007), Anthocoridae (Pitcher 1952, Wearing et al. 2013), Miridae (Wearing et al. 2013), and Syrphidae (Sampson et al. 2002), have been reported preying on other Cecidomyiidae. An example is the raspberry cane midge, *R. theobaldi*, the larvae of which are preyed upon by nymphs of *Anthocoris nemorum* L. (Hemiptera: Anthocoridae).

The goal of this thesis is to address the limited understanding of biological control agents for *R. maxima* in Minnesota. Chapter 1 explores the spatial and temporal dynamics of *R. maxima* parasitism in soybean fields, which provides valuable information about *R. maxima* and parasitoid distribution, as well as parasitism rates of *R. maxima* in Minnesota. Chapter 2 evaluates the predator community associated with *R. maxima* infestation in Minnesotan soybean fields, with the aim of identifying potential predators that could help control *R. maxima* populations. By identifying biological control agents, this study takes the first step towards developing a long-term solution to manage *R. maxima* infestation in Minnesotan soybean fields and will provide a foundation for future research on the biological control of *R. maxima.*

Chapter 1

Spatial and temporal dynamics of soybean gall midge (*Resseliella maxima*) parasitism by *Synopeas maximum*

Abstract

Soybean gall midge, *Resseliella maxima* Gagné (Diptera: Cecidomyiidae), is a pest that impacts soybean yield in the Midwest US. While biological control using parasitoids may be a promising approach for managing this pest, it is crucial to have a deep understanding of parasitism dynamics to ensure effective implementation. We investigated *R. maxima* parasitism using a combination of three methods: rearing of field-collected hosts, dissection, and molecular assays. We confirmed parasitism of *R. maxima* by the recently described wasp *Synopeas maximum* Awad & Talamas (Hymenoptera: Platygastridae) and used our combinatorial approach to observe the spatial and temporal relationships between *R. maxima* and its parasitoid in the field. The number of *R. maxima* adults was greater in symptomatic plants on field edges than the field interior, but such a pattern was inconsistent for parasitism rates. Parasitism rates were generally highest early and late in the season, and lower in the middle of the season when the number of *R. maxima* was highest. In Minnesota, overall season-long parasitism rates of *R. maxima* were low (<2%). To facilitate a wider investigation of the biological control of *R. maxima*, we designed a protocol for high throughput DNA extraction and real-time PCR that can be used across a broader geography. Further research should evaluate how parasitism rates by *S. maximum* could be promoted in production fields.

Introduction

The soybean gall midge, *Resseliella maxima* Gagné (Diptera: Cecidomyiidae) was described in 2019 as an important new pest of soybean plants (*Glycine max.* (L.) Merrill) in the Midwest United States (Gagné et al. 2019, McMechan et al. 2021b). Since then, *R. maxima* has been reported in five US states: Iowa, Minnesota, Nebraska, Missouri and South Dakota (McMechan et al. 2021b). These states accounted for 41% of US soybean production in 2021(USDA 2022). Furthermore, *R. maxima* was recently classified as a potential quarantine pest for the European Union (Bragard et al. 2023), an indication of the threat that this pest presents to soybean growers.

Resseliella maxima overwinter as larvae in silken cocoons in the soil, pupate in the spring, and emerge as adults in June before moving to nearby soybean fields to reproduce (McMechan et al. 2021b). *Resseliella maxima* females oviposit in the natural fissures (i.e., cracks) near the base of the soybean stem that develop once plants have two or more expanded trifoliate leaves (McMechan et al. 2021b). After hatching, *R. maxima* larvae burrow into the stem and feed on the plant phloem, eventually feeding as deep as the pith (Gagné et al. 2019). This internal feeding results in black coloration at the base of the stem: a canonical symptom of *R. maxima* infestation (McMechan et al. 2021b). Depending on the level of infestation, soybean plants can wilt and die in a few days after infestation starts, causing a yield reduction up to 31% in field interiors and 100% in field edges (McMechan et al. 2021b, Helton et al. 2022).

Options for *R. maxima* management are currently limited and chemical control may not be sufficient to manage infestations in soybean fields (Hodgson and Helton 2021, McMechan 2021, Castro 2022, Montenegro et al. 2022). Therefore, alternative methods of control, such as biological control, need to be evaluated. *Synopeas maximum* Awad & Talamas (Hymenoptera: Platygastridae) was recently identified as a parasitoid associated with *R. maxima* (Melotto et al. 2023b). Platygastrines are koinobiont endoparasitoids that primarily target cecidomyiids in their early larval or late embryonic stages, with wasp development delayed until the host's final instar or prepupal stage (Kim et al. 2011, Abram et al. 2012a, Chen et al. 2021, Melotto et al. 2023b). Although it remains unknown if *R. maxima* is native to North America, phylogenetic evidence suggests that *S. maximum* may be native to North America (Melotto et al. 2023b). The potential impacts of this parasitoid on *R. maxima* need to be further examined.

When studying parasitoid-host interactions for cecidomyiid pests, most researchers have employed one of two methods for detection of parasitoids: (1) rearing field-collected hosts in the laboratory for parasitoid emergence (Sampson et al. 2006, Abram et al. 2012b, Johnson et al. 2013, Matsuo et al. 2016), or (2) dissecting hosts and examining parasitoids microscopically (Smith et al. 2004, He and Wang 2011, Roubos and Liburd 2013). However, molecular techniques (e.g., real time PCR) are an extraordinarily sensitive and a useful tool to provide data not available from the previously listed methods alone (Greenstone 2006, Furlong 2015). A combination of these techniques could improve estimates of parasitism rates and potential impacts of these parasitoids on pest populations (Sampson et al. 2006, Furlong 2015, Magagnoli et al. 2022).

Here we performed the first investigation of parasitism of *R. maxima* through a combination of the three methods mentioned above (i.e., rearing, dissections, and molecular assays), with the goals of: (1) developing a protocol for high throughput DNA extraction and real-time PCR to detect parasitism, (2) implementing these protocols to quantify parasitism rates in field-collected *R. maxima*, and (3) investigating the spatial and temporal dynamics of parasitism. This study will advance biological control of *R. maxima* and integrated pest management (IPM) for this pest.

Materials and Methods

Field description and sampling

Field sampling was conducted during the summers of 2021 and 2022 in two soybean fields at a farm near the City of Luverne, Minnesota, United States. Each year, the sampled fields were approximately 0.8 km apart from one another. *Resseliella maxima* was first identified at this farm in 2018 (McMechan et al. 2021b). In 2021, fields were planted on 27 and 28 April with Pioneer® P22A28X seeds with Roundup Ready 2 Xtend® technology (RR2X) (Pioneer® Seeds) tolerant to glyphosate and dicamba herbicides. Seeds were treated with the fungicides oxathiapiprolin (Lumisena™, Corteva™ Agriscience), prothioconazole, penflufen, and metalaxyl (EverGol® Energy, Bayer SeedGrowth™), and the insecticide imidacloprid (Gaucho® 600 Flowable, Bayer SeedGrowth™) for both years. In 2022, fields were planted on 08 and 09 May with Pioneer® P18A73E seeds that were tolerant to white mold disease (*Sclerotinia*

sclerotiorum). In both years, seeds were planted with 76.2-cm row spacing. The production system used for these fields was an annual crop rotation between soybean and corn, with minimum tillage (i.e., strip-till).

In each field, we established 8 sampling locations that were located along two transects parallel to a field edge adjacent to a corn field (i.e., previous year's soybean field) (Figure 1.1). The edge transect was 5 meters from the adjacent corn field and the interior transect was 30 meters into the field from the edge transect. The ends of transects were at least 30 meters from any adjacent edge of the sampled field. Within each transect, sampling locations were spaced 20 meters apart from each other.

Field sampling started on 30 June 2021 and 06 July 2022, when soybean plants started to show symptoms of infestation (i.e., darkened lesions at the base of the stems). These initial sample dates were approximately fifteen days after the first collection of *R. maxima* adults in emergence traps placed in the previous year's soybean fields on the farm (B.D. Potter unpublished data). Field sampling continued every other week until *R. maxima* infestation was no longer detected (2021: 30 June, 13, and 29 July, 13 August, and 01 September; 2022: 06 and 19 July, and 03, 17 and 31 August).

Given that *R. maxima* parasitism had not been robustly investigated prior to our study, we sought to maximize the likelihood of detecting any parasitism by focusing sampling on soybean stems displaying symptoms of *R. maxima* infestation (i.e., darkened lesions at the base of the stems). It is important to note that this sampling approach may influence estimates of parasitism, depending on the parasitoid functional response, which remains unknown. On each sample date, 20 randomly selected symptomatic plants were collected from each sampling location in each field by pulling the entire plants from the soil for a total of 160 plants from each field. These plants were then trimmed above the first pair of unifoliate leaves and placed in zipper-locking plastic bags (17.7x18.8 cm, Ziploc®), which were placed in coolers until brought to the laboratory (approximately 5 hours). The 20 soybean stems from each sampling location on each sample date were divided into three sets for use in three different methods of parasitism assessment: emergence cages, larval dissections, and molecular assessment (described below).

Emergence cages

A set of ten stems from each sampling location were prepared as described in Melotto et al. (2023b). Briefly, the cut end of the stem was wrapped with a small piece of parafilm, and the roots were trimmed to fit in emergence cages. Emergence cages consisted of 5-liter clear containers with a 6-cm diameter hole cut in the side and a fine mesh (0.02-cm mesh size) sleeve 30-cm long was attached to the hole. In each emergence cage, the 10 stems from a sampling location were placed vertically into a 3-cm deep layer of potting soil. A total of 8 emergence cages were assembled for each of the 5 sampling dates from each field, resulting in 40 emergence cages per field over the course of each summer. The emergence cages were kept at room temperature with 16:8 (light:dark) h, watered as needed, and inspected daily for emergence of insect adults. Adult insects were collected manually into microcentrifuge tubes, freeze-killed, and then preserved in 95% ethanol and stored at -20°C.

Larval dissections

In 2021, a set of five soybean stems from each sampling location were dissected using #9 razor blades (STANLEY® Tools). Larvae from these stems were counted and dissected following the methods of Roubos and Liburd (2013). Briefly, live *R. maxima* larvae were placed in a drop of water on a microscope slide with the help of a fine-tipped brush, compressed under a glass coverslip to rupture the larvae, and carefully examined for evidence of parasitism (e.g., parasitoid eggs or larvae) under a stereo microscope with 0.8x - 3.5x magnification (Leica EZ4 W, Leica Microsystems).

Molecular assessment

In 2021, a set of five soybean stems from each sampling location and in 2022 a set of ten soybean stems from each location were dissected as described above. Larvae from each soybean stem were counted and placed into a microcentrifuge tube containing 95% ethanol and stored at -20°C for later processing.

DNA extraction

DNA was extracted from individual specimens following one of two versions of a modified HotSHOT protocol (Truett et al. 2000). The first HotSHOT protocol was used for identification of adult specimens from emergence cages and left the specimens intact (non-destructive). Non-destructive extraction from single specimens was performed in 0.2-mL PCR tubes (Olympus plastic, Cat# 27-125) by adding 100 µL of the lysis reagent (25 mM NaOH: 0.2 mM disodium EDTA) and incubating at 95°C for 30 minutes on a Mastercycler® nexus PCR cycler (Eppendorf®). Samples were then cooled to 4°C and 100 µL of neutralizing reagent (40 mM Tris-HCl) was added to each sample (a final volume of 200 µL). The aqueous solution containing DNA was moved to a fresh tube and 95% Ethanol was added to the specimen for preservation.

For detection of parasitism of *R. maxima* larvae, the HotSHOT protocol was adjusted to include mechanical lysis, facilitating detection of any parasitoid DNA within the larvae. Prior to the 95°C incubation, two metal beads (3.175 mm carbon steel eclipse balls, Abbott Company, Connecticut, USA) were added to each sample. Mechanical lysis was performed by bead beating for 30 s in a Harbil® 5-Gallon Heavy-Duty Paint Mixer after which the homogenate was incubated and neutralized following the non-destructive method above.

DNA barcoding

Parasitic wasps, cecidomyiids other than *R. maxima*, and randomly selected adult specimens of *R. maxima* from the emergence cages were chosen for DNA extraction and the cytochrome oxidase subunit I (COI) gene was amplified alongside negative controls using the universal primer pair LCO-1490/HCO-2198 (Folmer et al. 1994). The PCR reaction mix was prepared in a final volume of 20 µL with 1 µL of DNA template, Q5® Hot Start High-Fidelity 2X Master Mix (New England BioLabs), and 500 nM of each primer. Thermalcycling was conducted on a Mastercycler® nexus PCR cycler (Eppendorf®) with 2 min at 98°C, 40 cycles of amplification (10 s at 98°C, 30 s at 60°C, and 20 s at 72°C), and 2 min at 72°C. PCR products were separated by gel electrophoresis on a 1% agarose gel and imaged under ultraviolet (UV) light after staining with GelRed™10000X in water (Biotium). PCR-products were cleaned using the

Zymo DNA Clean & Concentrator® – 5 Kit (Zymo Research) according to the manufacturer's protocol and Sanger sequenced in both directions at ACGT DNA Sequencing Services (Wheeling, Illinois, USA). Sequences were inspected for peak quality, aligned, and trimmed of priming regions in SnapGene® (version 6.1.2).

Primer design

For the detection and identification of *S. maximum* DNA in *R. maxima* larvae, we designed a set of *Synopeas maximum*-specific primers (PSM_F and PSM_R) (Table 1.1) for high throughput screening of field-collected midge larvae with real time PCR.

The specificity of the primer sets was validated in two separate qPCR reactions with DNA template of *S. maximum* (n=12) and *R. maxima* (n=6), alongside negative controls (*Lestodiplosis* spp. (Diptera: Cecidomyiidae) that emerged from the cages; n=2) on a QuantStudioTM 3 Real-Time PCR System (Applied Biosystems™). Primer sensitivity was validated against a panel of samples in which we combined verified *S. maximum* and *R. maxima* DNA extractions. Ratios of *S. maximum* to *R. maxima* DNA were varied to test the impacts of low wasp DNA concentration and high *R. maxima* background on the detection of parasitism. Specific controls included: (1) 1:1, (2) 1:10, (3) 1:20, (4) 1:100, and (5) a 10X dilution of 1:10 *S. maximum*: *R. maxima* DNA template. The qPCR reaction mix was prepared in a final volume of 20 µL with 1 µL DNA template, Luna® Universal qPCR Master Mix (New England BioLabs), and 500 nM of each primer (PSM_F and PSM_R). Thermalcycling was performed on an QuantStudioTM 3 Real-Time PCR System (Applied Biosystems™) with an initial denaturation of 1 min at 95°C, followed by 40 cycles of amplification (15 s at 95°C, 30 s at 60°C). After amplification was complete, a final melting curve was recorded by heating to 95°C for 15 s and then cooling down (1.6°C per s) to 60°C for 1 min before heating slowly (0.15°C per s) to 95°C again for dissociation step. Fluorescence was measured in the dissociation step. The melting temperature was used as a tool to analyze if the correct target amplicon was produced.

Evaluation of pooling samples for qPCR assays

We used a multi-step screening protocol to assess rates of *R. maxima* parasitism by *S. maximum* in the field. After initial verification to ensure specificity and sensitivity of primers, we screened individual larvae for the presence of parasitism until we identified three positive samples. Then, we used these positive samples to evaluate a pooling method that would facilitate efficient screening of the large number of field-collected samples (n=2872 in 2021, n=4443 in 2022). We evaluated the sensitivity of our qPCR assay under pooled conditions by simultaneous amplification of one parasitism-positive larval sample combined with 11 other larval samples which were parasitism-negative. In all cases, the qPCR assay was highly sensitive and detected parasitism of a single larva within the 12-larva pool. In addition to testing the impact of pooling DNA samples, we assessed two strategies for loading DNA from the 12 extractions into a single qPCR reaction including (1) preparing a mix of the DNA from equal ratios of the 12 extractions, then adding 1µl of the mix to the qPCR reaction, and (2) simply dipping a p10 pipette tip into a DNA extraction and then into the appropriate well of the qPCR plate containing master mix (repeated with clean tips for the remaining 11 extractions of the qPCR pool). The “dip” method provided consistent sensitive and robust detection of parasitism with the added benefit of avoiding additional steps of multiple transfers of the DNA extractions which takes significant time and liquid handling, and risks contamination. Setting up pooled qPCR reactions leveraged an 8-well multichannel pipettor that allowed efficient processing wherein each row of extractions in a 96-well plate (n=12) was consolidated into a single qPCR reaction.

Pooled screening of larvae

After validating primer and pooling specificity and sensitivity, we screened all field-collected larval samples with the pooled method. Positive pools advanced to the fourth step, in which DNA extractions from the pool were individually screened. Finally, positive individual samples were screened again in technical duplicate for final validation. All reactions were run alongside positive (different ratios of *S. maximum* and *R. maxima* DNA) and negative controls (adult *R. maxima* DNA and no template), following the qPCR conditions of the “primer design” section.

Host verification

The sixth and final step was included to verify the host species for larval samples that screened positive for parasitism. For this, we designed a forward *R. maxima*-specific primer (SGM_F) with the information from the COI barcoding regions we obtained from the emergence cages samples. We used the COI sequences of these insects to select a conserved region of the COI gene that was specific to *R. maxima* and generated a 600-bp amplicon when combined with the HCO-2198 reverse primer (Table 1.1). Six parasitism-positive individual larva samples were randomly selected from each year and the COI gene was amplified alongside negative controls using the primer pair SGM_F/HCO-2198, following the PCR conditions of section 2.4.2 in exception of the 40 cycles of amplification that were: 10 s at 98°C, 30 s at 63°C, and 20 s at 72°C. PCR-products were analyzed, cleaned, and sequenced as described in "DNA barcoding" section.

Statistical analyses

All analyses were performed using RStudio version 2022.12.0 (Build 353). To investigate the number of *R. maxima* in symptomatic stems, we used generalized linear mixed models with negative binomial distribution (package: lme4 (Bates et al. 2015), code: glmer.nb, family= nbinom2). We analyzed the number of *R. maxima* per ten stems separately for each insect stage (larvae and adults) and year (2021 and 2022), resulting in four models. For each model, the fixed factors included date of sampling, position within the field (edge and interior), and their interaction. To account for the repeated measures design, we included sampling location nested in field as a random factor for each model. Pairwise comparisons were performed using estimated marginal means with Tukey's adjustment (package: emmeans (Searle et al. 1980); code: emmeans).

Proportion parasitism of *R. maxima* by *S. maximum* was calculated using two methods for each sample position on each sample date. For emergence cages, proportion parasitism of *R. maxima* by *S. maximum* was calculated as the total number of *S. maximum* adults emerged in a cage divided the sum of the number of *S. maximum* adults and *R. maxima* adults emerged in that cage (Sampson et al. 2002, 2006, Roubos and Liburd 2013). For the molecular assessment, proportion parasitism of *R. maxima* by *S.*

maximum was calculated as the total number of *R. maxima* larvae that screened positive for *S. maximum* from the ten stems collected from a sampling location, divided by the total number of *R. maxima* larvae collected from those stems (Sampson et al. 2002, Roubos and Liburd 2013).

To investigate the spatial and temporal dynamics of parasitism by *S. maximum* and compare the sensitivity of the parasitism assessment methods, we analyzed the proportion parasitism separately for each year using generalized linear mixed models with a binomial distribution (package: lme4 (Bates et al. 2015); code: glmer; family = binomial; link = logit). Due to the low rate of parasitism detection, we excluded dates where parasitism was not detected to avoid complete separation of the data. In 2021, because parasitism was so low in the field interior, we analyzed only data from sampling locations on field edges. In addition, we excluded the molecular data from 13 July and emergence cage data from 19 July and 01 September from the edge sampling locations. Therefore, we analyzed the proportion parasitism from 2021, with method of parasitism assessment (emergence cages or molecular assessment), date of sampling, and their two-way interaction as fixed factor and location nested in field as a random factor.

In 2022, because parasitism was so low in the emergence in cages, we analyzed only data from the molecular assessment, and excluded 19 July and 03 August from both positions in the field (edge and interior), and 06 July from field interiors. Therefore, we analyzed proportion parasitism from 2022, with date of sampling, position in the field (edge and interior), and their interaction as fixed factors, and location nested in field as a random factor. For both years, pairwise comparisons were performed using estimated marginal means with Tukey's adjustment (package: emmeans (Searle et al. 1980); code: emmeans).

For all models, the significance of each factor and their interaction was estimated with a Type II Wald chi-squared test (package: car (Fox and Weisberg 2019); code: Anova) and model fit was verified with diagnostic plots of scaled residuals against fitted values and with goodness-of-fit tests on the scaled residuals (package: DHARMa (Florian 2022); code: simulateResiduals).

Results

Seasonality of adult R. maxima and parasitoid emergence

A total of 2221 *R. maxima* adults emerged in 2021, and 1999 emerged in 2022. Emergence of *R. maxima* adults started as early as 5 days after emergence cages were set up (Figure 1.2a-b), with an average of 6.8 days across all dates of sampling for first emergence in 2021 and 7.8 days in 2022. Conversely, 50% of the total number of adults emerged after an average of 25 days in 2021 and after 28.8 days in 2022. The *R. maxima* adults kept emerging without any additional supply of host material, with the last adult emerging after 88 days in 2021 and after 123 days in 2022 (Figure 1.2a-b), with an average of 70 days in 2021 and 93 days in 2022.

In 2021, 18 *S. maximum* adults emerged from cages, while in 2022 we observed only one *S. maximum* adult emergence. In 2021, the first *S. maximum* to emerge took 22 days (Figure 1.2c) and the average time to first emergence across collection dates was 29 days. Fifty percent of the total *S. maximum* emergence occurred after an average of 37 days, and the last emergence occurred after an average of 44 days in 2021. In 2022, the single *S. maximum* emerged after 23 days (Figure 1.2d).

Number of R. maxima in symptomatic soybean stems

The number of *R. maxima* adults that emerged from symptomatic soybean stems in the cages varied significantly ($P<0.05$) depending on the location within the field, the date of sampling, and their interaction (Table 1.2). In 2021 and 2022, the number of *R. maxima* adults that emerged from symptomatic stems collected from locations near the field edge was greater than from stems collected in the field interior (Table 1.2, Fig. 1.3c-d and 1.4c-d). Furthermore, in 2021, the number of *R. maxima* adults that emerged from symptomatic soybean stems collected from field edges on 30 June, 13 July, and 29 July was greater than from stems collected on 13 August and 1 September (Figure 1.3a). The number of *R. maxima* adults that emerged from symptomatic soybean stems collected from the field interior on 29 July was greater than from stems collected on 30 June, 13 July, 13 August, and 1 September (Figure 1.3b). In 2022, the number of *R. maxima* adults that emerged from symptomatic soybean stems collected from field edges on 06 July and 03 August was greater than from stems collected on 17 and 19 July, and August 31 (Figure 1.4a). The number of *R. maxima* adults that emerged

from symptomatic soybean stems collected from field interiors on 06 July, 03 and 17 August was greater than from stems collected on 19 July, and August 31 (Figure 1.4b).

The number of *R. maxima* larvae collected from symptomatic soybean stems also varied significantly ($P<0.05$) depending on the date of sampling and the location within the field in both years, and in 2021 the interaction of these two was also significant (Table 1.2). In 2021, the number of *R. maxima* larvae from symptomatic soybean stems collected from field edges was greater on 30 June and 29 July than on 13 July, 13 August, and 01 September (Figure 1.3a). The number of *R. maxima* larvae from symptomatic soybean stems collected from field interiors was greatest on 29 July than all other dates of sampling (Figure 1.3b). In 2022, the number of *R. maxima* larvae from symptomatic soybean stems collected from field edges and interiors were greater on 06 July, 03 and 17 August than on 19 July, and 31 August (Figure 1.4a-b). We conclude that *R. maxima* larval number in the field was higher at the beginning (2021: 30 June; 2022: 06 July) and at mid-season (2021: 29 July; 2022: 03 August).

Parasitism of R. maxima by S. maximum

In addition to sampling wasps from emergence cages, we assessed parasitism by two additional methods. Dissection of 1767 *R. maxima* larvae in 2021 revealed no indication of parasitism and we chose not to dissect larvae in 2022. In contrast, we detected *S. maximum* in both 2021 and 2022 using our molecular approach. All randomly selected parasitism-positive samples were sequenced, and in all cases, the host was confirmed to be *R. maxima*.

Overall parasitism rates (across all sample dates and locations) in 2021 was estimated as 0.74% via emergence cages and 1.32% via molecular assessment, and in 2022 was 0.02% and 1.59%, respectively. The proportion parasitism of *R. maxima* by *S. maximum* varied significantly ($P<0.05$) depending on method of parasitism assessment and date of sampling (Table 1.3). In 2021 and 2022, molecular assessment was more sensitive to detecting *R. maxima* parasitism (Table 1.3, Fig 1.3c-d and 1.4c-d). In 2021, the mean proportion parasitism of *R. maxima* by *S. maximum* on symptomatic soybean stems collected from field edges on 30 June, 13 August, and 01 September was greater than from the stems collected on 13 and 19 July (Figure 1.3c). In 2022, the proportion parasitism of *R. maxima* by *S.*

maximum on symptomatic soybean stems was not significantly different between positions within the field (Table 1.3). Finally, the proportion parasitism of *R. maxima* by *S. maximum* on symptomatic soybean stems collected on 17 and 31 August was greater than in the stems collected on 06 July (Fig 1.4c-d).

Discussion

Our study represents the first successful attempt to mass rear *R. maxima,* and its parasitoid(s), from field-collected soybeans and assess the potential for biological control of this pest. We found that the first emergence of *R. maxima* and its parasitoid *S. maximum* in cages occurred on average 7 and 26 days after sample collection, respectively. Similarly, the swede midge, *Contarinia nasturtii* (Kieffer), and its parasitoids (i.e., *Synopeas myles, Macroglenes chalybeus, Inostemma opacum, and Synopeas osaces*) started to emerge 10 and 20 days after sample collection, respectively (Abram et al. 2012b). The emergence curves of *R. maxima* and *S. maximum* in cages exhibited a long tail, extending up to 120 and 58 days, respectively. Under field conditions, emergence of adult *R. maxima* in Nebraska can extend over long periods of time (McMechan et al. 2021a). In contrast, *C. nasturtii* and its parasitoids exhibited a shorter period of emergence from cages: only 5-10 days (Abram et al. 2012b). The long tail of emergence of some cecidomyiids, like *R. maxima*, might be explained by the differential behavior and response of these species to climatic variations, as well as the presence of suboptimal conditions such as a low-quality food source (Gagné 1989, Nguyen et al. 2022).

Previous work reported *S. maximum* as a putative parasitoid of (i.e., associated with) *R. maxima* (Melotto et al. 2023b). *Synopeas* are known to attack only cecidomyiids (Awad et al. 2021) and stem associated cecidomyiids, other than *R. maxima*, were rare in our samples. From the rearing buckets, detection of non-target cecidomyiids was infrequent and comprised only *Lestodiplosis* spp. (Diptera: Cecidomyiidae) (Melotto et al. 2023b). Furthermore, the only other cecidomyiid known to infest soybean stems in Minnesota is the white-mold gall midge, *Karshomyia caulicola* (Coquillett) (Diptera: Cecidomyiidae), which occurs only in stems infected with the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary (Koch et al. 2020), but the soybean sampled for this work was resistant to this disease in the 2022

season, and no evidence was observed for infection of the soybean plants by this disease. The present work made the important contribution of confirming *S. maximum* parasitism of *R. maxima*, via barcoding of *R. maxima* larvae that were positive for *S. maximum* DNA.

The number of *R. maxima* in symptomatic soybean stems in the field was highest in early July and early August (i.e., larvae from overwintering and first-generation adults), and with higher numbers on field edges than the field interiors in both years. Such an edge effect has been seen for other cecidomyiid pests of annual host plants, such as the lentil gall midge, *Contarinia lentis* Aczél, the sunflower midge, *Contarinia schulzi* Gagné, and *C. nasturtii* (Kolesik 2000, Hodgson et al. 2004, Roubos and Liburd 2010, Hallett 2017). In contrast, an edge effect was not observed in the blueberry gall midge, *Dasineura oxycoccana* (Johnson), which infests a perennial plant host (Roubos and Liburd 2010, Rhodes et al. 2014).

Our findings indicate that rates of parasitism by *S. maximum* were higher in field edges than the field interior in 2021, but not in 2022 when parasitism was also detected in field interiors at the end of the season. A recent study on the parasitoids of the olive leaf gall midge, *Dasineura oleae* (Angelini), also showed no differences in the abundance of *Platygaster demades* Walker between the interiors and edges of the fields (Tondini et al. 2023). Despite the higher number of *R. maxima* in early August (mid-season), very little parasitism was detected during this period in both years. *Synopeas maximum* was mostly detected in the fields in early July and then again in mid-August until early September (beginning and end of the season), while *R. maxima* was active from mid-June until early September. However, parasitism by *S. maximum* was detected in small white (presumed 1st or 2nd instar) through large orange (presumed 3rd instar) *R. maxima* larvae. This pattern suggests either a low level of synchrony between parasitoid and midge generations or possibly a negative density dependence in parasitism. Asynchrony has been documented for the apple leaf curling midge, *Dasyneura mali* Kieffer, and its parasitoid *Platygaster demades* (Walker), in which the parasitoid was almost entirely absent when they observed the greatest larval damage (Todd 1959, Shaw et al. 2005). Additionally, *P. demades* adults increased in abundance late in the season at a time when there is decline in the incidence of the *D. mali* (Todd 1959). The asynchrony between *D. mali* and its parasitoid was explained by laboratory trials that showed *P. demades* emerged approximately 25 days after *D. mali* (Shaw et al. 2005). We hypothesize that a similar

asynchrony may be occurring between *R. maxima* and *S. maximum* and may be contributing to the low parasitism rates of *R. maxima* found in Minnesota. An alternative explanation for this pattern may be negative density dependence between *R. maxima* and *S. maximum*. Negative density-dependent patterns in parasitism can arise due to various mechanisms, such as parasitoids leaving patches to prevent self-superparasitism, deceleration of functional responses influenced by factors like handling time or group defenses, and interference among parasitoids (Walde and Murdoch 1988, Ives 1992, Rosenheim and Mangel 1994, Hunter 2000, Umbanhowar et al. 2003).

Parasitism of *R. maxima* by *S. maximum* was detected through molecular assays and emergence cages, but dissection of *R. maxima* larvae did not reveal evidence of parasitism. However, other studies using dissections have successfully detected parasitism of cecidomyiid larvae by various parasitoids, such *as S. myles*, *M. penetrans*, and other eulophid and platygastrid wasps (Smith et al. 2004, Sampson et al. 2006, Abram et al. 2012a, Roubos and Liburd 2013). Our inability to detect the presence of parasitoids in *R. maxima* larvae through dissection may be attributed to the developmental delay of parasitoids belonging to the Platygastridae family. They typically complete their development during the final instar or prepupal phase of their host (Chen et al. 2011, Kim et al. 2011, Abram et al. 2012a), which can make it challenging to identify them during the earlier larval stages of the host. Another reason can be the relatively low parasitism rates and/or the use of a stereo microscope with insufficient capabilities (i.e., 0.8x - 3.5x magnification). In contrast to our results, successful parasitoid detection was observed with 600x magnification (Abram et al. 2012a), 4–100x magnification (Roubos and Liburd 2013), and 10–15x magnification (Smith et al. 2004). As dissections were time consuming and did not yield evidence of parasitism, we decided not to include dissections in 2022 study.

Although *R. maxima* parasitism was detected by both molecular assays and emergence cages, greater parasitism rates were detected by the molecular method. The lower parasitism rates from emergence cages might be explained by a failure of some parasitized hosts to produce adult parasitoids due to host defenses or other causes of egg or larval parasitoid mortality (Greenstone 2006). Overall, molecular screening appeared more efficient than rearing host material for assessment of parasitism of *R. maxima* by a known parasitoid. However, molecular screening does require specialized training and equipment, and the emergence cages facilitate identification of new species of parasitoids as well as

additional insights into the behavior and ecology of the host and parasitoid. The use of both methods was essential for this study to identify the parasitoid attacking *R. maxima* larvae and to improve estimates of parasitism rates.

Conclusion

We successfully reared *R. maxima* and *S. maximum* adults from field-collected symptomatic soybean stems and used molecular methods to confirm parasitism of *R. maxima* larvae by *S. maximum*. Although an edge effect was observed for *R. maxima* abundance, it was inconsistent for parasitism rates by *S. maximum*, indicating the need for further research to understand parasitism dynamics in the field. The combination of molecular assays and emergence cages provided a comprehensive understanding of parasitism rates and dynamics for populations of these insects. While emergence cages offered crucial insights into the ecology of *S. maximum* and *R. maxima*, molecular assays provided a more sensitive assessment of parasitism rates. Our protocol for high throughput DNA extraction and real-time PCR to detect parasitism by *S. maximum* will allow investigation of biological control of *R. maxima* across a broader geography. Further research should evaluate how parasitism rates by *S. maximum* could be promoted in production fields (Lee and Heimpel 2005, Lahiri et al. 2017).

	Forward Primer	Reverse Primer
Synopeas maximum	PSM_F: TCCTCTTATGCTAAGAGCC	PSM_R: GTTAAAAGTGATAATGGAGG
Resseliella maxima	SGM_F: GCTCATACAGGATCTTCTGTAG	HCO-2198: TAAACTTCAGGGTGACCAAAAAATCA
Universal primers (Folmer et al. 1994)	LCO-1490: GGTCAACAAATCATAAAGATATTGG	HCO-2198: TAAACTTCAGGGTGACCAAAAAATCA

Table 1. 1. Primer sequences used for *S. maximum* detection and *R. maxima* identification.

Source	χ^2	df	P
Adults in 2021			
Date	204	4	< 0.0001
Position	27	1	< 0.0001
Date x Position	20	4	0.0004
Larvae in 2021			
Date	82	4	< 0.0001
Position	26	1	< 0.0001
Date x Position	12	3	0.008
Adults in 2022			
Date	62	4	< 0.0001
Position	18	1	< 0.0001
Date x Position	34	4	< 0.0001
Larvae in 2022			
Date	96	4	< 0.0001
Position	47	1	< 0.0001
Date x Position	5	4	0.25

Table 1. 2. ANOVA table for the effects of date of sample collection and position in the field (edge or interior) on the number of *R. maxima* adults reared from symptomatic soybean stems in emergence cages and larvae dissected from symptomatic soybean stems 2021 and 2022.

Source	χ^2	df	P
2021			
Method	6	1	0.017
Date	31	4	< 0.0001
Method x Date	1	1	0.29
2022			
Date	82	4	< 0.0001
Position	26	1	0.58
Date x Position	12	3	0.91

Table 1. 3. ANOVA table for effects of date of sample collection and method of parasitism assessment for 2021 or position in the field for 2022 on proportion parasitism of *R. maxima* by *S. maximum* in symptomatic soybean stems.

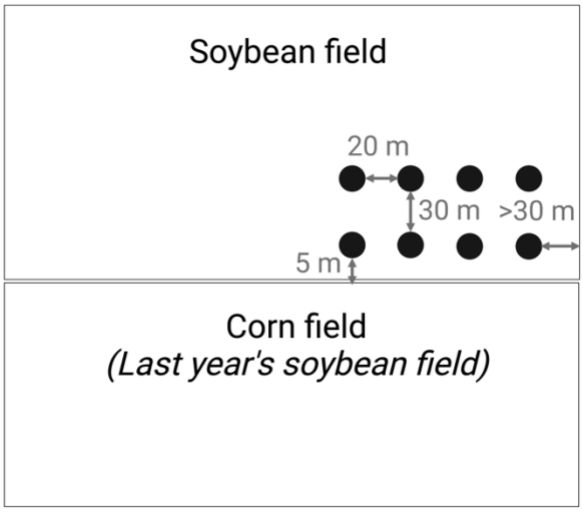

Figure 1. 1. Schematic of fields and locations of sample location.

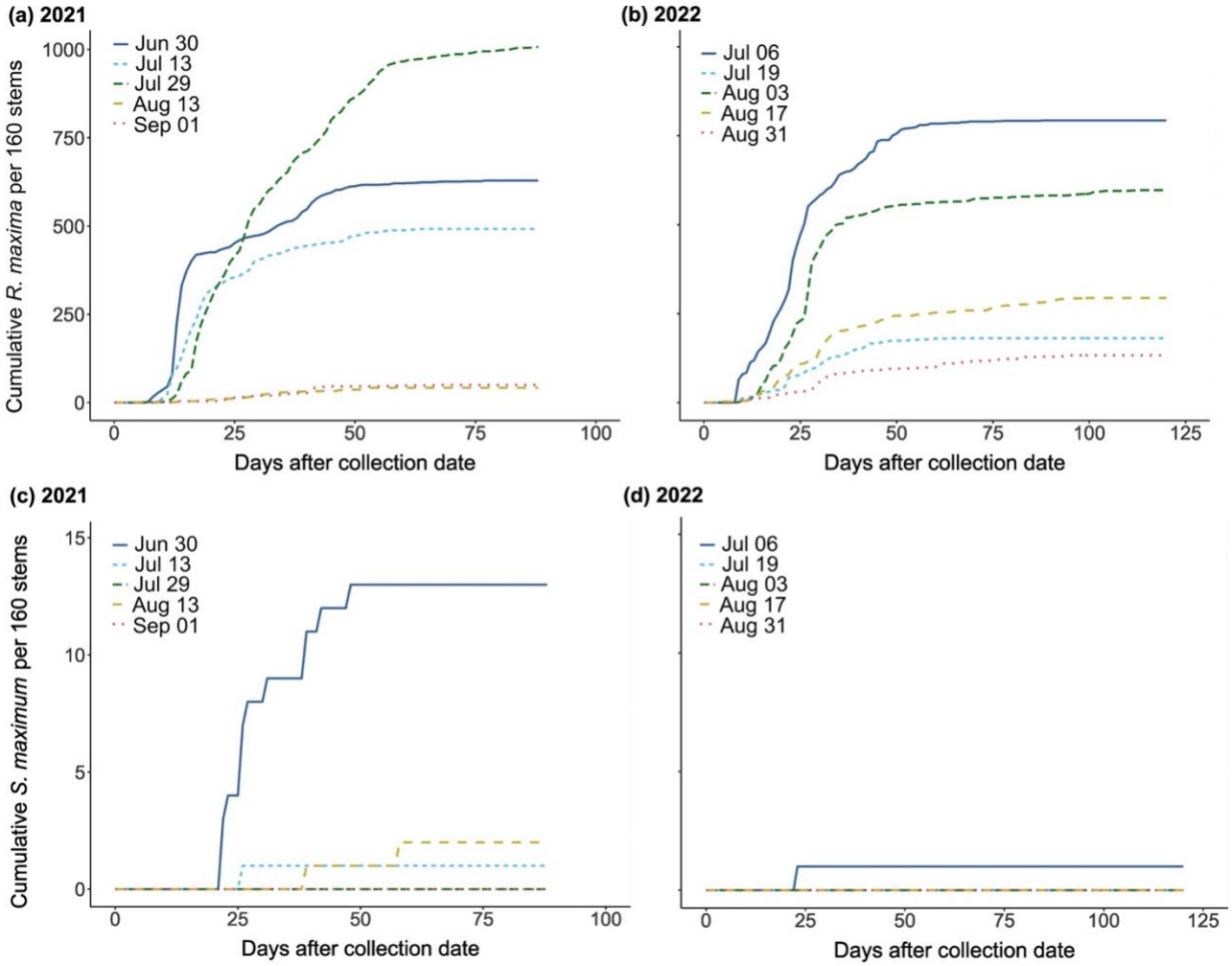

Figure 1. 2. Cumulative emergence of *R. maxima* (a, b) and *S. maximum* (c, d) adults from symptomatic soybean stems in emergence cages in 2021 (a, c) and 2022 (b, d) for each date of sample collection (across all sample locations in each field).

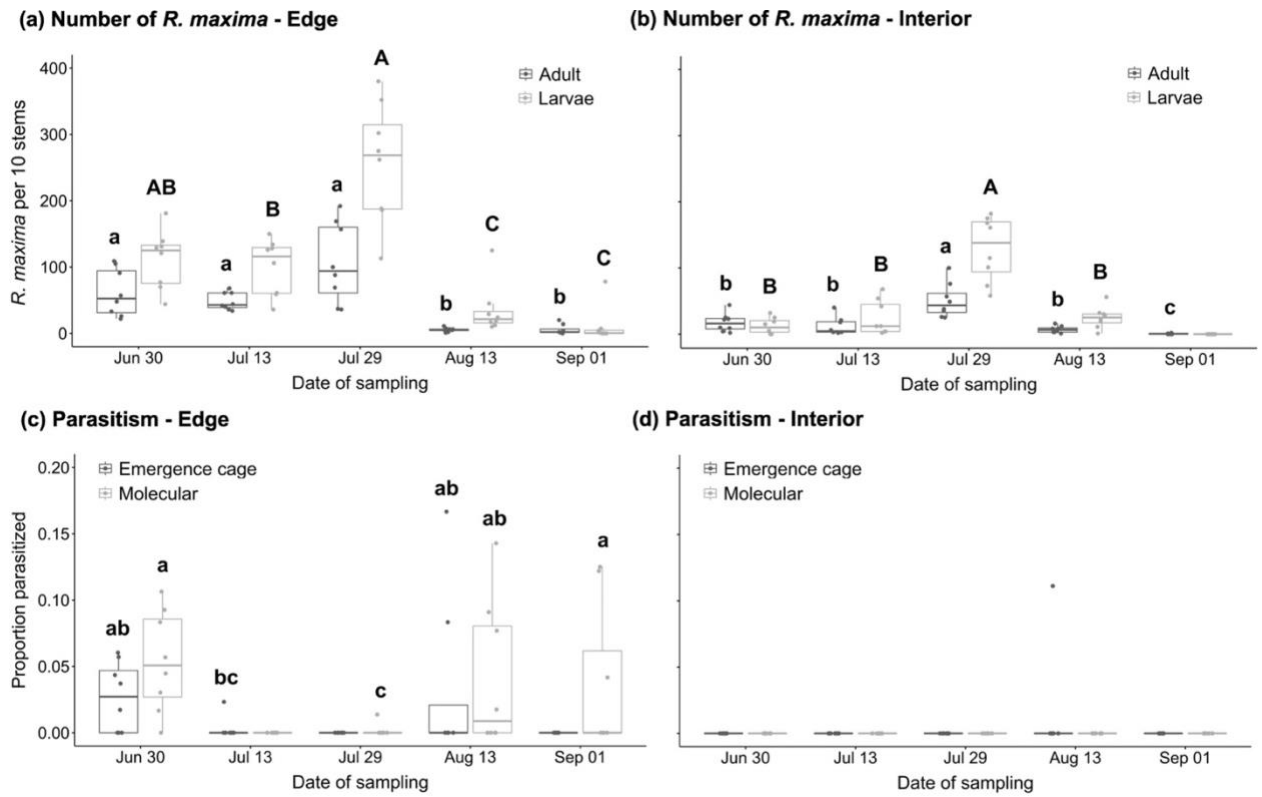

Figure 1. 3. Number of *R. maxima* adults and larvae from symptomatic soybean stems collected from (a) field edges and (b) field interiors, and proportion parasitism of *R. maxima* by *S. maximum* collected from (c) field edges and (d) field interiors in 2021. For panels a and b, different lowercase letters represent differences (P<0.05) in mean number of *R. maxima* adults among dates of sample collection within positions in the field (edge or interior); and different uppercase letters represent differences (P<0.05) in mean number of *R. maxima* larvae among dates of sample collection within positions in the field (edge or interior). For panels c and d, different lowercase letters represent differences (P<0.05) in mean proportion parasitism among combinations of methods of parasitism assessment (emergence cages or molecular assays) and

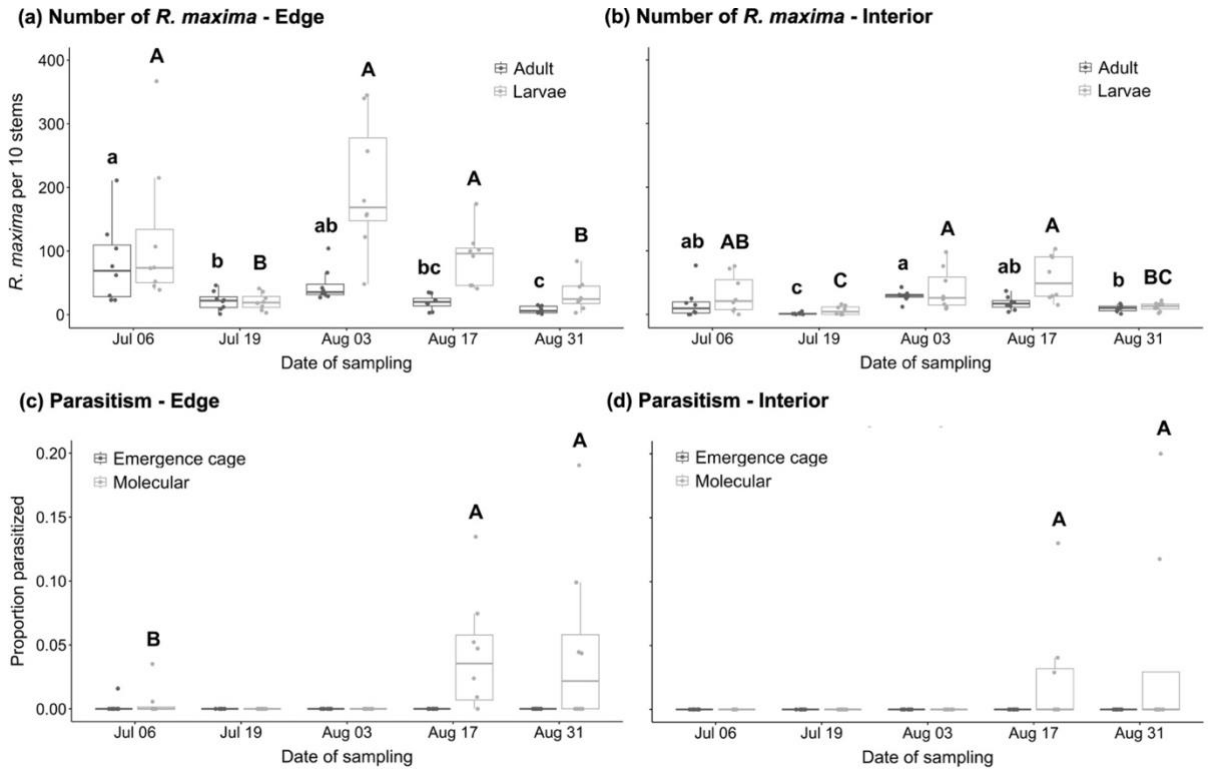

Figure 1. 4. Number of *R. maxima* adults and larvae from symptomatic soybean stems collected from (a) field edges and (b) field interiors, and proportion parasitism of *R. maxima* by *S. maximum* collected from (c) field edges and (d) field interiors in 2022. For panels a and b, different lowercase letters represent differences (P<0.05) in mean number of *R. maxima* adults among dates of sample collection within positions in the field (edge or interior); and different uppercase letters represent differences (P<0.05) in mean number of *R. maxima* larvae among dates of sample collection within positions in the field (edge or interior). For panels c and d, different lowercase letters represent differences (P<0.05) in mean proportion parasitism from the molecular assays among combinations across positions within the fields (edge and interior) and dates of sample collection. Analyses were not performed for proportion parasitism in the field interior (d).

Chapter 2

Arthropod predator community associated with soybean gall midge, *Resseliella maxima* (Diptera: Cecidomyiidae) in Minnesotan soybean fields.

Abstract

The soybean gall midge, *Resseliella maxima* Gagné (Diptera: Cecidomyiidae), is a pest that injures soybeans in the Midwest US. Little is known about the natural enemies of *R. maxima* or the potential for biological control. Therefore, we performed a two-year survey in Minnesota to examine the predator community associated with *R. maxima* infestations. We found that *Orius insidiosus* (Say) (Heteroptera: Anthocoridae) and *Pterostichus melanarius* (Illiger) (Coleoptera: Carabidae) were the most common foliar- and ground-foraging predators, respectively. Moreover, some of the commonly encountered predator species were tested in laboratory predation experiments. *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) and *O. insidiosus* represented the foliar-foraging predators tested, and *H. axyridis* consumed significantly more *R. maxima* larvae than *O. insidiosus*. Among the ground-foraging predators, four carabids were tested. *Poecilus lucublandus* (Say) and *Pt. melanarius* consumed significantly more *R. maxima* larvae than *Poecilus chalcites* (Say) and *Bembidion quadrimaculatum oppositum* (Say). We conclude that *Pt. melanarius* should receive further attention as a potential biological control agent of *R. maxima*, due to its high abundance in the soybean fields in this study, temporal overlap with the pest, and high propensity to feed on the pest.

Introduction

The soybean gall midge, *Resseliella maxima* Gagné (Diptera: Cecidomyiidae), is a new pest attacking soybean plants in the midwestern US (Gagné et al. 2019). Nearly 40% of US soybean production (i.e., 34.2 million acres) occurs in states where *R. maxima* is present (i.e., Minnesota, South Dakota, Iowa, Nebraska and Missouri) (USDA 2022). *Resseliella maxima* larvae overwinter as larvae in silken cocoons in the soil, pupate in the spring, and emerge as adults around mid-June in Minnesota (McMechan et al. 2021b). Adult females lay their eggs in fissures that develop on the stems of soybean plants during early vegetative growth stages. Larvae feed on plant tissue inside the stems before reaching maturity and dropping to the ground to pupate or overwinter (McMechan et al. 2021b). Infested plants exhibit a dark discoloration at the base of the stem that may lead to wilting, lodging, and plant death in severe infestations (McMechan et al. 2021b). Ultimately, *R. maxima* feeding can have a significant negative impact on soybean yield (McMechan et al., 2021).

The current lack of effective management tactics (Hodgson and Helton 2021, McMechan 2021, Montenegro et al. 2022) highlights the crucial need for further development of integrated pest management (IPM), including biological control. A new species of parasitoid wasp was found to parasitize *R. maxima* (Melotto et al. 2023b), albeit at relatively low rates in Minnesota (Melotto et al. 2023a). However, there is a need to examine the role of other groups of natural enemies in the potential suppression of this pest. Predatory arthropods, particularly generalists, contribute to suppression of other pests in soybean fields (Koul and Dhaliwal 2003, Fox et al. 2005, Rutledge and O'Neil 2005, Stiling and Cornelissen 2005, Costamagna and Landis 2007, Hajek et al. 2007). Although *R. maxima* larvae are likely well protected within soybean stems, they become more vulnerable to predators when they drop from plants to pupate in the soil.

Several ground-foraging and/or plant-foraging predatory taxa, such as Araneae (Xia et al. 2021), Carabidae (Floate et al. 1990), Coccinellidae (Corlay et al. 2007, Gardiner and Landis 2007), Anthocoridae (Pitcher 1952, Wearing et al. 2013), Miridae (Wearing et al. 2013), and Syrphidae (Sampson et al. 2002), have been reported preying on other species of Cecidomyiidae. Additionally, understanding the temporal abundance of insect pests and their predators is crucial for predicting the dynamics of predator-prey interactions and developing effective pest management strategies (Losey and

Denno 1999, Rutledge et al. 2004, Corlay et al. 2007, Lee et al. 2022). Despite the well-studied predator communities associated with other soybean pests in the midwestern U.S. (e.g., Rutledge et al. 2004, Ragsdale et al. 2011), information on predator communities associated with *R. maxima* infestations is currently unavailable. In this study we surveyed for potential predators of *R. maxima* and assessed their seasonal dynamics across two growing seasons. In addition, laboratory predation experiments were performed to evaluate the potential of various predators to prey on *R. maxima* larvae. The identification of key predator species and their temporal abundance in relation to pest abundance is an important first step in development of a biological control program.

Materials and Methods

Field description and sampling

Field sampling for arthropod predators coincided with sampling for *R. maxima* and its potential parasitoids, as described by (Melotto et al. 2023a). Briefly, sampling was performed in two fields that were 0.8 km apart from one another near Luverne, Minnesota, USA, during the summers of 2021 and 2022. Due to crop rotation, the specific field locations varied between years. For the soybean fields sampled in our study, corn was grown the previous year, followed by strip-tillage. In 2021, fields were planted in late April with Pioneer® P22A28X RR2X soybean seeds. In 2022, fields were planted in early May with Pioneer® P18A73E soybean seeds. In both years, soybean seeds were treated with an insecticide (imidacloprid) and fungicides (oxathiapiprolin, prothioconazole, penflufen and metalaxyl). Soybean row spacing in both years was 76.2 cm.

Eight sampling locations were established in each field. The locations were placed along two transects (i.e., one on the field edge and one in the interior) that were parallel to the edge bordering a corn field (i.e., the previous year's soybean field). The edge and interior transects were 5 and 35 meters, respectively, from the adjacent corn field. Along each transect, locations were at least 20 meters apart from one another. The ends of transects were at least 30 meters from the adjacent edges of the field.

To assess predator communities, two different methods of sampling (pitfall trapping and whole-plant visual inspection) were conducted every other week at these sampling locations. Each sampling occurred over 2 days. On the first day, we set up pitfall traps at the center of each location, and on the following day, we conducted plant inspections and collected the pitfall traps. The plant inspections covered a 10-meter radius around each pitfall trap, excluding plants within 2-meters of the traps. Sampling began after soybean plants started to exhibit symptoms of *R. maxima* infestation and continued until infestation was no longer detected. Sampling was performed on five dates each year (2021: June 30, 13, and 29 July,13 August, and 01 September; 2022: 06 and 19 July, and 03, 17 and 31 August).

Plant inspections for predators

The community of foliar-foraging predators was assessed through visual whole-plant inspections (modified from Tran and Koch, 2017). On each sample date, 10 plants were selected from each location. Selected plants were first visually inspected when plants were still in the soil to minimize startling active predators. Plants were inspected again after pulling the entire plants from the soil to search more carefully for small predators. Predators were collected and individually placed into 1.5-mL microcentrifuge tubes filled with 95% ethanol and placed in a cooler with ice for transport to the laboratory. In the laboratory, predators were stored at -20°C for later identification and quantification. We recognize that predator counts from the plants represent 'density', but for consistency throughout the text we refer to these counts as 'abundance'.

Pitfall trapping for predators

The community of ground-foraging predators was assessed through pitfall trapping (modified from Daniel, 2021). One pitfall trap per location was assembled by digging a hole in the soil with a depth of 15 cm and diameter of 12 cm. An outer cup (Mountain mixing cup, 946.37 ml) with four holes in its base to allow water to drain was placed into the hole in the soil. An inner cup (Pactiv Delitainer, 946.37 ml) was placed into the outer cup so that the rim of the inner cup was level with the soil surface. Then, 200 mL of propylene-glycol-based marine antifreeze (SPLASH RV/Marine Antifreeze) was added to the inner cup.

Finally, two 23-cm diameter styrofoam plates stacked one on top of the other were secured over each cup as a roof with two 20-cm long nails driven into the soil, leaving a gap of approximately 2 cm for insects to pass under the roof.

On each sampling date, each trap was deployed for 24 hours. After this period, trap contents were poured into a strainer and rinsed with 95% ethanol to remove the antifreeze liquid and impurities (e.g., debris and soil). Arthropods were then transferred to a Whirl-Pak® Write On bag (Whirl-Pak®, 384 mL) filled with 95% ethanol. Samples were placed in coolers with ice for transportation to the laboratory (approximately 5 hours) where they were stored at -20°C for further identification and quantification of specimens. We recognize that predator counts from pitfall traps represent 'activity density', but for consistency throughout the text we refer to these counts as 'abundance'.

Predator identification

Carabidae were identified to species according to Ball and Bousquet (2000) and Bousquet (2010). Anthocoridae and Coccinellidae were identified according to Herring (1966) and (Koch 2003), respectively, and reference specimens in the Insect Museum of the Department of Entomology, University of Minnesota. Gryllidae was included as predators due to the predatory or omnivorous behavior of some species in this family (Burgess and Hinks 1987, Rubia and Shepard 1987).

Stem dissections for prey

The abundance of *R. maxima* larvae was assessed through dissection of soybean stems presenting symptoms of *R. maxima* infestation, as described in Melotto et al. (2023a). Briefly, ten randomly selected symptomatic soybean stems were collected per location at each sampling date. Soybean stems from the same location were placed in Ziploc® bags and brought back to the laboratory. At the laboratory, stems were dissected using #9 razor blades (STANLEY® Tools) to expose and count *R. maxima* larvae.

Predation experiments

Laboratory predation experiments were performed to determine if insect predators that occurred in soybean fields would consume *R. maxima* larvae. To obtain live *R. maxima* to serve as prey for these trials, larvae were collected as described above. Adult ground-foraging predators (i.e., *Pterostichus melanarius* (Illiger) (Coleoptera: Carabidae), *Poecilus lucublandus* (Say) (Coleoptera: Carabidae), *Poecilus chalcites* (Say) (Coleoptera: Carabidae), and *Bembidion quadrimaculatum oppositum* (Say) (Coleoptera: Carabidae)) were obtained by dry pitfall trapping at the same fields described above. Pitfall traps were assembled as described earlier, except the propylene glycol-based marine antifreeze was not added to the traps. Adult foliar-foraging predators (i.e., *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) and *Orius insidiosus* (Say) (Heteroptera: Anthocoridae)) were obtained by sweep net sampling a soybean field in Saint Paul, Minnesota, USA.

All predators were maintained in coolers and brought to the laboratory in containers (Pactiv Delitainer, 946.37 ml). Feeding experiments were initiated about 6 and 2 hours after collection for the ground-foraging and foliar-foraging predators, respectively. Each predator was transferred to an individual Petri dish (1.5 x 9 cm) containing seven 3rd instar *R. maxima* larvae (as described by Gagné et al. (2019)) for each of the ground-foraging predators, or six 3rd instar *R. maxima* larvae for each of the foliar-foraging predators. Each dish contained a single adult predator individual and was replicated as follows: *Pt. melanarius* (n=9), *Po. chalcites* (n=7), *Po. lucublandus* (n=9), *B. q. oppositum* (n=6), *H. axyridis* (n=5), and *O. insidiosus* (n=8). Dishes were maintained on a laboratory bench at approximately 25°C and were monitored continuously for 1 hour to record the number of *R. maxima* larvae consumed by all the predators. The dishes with ground-foraging predators were checked again after 24 hours for the number of *R. maxima* larvae consumed.

Statistical analyses

We performed all statistical analyses using RStudio version 2022.12.0 (Build 353). To determine which predators were most prevalent throughout the season, we used generalized linear mixed models with a negative binomial distribution (package: lme4 (Bates et al. 2015), code: glmer.nb, family = nbinom2). We analyzed each sampling method separately for each year, resulting in a total of four

models. For each model, we used the season-long cumulative number of predators per location as the response variable, predator taxon as a fixed factor, and sampling location nested within field as a random factor to account for the repeated measures design. Except for Gryllidae, we identified all insects to species. Non-insect arthropods were identified at the order level. Thus, each predator group was referred to as a "taxon" in our analyses. While carabids were exclusively found as adults, we found juveniles of *H. axyridis*, *O. insidiosus*, Opiliones, Araneae, and Gryllidae and abundance counts for those taxa include all life stages.

To examine temporal and spatial effects on abundance of the most prevalent predators, we used generalized linear mixed models with negative binomial distribution for the most abundant predator from each sampling method and year (package: lme4 (Bates et al. 2015), code: glmer.nb, family = nbinom2). We treated the number of predators per location (i.e., per 10 plants or per trap) per sample date as the response variable, and incorporated sample date, position in the field (edge and interior), and their interaction as fixed factors. We accounted for the repeated measures design by including location nested within field as a random factor.

For the laboratory feeding trials, we compared the voracity of different predator species within each guild (i.e., foliar and ground) using generalized linear models with the bias reduced fitting method brglmFit (Kosmidis 2014). We analyzed voracity of both predator guilds after 1 h, and for the ground-foraging predators, we also analyzed their voracity after 24 h, resulting in a total of three models. For each model, we had the proportion of prey consumed per dish as the response variable and predator species as the predictor (package: brglm2 (Kosmidis 2023), code: glm, method=brglmFit).

For all models mentioned above, the significance of each factor and their interaction (if present) was estimated with a Type II Wald chisquare test (package: car (Fox and Weisberg 2019); code: Anova) and model fit was verified with diagnostic plots of scaled residuals against fitted values and with goodness-of-fit tests on the scaled residuals (package: DHARMa (Florian 2022); code: simulateResiduals). Furthermore, we conducted pairwise comparisons using estimated marginal means with Tukey's adjustment, which was carried out using the "emmeans" package (Searle et al. 1980) (code: emmeans).

Results

Community of foliar-foraging predators

We collected a total of 193 individual foliar-foraging predators (n=67 in 2021, n=126 in 2022) through plant inspections. Predator taxa recovered included: *O. insidiosus*, Opiliones, Araneae and *H. axyridis.*

In each year, the cumulative number of foliar-foraging predators per location varied significantly by predator taxon (P<0.0001) (Table 2.1). In both years, the cumulative number of *O. insidiosus* was significantly greater than any other foliar-foraging predator (Fig 2.1).

Community of ground-foraging predators

We collected a total of 9988 ground-foraging predators (n=4905 in 2021, n=5083 in 2022) using pitfall traps. Predator taxa recovered included *Pt. melanarius*, *Po. chalcites*, *Po. lucublandus* and *B. q. oppositum,* as well as Araneae, Opiliones and Gryllidae.

In each year, the cumulative number of ground-foraging predators per location varied significantly by predator taxon (P<0.0001) (Table 2.1). In 2021, the cumulative number of *Pt. melanarius* was 16 times greater than that of the second most abundant predator group, Opiliones (Fig 2.2a). In 2022, the cumulative number of *Pt. melanarius* was 18 times greater than the cumulative number of the second most abundant predators, Opiliones and Gryllidae (Fig 2.2b).

Seasonal abundance of the most prevalent predators and prey

The abundance of the most prevalent foliar-foraging predator, *O. insidiosus,* varied significantly (P<0.0001) across the season in both years (Table 2.2, Fig 2.3a-b). In 2021, the abundance of *O. insidiosus* on 30 June and 13 July was significantly greater than on 29 July and 13 August (Fig 2.3a). In 2022, the density of *O. insidiosus* was greatest on 31 August (Fig 2.3b). In both years, the abundance of *O. insidiosus* did not differ significantly (P>0.05) according to position in the field (edge vs. interior) and the interaction between sample date and position was not significant (P>0.05) (Table 2.2).

The abundance of the most prevalent ground-foraging predator, *Pt. melanarius*, varied significantly (P<0.0001) across the season in both years (Table 2.2, Fig 2.3c-d). In 2021, the abundance of *Pt. melanarius* decreased across the season, with greatest abundance 30 June (Fig 2.3c). In 2022, the abundance of *Pt. melanarius* was significantly greater on 19 July and 17 August than on 6 July, 3 August or 31 August (Fig 2.3d). In both years, the abundance of *Pt. melanarius* did not differ significantly (P>0.05) according to position in the field (edge vs. interior) and the interaction between sample date and position was not significant (P>0.05) (Table 2.2).

The abundance of the potential prey (*R. maxima* larvae) peaked on 29 July in 2021 (Fig 2.3a-d) and on 6 July and 3 August in 2022 (Fig 2.3b and d).

Feeding trials

We tested two species of foliar-foraging predators (*O. insidiosus* and *H. axyridis*) and four species of ground-foraging predatory beetles (*Pt. melanarius*, *Po. chalcites*, *Po. lucublandus,* and *B. q. oppositum*) to determine if they would feed on *R. maxima* larvae. We found that all predator taxa fed on *R. maxima* larvae within 1 hour. The voracity of the foliar-foraging predators varied significantly (P<0.0001) depending on the predator taxon (Table 2.3, Fig 2.4a). After 1 h, adults of *H. axyridis* consumed a significantly greater proportion of the *R. maxima* larvae than did adults of *O. insidiosus* (Fig 4a). The voracity of the ground-foraging predators also varied significantly (P=0.01) depending on the predator taxon after 1 and 24 h (Table 2.3, Fig 2.4b). After 1 h, adults of *Po. lucublandus*, consumed a significantly greater proportion of the *R. maxima* larvae than adults of *B. q. oppositum*, and consumption by *Pt. melanarius*, and *Po. chalcites* was intermediate (Fig 2.4b). After 24 h, the voracity of the ground-foraging predators also varied significantly (P<0.0001) depending on the predator (Table 2.3, Fig 2.4c). Adults of *Po. lucublandus* and *Pt. melanarius* consumed a significantly greater proportion of *R. maxima* larvae than adults of *Po. chalcites* and *B. q. oppositum* (Fig 2.4c).

Discussion

To address the lack of information about the predator community associated with *R. maxima* infestation, we sampled for ground- and foliar-foraging predators in *R. maxima*-infested soybean fields in Minnesota. We found that carabids dominated the ground-foraging predator community, with *Pt. melanarius* as the most prevalent species. These findings align with previous studies in soybean fields in Québec, Canada, where *Pt. melanarius* was the most prevalent carabid, constituting up to 84.5% of all individuals collected (Firlej et al. 2012, 2013). However, other studies that found carabids to be the most abundant ground-foraging predators in Iowa, Michigan, New York, and Missouri had more diverse species compositions and did not have *Pt. melanarius* as the most prevalent predator (Rutledge et al. 2004, Fox et al. 2005, Hajek et al. 2007, O'Rourke et al. 2008).

The foliar-foraging community of predators was dominated by *O. insidiosus*. This finding is consistent with a previous report from soybean fields in Minnesota (Tran and Koch 2017). Similarly, *O. insidiosus* and Araneae were the most prevalent foliar-foraging predators in soybean fields in southeastern Virginia (Whalen et al. 2016). In contrast, a study assessing the foliar-foraging predator community associated with *Aphis glycines* Matsumura (Hemiptera: Aphididae) in soybean fields in Iowa (Schmidt et al. 2008) and Michigan (Costamagna et al. 2008) found that the overall abundance of *H. axyridis* was greater than *O. insidiosus* and Aranae. The diversity and abundance of foliar-foraging predators in our fields was relatively low, which may be explained by the low abundance and diversity of alternate prey in the fields (e.g., Schmidt et al. 2008). For example, though *A. glycines* has remained a significant pest of Minnesota soybean (Koch et al. 2018), the abundance of this pest (and thus potential role as alternate prey) was very low in the fields we sampled (i.e., no plant observed had more than five *A. glycines*) (Melotto, personal observations). Further work is needed to evaluate these predator communities across a broader geography.

The co-occurrence of predators with pest populations is important for effective pest suppression (Rutledge et al. 2004, Lee et al. 2022). Understanding the temporal abundance of the most prevalent predators can help identify the species with the greatest potential impact on *R. maxima* populations. In this study, we investigated the abundance of the two most prevalent predators, *O. insidiosus* and *Pt. melanarius*, and their relationship with *R. maxima* infestation in Minnesotan soybean fields. Larval abundance of *R. maxima* was generally greater in early and mid-season, *O. insidiosus* were more

abundant in early and late season, while *Pt. melanarius* adults were more abundant in early and mid-season. It is worth noting that during two sampling dates in 2022, rainfall events (77 mm on 6 July and 13 mm on 3 August) occurred while the pitfall traps were deployed, which likely resulted in decreased capture of *Pt. melanarius* on those dates. Accordingly, we hypothesized that *Pt. melanarius* (which occurred in much higher numbers early in the season) may have more potential to curb outbreaks of *R. maxima* compared to *O. insidiosus* that occurred in lower numbers and generally later in the season. Because the *R. maxima* become most available to predators after they have injured the plants and drop to the soil, it is important to note that predators will likely be most impactful on reducing plant injury from *R. maxima* in subsequent generations that season or the following season.

Many of the predators that we investigated in our laboratory feeding experiments (e.g., *H. axyridis*, *O. insidiosus*, *Pt. melanarius*, and *Bembidion* spp.) have been previously documented to feed on cecidomyiids or other Diptera (Pitcher 1952, Floate et al. 1990, Corlay et al. 2007, Renkema et al. 2012). *Resseliella maxima* larvae were consumed by all predator taxa tested, regardless of their foraging behavior (foliar or ground). Among the foliar-foraging predators tested, *H. axyridis* was more voracious than *O. insidiosus* when consuming *R. maxima* larvae. Similarly, Rutledge et al. (2004) documented higher consumption of *A. glycines* by *H. axyridis* than *O. insidiosus* in a no-choice feeding experiment. The difference in predation rates between these two predator species could be attributed to the differences in their body sizes and the mouthparts of the predators, with *H. axyridis* being larger and having chewing mouthparts while *O. insidiosus* has piercing-sucking mouthparts. For ground-foraging predators, *Po. lucublandus* and *Pt. melanarius* generally consumed more *R. maxima* larvae than did *Po. chalcites* and *B. q. oppositum*, despite some subtle difference between the 1 and 24 h intervals. These findings are consistent with those reported by Floate et al. (1990), who found that *Bembidion quadrimaculatum* consumed fewer *Sitodiplosis mosellana* Géhin (Diptera: Cecidomyiidae) larvae compared to other carabids. The lower predation rates for *Bembidion* spp. may be due to their smaller size than the other carabids (Floate et al. 1990).

Although foliar-foraging predators occurred in fields with *R. maxima* and fed on *R. maxima* larvae in our predation experiments, they may have less opportunity to encounter *R. maxima* in the field. Early instars of *R. maxima* larvae remain within the soybean stems, making them less accessible to foliar-

foraging predators. As the *R. maxima* larvae mature and drop to the soil to pupate, they likely become more vulnerable and accessible to ground-foraging predators. Therefore, based on *R. maxima* biology, the high abundance of *Pt. melanarius* in our fields (especially early and mid-season when *R. maxima* abundance is high), and the known importance of carabids in biological control of other cecidomyiids (Floate et al. 1990), we hypothesize that ground-foraging predators like *Pt. melanarius*, could play an important role in biological control of *R. maxima* populations. However, further research is needed to determine if *Pt. melanarius* and the other predators will feed on *R. maxima* under field conditions. Furthermore, this research was performed at only one farm, so further research is needed at multiple locations to examine geographic variability in the predator communities associated with *R. maxima*. Our findings provide a foundation for the development of biological control programs for *R. maxima*.

Source	χ^2	Df	P
Plant inspection, 2021			
Predator taxa	64.43	3	< 0.0001
Plant inspection, 2022			
Predator taxa	89.83	2	< 0.0001
Pitfall trap, 2021			
Predator taxa	749.1	6	< 0.0001
Pitfall trap, 2022			
Predator taxa	1073.8	6	< 0.0001

Table 2. 1. ANOVA table for the effects of predator taxon on the cumulative number of predators per location for 2021 and 2022.

Source	χ^2	Df	P
O. insidiosus, Plant inspection 2021			
Date	19.113	4	< 0.0001
Position	1.23	1	0.268
Date x Position	3.413	4	0.491
O. insidiosus, Plant inspection 2022			
Date	63.535	4	< 0.0001
Position	0.043	1	0.835
Date x Position	0.972	4	0.914
Pt. melanarius, Pitfall trap 2021			
Date	149.711	4	< 0.0001
Position	3.572	1	0.059
Date x Position	1.894	4	0.755
Pt. melanarius, Pitfall trap 2022			
Date	58.666	4	< 0.0001
Position	0.823	1	0.364
Date x Position	3.343	4	0.502

Table 2. 2. ANOVA table for the effects of date of collection and position in the field (edge vs. interior) on the abundance of predators collected in the different methods of sampling in 2021 and 2022.

Source	χ^2	Df	P
Foliar-foraging predators (1 h)			
Predator taxa	22.29	1	< 0.0001
Ground-foraging predators (1 h)			
Predator taxa	11.19	3	0.011
Ground-foraging predators (24 h)			
Predator taxa	33.9	3	<0.0001

Table 2. 3. ANOVA table for the effects of predator taxon on the predation of *R. maxima* larvae in feeding trials.

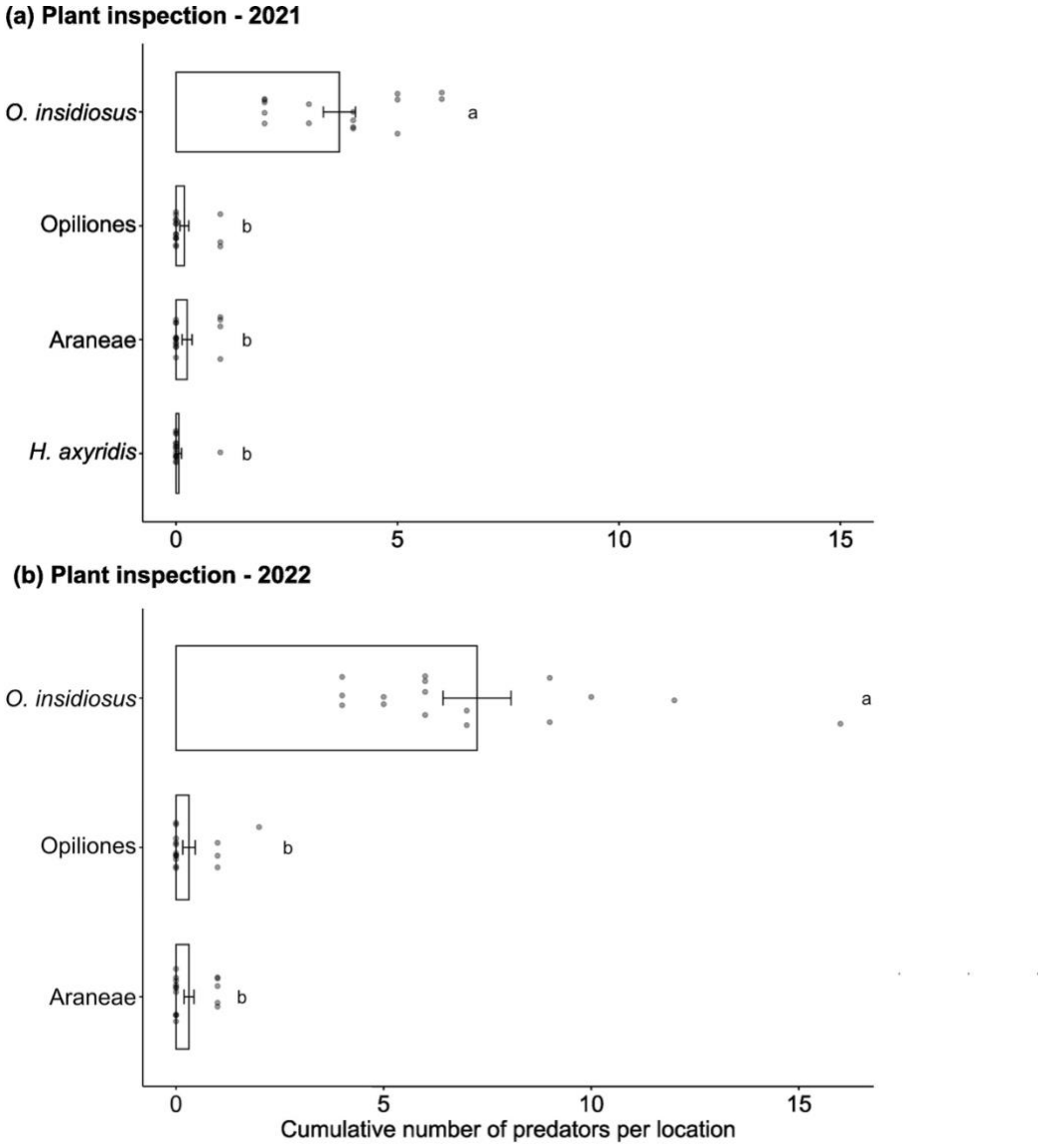

Figure 2. 1. Cumulative number (mean ± SE) of foliar-foraging predators collected over the season per location (n=16) through visual whole-plant inspections in 2021 (a) and 2022 (b). Different letters represent significant differences among the means ($P<0.0001$).

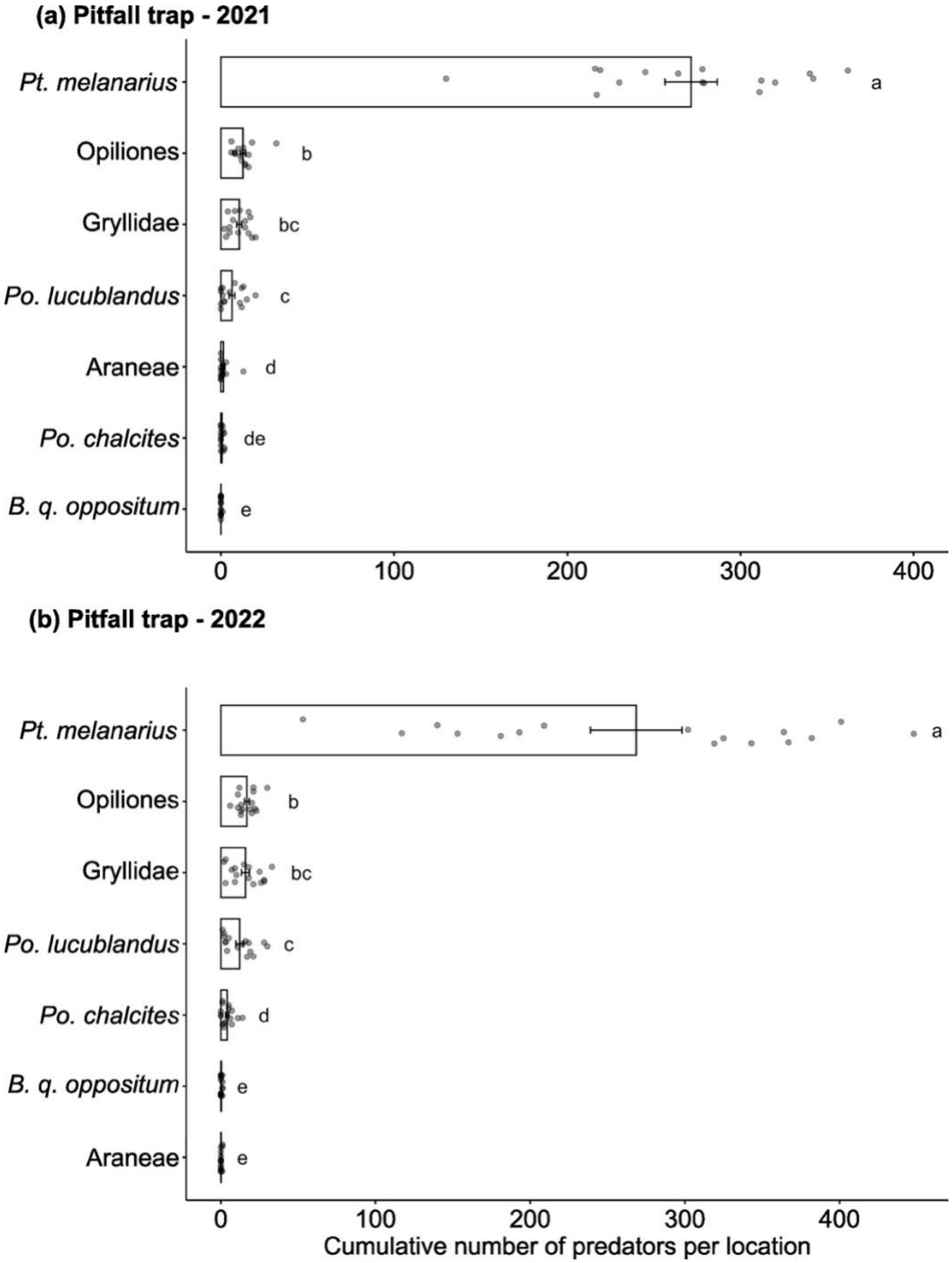

Figure 2. 2. Cumulative number (mean ± SE) of ground-foraging predators collected over the season per location (n=16) through pitfall traps in 2021 (a) and 2022 (b). Different letters represent significant differences among the means (P<0.0001).

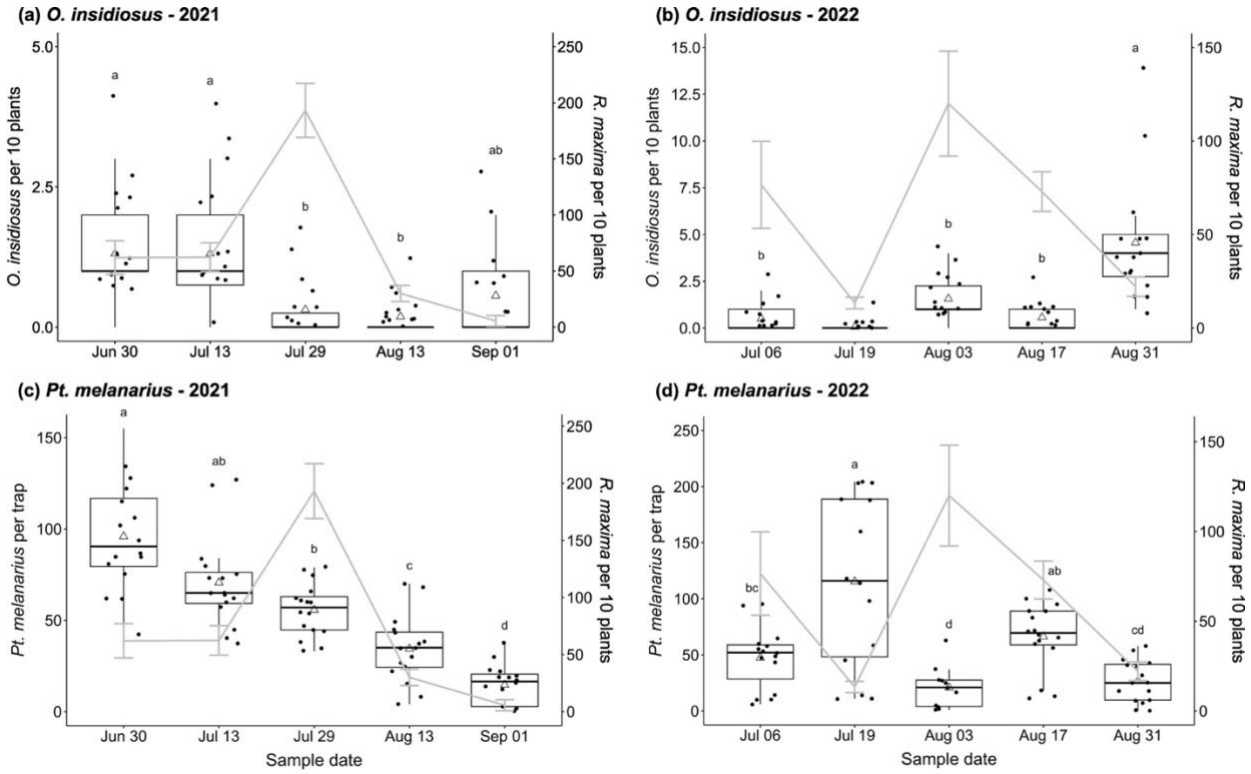

Figure 2. 3. Temporal abundance of most prevalent predators and *R. maxima* larvae in 2021 and 2022. *O. insidiosus* collected through plant inspections (i.e., boxplot/left axis) and *R. maxima* larval abundance through soybean stem dissections (i.e., gray line/right axis) in 2021 (a) and 2022 (b). *Pt. melanarius* collected through pitfall traps (i.e., boxplot/left axis) and *R. maxima* larval abundance through soybean stem dissections (i.e., gray line/right axis) in 2021 (c) and 2022 (d). Black triangles on boxplots represent means. Different letters represent significant differences among the means (P<0.0001).

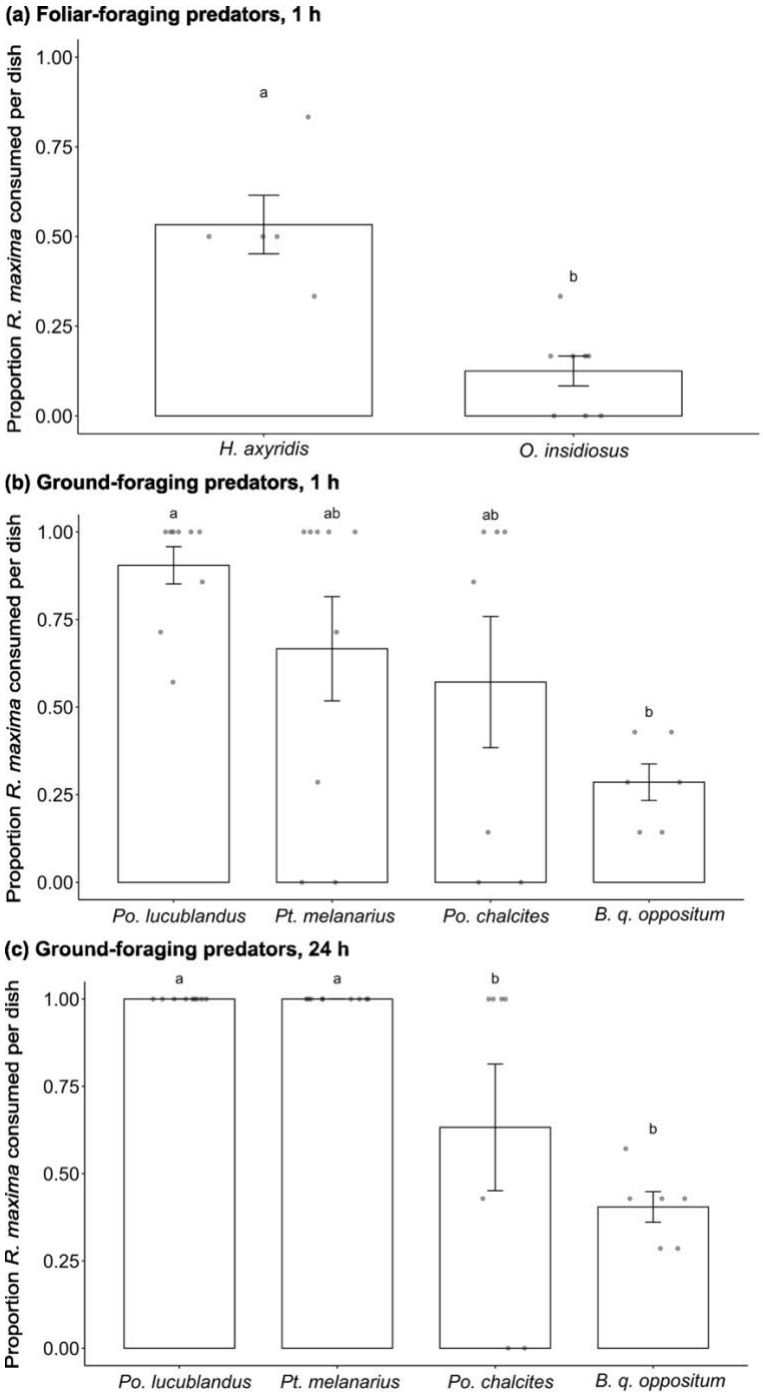

Figure 2. 4. Voracity (mean ± SE) of the different predator taxa offered *R. maxima* larvae for 1h or 24 h in laboratory predation experiments. (a) Foliar-foraging predators for 1 h, (b) ground-foraging predators for 1 h, and (c) ground-foraging predators for 24 h. Different letters represent significant differences among the means ($P<0.0001$).

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