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RESEARCH ARTICLE

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Natural enemies of *Lemna minuta* in its native range and their potential as biological control agents for Europe

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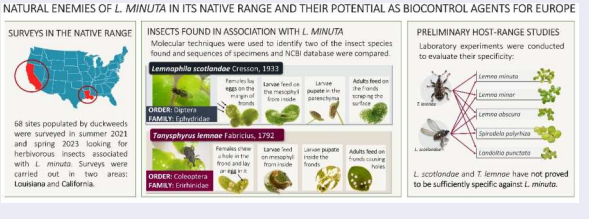
ABSTRACT

Lemna minuta is native to North America but an invasive alien plant in Europe, where it poses significant threats to freshwater ecosystems. Explorations for biological control agents were conducted in two regions of the United States (Louisiana and California), revealing three candidate species. Subsequent laboratory investigations focused on the weevil *Tanysphyrus lemnae* and the fly *Lemnaphila scotlandae* as the most promising agents and confirmed that both the herbivorous insects attack *L. minuta* plants. To evaluate the host specificity of these two potential agents, insects were exposed to five species of duckweed commonly occurring in North America and Europe. Preliminary host-range testing indicated that *T. lemnae* develops on all evaluated duckweed species, including those from different genera (*Lemna*, *Spirodela*, *Landoltia*). Conversely, data revealed that feeding and development of the dipteran *L. scotlandae* are limited to species in the genus *Lemna*, but this includes *L. minor*, a native European species threatened in Europe due to the spread of *L. minuta*. No-choice and choice tests confirmed that neither larvae nor adults of *L. scotlandae* discriminate between the two *Lemna* species, except for pupation, which occurs more frequently in *L. minor* under choice conditions. We conclude that the broad host-range of *T. lemnae* and *L. scotlandae* render them unsuitable as biological control agents of *L. minuta* in Europe.



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#The data that support the findings of this study are available on request from the corresponding author.

1. Introduction

Invasive free-floating aquatic plants pose substantial threats to freshwater ecosystems (Stiers et al., 2011; Hussner et al., 2021). Their invasiveness is driven by a complex interplay of physiological and ecological factors (Vilà et al., 2011; Hussner, 2012), including adaptations for both sexual and asexual reproduction (Li, 2014; Eckert et al., 2016), buoyancy facilitating swift colonisation (Heidbüchel et al., 2020) and hydrological connectivity aiding dispersal via water currents (Gurnell et al., 2008). Additionally, animal vectors (Coughlan et al., 2015) and human activities (Brunel, 2009; Hussner et al., 2010) contribute to their spread. Their establishment can be further facilitated by limited competition with native plants (Chadwell & Engelhardt, 2008; Thouvenot et al., 2019), disturbances such as eutrophication (Coetzee & Hill, 2012), and release from natural enemies (Petruzzella et al., 2017; Pulzatto et al., 2018). The resulting mats outcompete native vegetation, leading to reduced biodiversity and habitat alterations, with cascading effects on fauna, nutrient cycling, and sediment stability (Hill, 2003; Keller et al., 2018). Moreover, they can disrupt irrigation systems, degrade water quality, hinder navigation (Habib & Yousuf, 2014), and interfere with fishing and recreational activities (Lancar & Krake, 2002).

Efforts to mitigate the effects of floating exotic aquatic weeds have led to classical biological control strategies targeting species such as *Alternanthera philoxeroides* (Mart.) Griseb., *Pistia stratiotes* L., *Azolla filiculoides* Lam., *Pontederia crassipes* (Mart.) and *Salvinia molesta* D.S. Mitch. (Forno & Julien, 2000). The introduction of *Neochetina eichhorniae* Warner significantly reduced *P. crassipes* in Africa, while *Cyrtobagous salviniae* Calder & Sands effectively controlled *S. molesta* in subtropical regions (Sullivan et al., 2011).

However, no classical biological control attempts have targeted duckweeds (Lemnaceae). Duckweeds are the smallest and fastest-growing flowering plants (Acosta et al., 2021), floating on slow-moving or stagnant water bodies (Preston & Croft, 1977). Among these, the American duckweed *Lemna minuta* Kunth (Araceae), introduced to Europe in the 1940s (Ceschin et al., 2018), has rapidly spread across broad climatic ranges (Roy et al., 2020) and is now considered invasive in Italy (Celesti-Grapow et al., 2009). Enabled by its dispersal mechanism (Bramley et al., 1995; Coughlan et al., 2015) and high reproductive rate (Landolt, 1986; Ceschin et al., 2016a), *L. minuta* quickly forms dense mats that obstruct light and gas exchanges (Dussart et al., 1993; Janes et al., 1996). These mats negatively impact macroinvertebrates and reduce the cover and richness of native macrophytes, including the native duckweed in Europe *Lemna minor* L. (Ceschin et al., 2016b, 2020a).

Due to the rapid spread of *L. minuta* across Europe, there is an urgent need for effective management strategies (Gassmann et al., 2006; Baars, 2012). While classical biological control is a promising option, it remains largely unexplored, with limited information available on natural enemies in the plant's native range (Gassmann et al., 2006). North America boasts a rich array of insect herbivores that target various duckweed species (Buckingham, 1989; Center et al., 2002), making them viable candidates for assessing their potential as biological control agents against *L. minuta* in its non-native range. Among these, the weevil *Tanysphyrus lemnae* (Fabricius) is one of the most common herbivores of duckweeds (Center et al., 2002), with documented impacts on

Lemna minor and *Lemna perpusilla* Torr. (Lee et al., 2022). The native shore-fly *Lemnaphila scotlandae* Cresson, widely distributed in the United States (Buckingham, 1989) has been observed attacking *Lemna valdiviana* Phil., *Lemna gibba* L. and *L. minor* (Scotland, 1940; Mansor & Buckingham, 1989). A cosmopolitan herbivore of duckweeds, water lily aphid *Rhopalosiphum nymphaeae* Linnaeus (Scotland, 1940), has been found in experimental studies to target *Spirodela polyrhiza* (L.) Schleid. and *L. minor*, displaying a preference for the former (Subramanian & Turcotte, 2020). Additionally, larvae of aquatic moth species contribute to herbivory on duckweeds in North America (Scotland, 1940). Common native species in the United States include the black duckweed moth, *Elophila tinealis* Munroe and the water lily leaf-cutter moth, *Elophila obliteralis* Walker (Munroe, 1972), whose larvae feed on a wide range of aquatic plants, including *Lemna*, *Sagittaria*, and *Myriophyllum* species (Stoops et al., 1998).

Unfortunately, none of these widespread North American insects have demonstrated specific attacks on *Lemna minuta*. To develop a biological control program for *L. minuta*, it is crucial to address this knowledge gap by characterising the insect herbivores associated with *L. minuta* in its native range and assessing their specificity concerning closely related non-target species. Therefore, this study aims to (i) identify herbivorous insects associated with *L. minuta* in selected regions of the United States and (ii) examine the life history and host specificity of a selected group of herbivores within the framework of a European perspective.

2. Materials and methods

2.1. Native range surveys

Surveys for insect herbivores associated with *L. minuta* were conducted during the summer of 2021 and the spring of 2023 in Louisiana and California. The selection of survey areas was based on the ecological and climatic characteristics of southern Louisiana, which is abundant in wetlands that support a high diversity of duckweed species, and California, characterised by a Mediterranean climate similar to Italy. A total of 68 sites populated by various duckweed species were surveyed. Sites were identified by first reviewing publicly available satellite imagery to locate water bodies, followed by on-site verification of both the sites and their aquatic flora. This method was chosen over consulting herbarium-preserved samples as duckweed populations fluctuate with changes in water levels due to rainfall, flooding, and hurricanes, which are common phenomena in these areas.

In Louisiana, sampling was carried out in various types of water bodies (ditches, canals, lakes, ponds, swamps) distributed across diverse environments including the Mississippi River floodplain, and the deltaic coastal freshwater marshes. In California, surveys were conducted in wetlands associated with the San Francisco Bay and Sacramento-San Joaquin Delta, an extensive tidal, freshwater region situated at the landward end of the San Francisco Estuary (Conrad et al., 2023). Data collected at each site included information on the date, site coordinates, type of water body, and free-floating aquatic plant species present at the site. When *L. minuta* was recorded, plants underwent inspection for natural enemies and signs of herbivory. Samples of both the plant and herbivorous insects were collected and brought to the laboratory for

identification. Plant specimens were identified under the stereoscope using Landolt (1986) and the Flora of the Southeastern United States (Weakley, 2020).

2.2. Identification of herbivorous insects

Collected insects were preserved in 90% to 100% ethyl alcohol, and specimens or photographs of them were sent to experts (Dr. Christopher Carlton, LSU AgCenter, Louisiana; Dr. Tadeusz Zatwarnicki, Opole University, Poland) for morphological identification. For the identification of the specimens using molecular method, the Dneasy Blood & Tissue kit (Qiagen, Hilden, Germany) was utilised to extract genomic DNA from the sample specimens. The entire body of each insect was ground using Fisherbrand™ Rnase-Free Disposable Pellet Pestles (Fisher Scientific, Hampton, NH, USA) in 180 µL of ATL buffer and 20 µL of Proteinase K solution (Qiagen, Hilden, Germany) and each sample was incubated overnight at 56°C. The manufacturer's protocol was followed in the subsequent steps. PCR was conducted using a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). The total volume of each PCR sample was 25 µL, containing 12.5 µL of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, Waltham, MA, USA), 1 µL of template genomic DNA, 8.5 µL of nuclease free water, 0.5 µL MgCl2 (Thermo Scientific, Waltham, MA, USA), and 1.0 µL of each primer at 5 µM resuspended in a low TE buffer (10 mM Tris, 0.05 mM EDTA, pH 8). A primer set, LCO1490 (forward) and HCO2198 (reverse) (Folmer et al., 1994), was used to amplify 658 bp of the target COI DNA gene region. The PCR conditions were 95°C for 3 min; 5 cycles of 95°C for 30 s, 45°C for 30 s and 72°C for 1 min and 35 cycles of 95°C for 30 s, 51°C for 30 s and 72°C for 1 min; and a final elongation at 72°C for 7.5 min. Using a 2.0% agarose gel stained with 1X SYBR™ Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA), the amplification of PCR products was confirmed. Crude PCR products were cleaned by a primer depletion clean-up method developed at the LSU Genomics facility (Baton Rouge, LA, USA) and sequenced on the Applied Biosystems 3130xl Genetic Analyzers (Thermo Scientific, Waltham, MA, USA) using BigDye Terminator v 3.1 Cycle Sequencing Kit (Thermo Scientific, Waltham, MA, USA) at the same facility. Utilising Geneious Prime software (version 2022.0.1) (https://www.geneious.com), DNA sequences were edited and assembled via De novo assembly. The obtained sequences were compared with COI sequences deposited in the GenBank database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST).

2.3. Plant and insect colonies

2.3.1. Duckweeds

Monospecific colonies of five duckweed species were established from field populations: *L. minuta* (target duckweed), *L. minor* and *Spirodela polyrhiza* (native to Italy), *Lemna obscura* (Austin) Daubs and *Landoltia punctata* (G.F.W. Meyer) Les & D.J. Crawford (see Table 1). Plant colonies were maintained in an indoor greenhouse with 12:12 light/dark cycle, at 33 (± 3)°C and 40% humidity. Before starting the experiments, a minimum period of one month was observed to ensure that the plants were free from herbivores. Plant colonies were housed in $30 \times 25 \times 10$ cm (length × width × height)

Table 1. Duckweed species, geographical coordinates of collection sites in Louisiana and California, native range of the species, and their status as native or alien in North America and Europe.

Name	Collection coordinates	Native range	North America	Europe
Lemna minuta Kunth	37.9365; −121.4340 30.8187; −91.5801	Northern America	Native	Alien
Lemna minor L.	30.6106; −91.4679 30.3677; −91.1840	Subcosmopolitan	Native	Native
Lemna obscura (Austin) Daubs	30.3677; −91.1840	Southeastern U.S.A.	Native	Alien
Spirodela polyrhiza (L.) Schleid.	30.3679; −91.1822	Southeastern U.S.A./ Middle Europe	Native	Native
Landoltia punctata (G.F.W. Meyer) Les & D.J. Crawford	30.3679; −91.1822	Australia and Southeast Asia	Alien	Alien

Source: DAISIE – Inventory of alien invasive species in Europe. Checklist dataset: https://doi.org/10.15468/ybwd3x.

plastic containers filled 10 cm deep with distilled water and 0.015 g/L of Miracle-Gro ® (Scotts Miracle-Gro Company, Marysville, OH, USA) fertiliser (24-8-16, N-P-K). The containers were refilled with the same solution twice a week to ensure the water level remained constant. The greenhouse was located in the Life Sciences Building at Louisiana State University in Baton Rouge, LA, USA.

*2.3.2. Flies (*Lemnaphila scotlandae *Cresson)*

Fly colonies were established in September 2021 from an initial population consisting of adults and pupae collected from a ditch in French Settlement, LA, USA (30.3300; −90.8136). The colonies were kept in an indoor greenhouse at 31.5 (± 2)°C, 30% humidity and 12:12 light/dark cycle. Flies were inoculated into pop-up mesh cages (40 × 40 × 40 cm; length × width × height) in which plastic containers (30 × 25 × 10 cm) filled with distilled water and a mixed colony consisting of the five duckweed species were placed. All life stages were maintained in cages (Figure 1). New plants from the duckweed colonies were added in excess of the flies' dietary needs at weekly intervals.

*2.3.3. Weevils (*Tanysphyrus lemnae *Fabricius)*

Given the difficulty encountered in establishing a laboratory colony of weevils, it was decided to use field-collected adults for the experiment. Biomass samples of duckweeds were collected at two roadside ditches in St. Amant, LA, USA (30.2188; −90.8149 and 30.2615; −90.7619) and transferred to the laboratory in 18 × 25 cm Ziploc® plastic bags (SC Johnson, Racine, WI, USA). Live adults were retrieved by drying plant biomass samples in Berlese funnels equipped with 60 W lamps and fitted with Whirl-Pak® bags (Nasco, Fort Atkinson, WI, USA) at the bases. Prior to the start of the experiment, weevils were kept in an indoor greenhouse at 31.5 (± 2)°C, 30% humidity and a 12:12 light/dark cycle, where they were allowed to acclimate for 7 days in 30 × 25 × 10 cm (length × width × height) transparent plastic containers filled with distilled water and a mixed colony of the five duckweed species so as not to influence their behaviour in the host-range test.

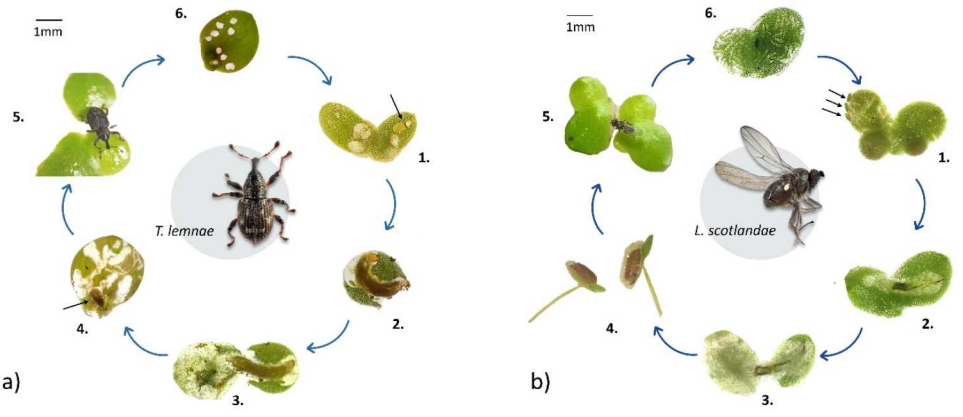

Figure 1. Life cycle of *T. lemnae* (a) and *L. scotlandae* (b) on *L. minuta*. *T. lemnae*: The female chews a hole in the plant, lays an egg in the hole and seals it with frass (a1). Newly hatched larvae inside the plants feed on mesophyll (a2), and when the plant is entirely consumed, larvae migrate to another plant (a3). The larvae pupate inside the plant (a4), and upon emergence, adult weevils feed on the plant's surface (a5), resulting in characteristic round holes (a6). *L. scotlandae*: Females lay yellowish ovoid eggs on the margin of the upper surface (b1). Neonate larvae burrow into the plant, consuming it until depletion (b2), and then move to adjacent plants (b3). Pupation takes place in the parenchyma (b4), and adult flies emerge from the pupae (b5). The flies feed on the plant by scraping its upper surface (b6).

2.4. Preliminary host-range testing

2.4.1. Oviposition and larval development of L. scotlandae *and* T. lemnae

An experiment was designed to assess the host-range of the two insect species found in surveys. Five plant species (detailed above and in Table 1) were tested for each insect species. Each of the duckweed species was individually assigned to five replicate containers, resulting in a total of 50 replicates across the experiment (2 insect species × 5 plant species × 5 replicates). Round plastic containers (7 × 3 cm; height × diameter) were filled with 15 ml of distilled water, and plants were transferred using a camel's hair paintbrush, forming a plant monolayer on the water surface. Mesh lids were placed on each container to allow oxygenation while confining the insects within the experimental arenas. Six adult insects of either the fly or the weevil were collected from the laboratory population and added to each of the replicated plastic containers. Flies used in the experiment were 24–48 h old, while the weevils' age was unknown. Sex ratio was determined prior to the experiment by randomly selecting 10 adults from each laboratory population and determining the gender of each individual. This process was repeated three times, and the observed sex ratio was 1:1 for both insect colonies, which was then inferred to the six individuals used for the experiment. During the experiment, plants were exposed to adult insects for 96 h, after which the insects were removed and photographs of the plants in each replicate were taken under the stereoscope. For flies, the number of eggs laid in 96 h was counted. The eggs were recognisable by their elliptical shape and yellowish colour, with parallel surface ridges running lengthwise (Center et al., 2002). Eggs were kept under the same experimental conditions and checked at 24-hour intervals until eclosion. Developmental times of each stage and number of adults emerging from

the initial laid eggs were recorded. For weevils, whose eggs were laid inside the mesophyll of the plant, and therefore, difficult to detect, the plants were checked daily until egg hatching occurred, and number of neonate larvae was counted.

2.4.2. Feeding damage by L. scotlandae larvae

A second series of experiments were designed to quantify herbivory levels from the fly on the target duckweed *L. minuta* as compared to the non-target congener and European native *L. minor*. This set of experiments did not include *T. lemnae*, since results from the preliminary host-range test showed that the weevil attacked all provided duckweed species. The experimental setup mirrored the previous trials, with the containers filled with 15 ml of distilled water and a monolayer of plants, 260 (± 9.71, $n = 10$) for *L. minuta* and 155 (± 11.84, $n = 10$) for *L. minor*. There were five replicate containers for each test plant species (2 plant species × 5 replicates). In the no-choice test, five eggs approaching eclosion were collected from the fly colonies and transferred with a camel-hair paint brush in each container. Once hatched, the larvae were allowed to feed and grow for nine days until pupation. The larvae of *L. scotlandae* are known to tunnel inside the duckweeds, consuming the mesophyll while leaving the epidermis intact (Scotland, 1940). When larvae pupate, brownish pupa inside the mesophyll can be seen through the epidermis which is transparent. Photographs of the surface of the plants in each replicate container were taken prior to adding the larvae and again at the end of the experiment. Number of initial and final plants, as well as plants with tunnels, indicative of larval feeding, were counted.

A dual-choice test was conducted to quantify feeding and pupation site preferences when both *Lemna* species were present. The test design closely followed the previous experiments, except 100 (±4.52, $n = 10$) plants of *L. minuta* and 60 (±1.38, $n = 10$) of *L. minor* were added to each container to ensure equal leaf surface area (LSA) of both species. In this test, 9 replicates were conducted. A thin plastic divider was placed in the centre of the container and the two *Lemna* species were added separately to either side of the divider, to cover the water surface without overlapping plants. Subsequently, the divider was removed, and the plants of the two species were mixed using a camel-hair paint brush. Pictures were taken nine days following inoculation and the number of initial, final, and damaged plants for each species were recorded. Additionally, the number of fly pupae and the plant species in which they pupated were also noted.

2.4.3. Feeding damage by L. scotlandae adults

Herbivory damage of *L. scotlandae* on *L. minuta* and *L. minor* was quantified by counting the number of dead plants (white in colour), plants with feeding gouges, and duckweed growth, in the presence or absence of adult insects. In the no-choice test, two-thirds of the water surface in each container was covered with either *L. minuta* or *L. minor*, allowing space for plant growth. Subsequently, five adult of *L. scotlandae* individuals were added into each container. As before, containers were covered with a fine mesh to prevent escape. Each treatment was replicated five times and an insect-free control set with five replicates was also established to monitor plant growth rates during the experimental time frame in absence of the herbivore. To assess feeding damage the percentage of non-viable or damaged plants was quantified and plant growth was measured after 72-hours of exposure to adults. For the choice test, the experiment was repeated under the

same conditions as described above but with both species separated by a plastic divider, providing equal LSA for both. A set of five replicate containers without the adult insects were used as a control as described previously.

2.5. Statistical analyses

Assumptions of normality and homoscedasticity were verified on model residuals using the appropriate tests (Shapiro–Wilk for normality and Levene for homoscedasticity). One-way ANOVAs were used to compare the means of the number of eggs laid/newborn larvae, the adults emerged from the eggs, the eggs developed to the adult stage and the duration of each life cycle stage. When the assumptions were not met, a non-parametric alternative was used (Kruskal–Wallis). Post-hoc pairwise comparisons among the test plants were conducted using Tukey's HSD test, with the Dunn's test serving as a non-parametric alternative to identify significant differences ($\alpha = 0.05$).

An independent samples *t* test was used to compare means of relative growth rate among plant species and damaged plants in no-choice tests, while a paired *t* test was used for comparing the number of pupae and damaged plants in the choice tests.

The parameters expressed as percentages, such as pupated larvae, adult survivorship, and damaged plants, were analyzed using a generalised linear model (GLM) with binomial error distribution and logit link function. Plant species served as a fixed effect factor in all statistical models. The analyses were performed using R software, version 4.2.1.

3. Results and discussion

3.1. Native range surveys

We observed an uneven distribution of *L. minuta* in the surveyed areas of Louisiana and California. In Louisiana, its presence was found at only 3 of the 44 sites surveyed (7%). This infrequent occurrence was unexpected, given the diversity and abundance of other duckweed species in Louisiana sites, where up to 15 species have been documented. Native populations of *L. minuta* at these sites may be affected by competition with alien duckweed species, such as the Asian *L. punctata.* Interestingly, the reverse occurs in Europe, where *L. minuta* outcompetes native duckweeds (Ceschin et al., 2016b). In Louisiana, *L. minuta* was solely observed in the most natural sites with minimal anthropogenic disturbance (Figure A1), whereas the exotic *L. punctata* was never recorded in these locations. Conversely, the latter abounded in highly disturbed sites (F. Mariani personal observation), providing additional support for the established correlation between anthropogenic disturbance and the presence of alien plants (Meyer et al., 2021).

On the other hand, in California *L. minuta* exhibited widespread distribution, occurring at 14 of the 19 sites surveyed (74%); however, no herbivorous insects were detected during our survey. This temporary lack of herbivores might be due to seasonal changes, as insects may follow specific life cycles or be active only during certain periods. Our surveys in August may have coincided with a time when *L. minuta* herbivorous insects were not actively present, or the absence of herbivores could be influenced by environmental factors like climatic conditions or the presence of natural predators or parasites. Another reason could be the occurrence of pesticides in the water; in

fact, the Sacramento-San Joaquin River Delta is known to be exposed to mixtures of pesticides that flow into Delta waterways from various sources (Kuivila & Orlando, 2012), leading to negative consequences for aquatic invertebrate communities (Weston & Lydy, 2014). To obtain a more comprehensive understanding of the insects associated with *L. minuta*, additional surveys in California should be conducted at various times throughout the year and in areas with minimal anthropogenic disturbance. Given the absence of herbivorous insects during the California survey, the herbivorous insects used in the laboratory tests in this study were exclusively collected during the Louisiana surveys.

3.2. *Herbivorous insects associated with* L. minuta

Three herbivorous insects in the orders Coleoptera, Lepidoptera, and Diptera were found in Louisiana, representing the first known survey for natural enemies targeting *L. minuta* in its native range. The herbivores discovered during our surveys include: *Elophila tinealis, Tanysphyrus lemnae* and *Lemnaphila scotlandae.*

3.2.1. Elophila tinealis *(Lepidoptera: Pyraloidea: Crambidae)*

Morphological identification revealed that the moth found associated with *L. minuta* at one location was *Elophila tinealis*. This moth is native to North America and can be found in swamps and wetlands, where it completes its life cycle on *S. polyrhiza*, *Lemna valdiviana* and *Lemna perpusilla* (Kinser & Neunzig, 1981). However, there is no evidence in scientific literature that *L. minuta* is a host plant of *E. tinealis*. Field observations revealed that larvae of this moth use *L. minuta* to construct protective cases for pupation, similar to *Cataclysta lemnata* (Linnaeus), a lepidopteran native to Europe that adapted its biology to utilise American *L. minuta* as a host plant (Mariani et al., 2020a, 2020b). However, due to the broad host range of *E. tinealis* (Kinser & Neunzig, 1981), this species was not considered suitable as a potential biological control agent for *L. minuta* and was therefore not included in further host-range tests.

3.2.2. Tanysphyrus lemnae *(Coleptera: Curculionoidea: Erirhinidae)*

Morphological and genetic identification confirmed the presence of *Tanysphyrus lemnae* (GenBank Accession Number: OR459815) among sampled duckweeds (Figure A2). This weevil was found at multiple sites explored in Louisiana, but it was associated with *L. minuta* at only two of these sites. This insect is the most prevalent and widely distributed herbivorous arthropod known to attack duckweeds (Center et al., 2002). The female chews a hole into the plant's upper surface and deposits eggs individually within the plant parenchyma, sealing the chamber with frass – a mixture of macrophyte fragments and excrement (Figure 1(a1)). The eggs undergo maturation within the plant, and upon hatching, larvae remain inside, consuming parenchyma while preserving the epidermis (Figure 1(a2)). Once the content of one plant has been completely consumed, the larvae migrate to the next (Figure 1(a3)), continuing to feed until pupation, which occurs within the plant (Figure 1(a4)). Adults emerged from pupae feed on plant surface (Figure 1(a5)), creating large circular perforations (Figure 1(a6)) (Center et al., 2002).

3.2.3. Lemnaphila scotlandae *(Diptera: Ephydroidea: Ephydridae)*

Lemnaphila scotlandae (Figure A3) was identified through morphological and genetic analysis (GenBank Accession Number: OR462835.1). This species was discovered in a roadside ditch in a mixed stand of *L. minor* and *L. minuta*, where the former was most abundant compared to the latter. *Lemnaphila scotlandae* is native to the United States, and it is among the few insects known to attack duckweeds in North America (Buckingham, 1989). Although this insect was previously found to be specific to the genus *Lemna* (Mansor & Buckingham, 1989), our findings provided the first evidence that *L. minuta* is a host of *L. scotlandae*. Observation of plants under stereoscope revealed that both *Lemna* species displayed typical feeding damage caused by this fly, as they had their upper surface scraped (Cresson, 1933). Female adults lay yellowish ovoid eggs on the margin of the upper surface of the plant (Figure 1(b1)). Upon hatching, the larvae burrow inside the plant, feeding on the spongy parenchyma (Figure 1(b2)). When the plant is entirely consumed, the larvae move to the adjacent plants to continue feeding (Figure 1(b3)). Pupation takes place in the parenchyma of duckweeds (Figure 1(b4)) and the larvae emerge as flies (Figure 1(b5)), which also feed on the duckweeds by scraping their upper surface (Figure 1(b6)) (Scotland, 1940).

3.3. *Life history of* Tanysphyrus lemnae

Preliminary host-range tests demonstrated that *T. lemnae* successfully fed on, laid eggs and hatched on all duckweed species tested. Within 24 h of the experiment's start, eggs and characteristic feeding damage holes, indicative of feeding activity (Center et al., 2002), were observed on all tested species. Differences emerged among host plants in the time elapsed from the start of the experiment to egg hatching ($F = 5.46$; $df = 4, 15$; $p = 0.0042$; Figure 2(a)), indicating variable reluctance to oviposition among the tested plants. Post-hoc tests revealed that the egg developmental times on *L. minor* (10 ± 0.6 days) and *L. minuta* (9.7 ± 0.4 days) differed significantly from *L. obscura* (7.2 ± 0.2 days).

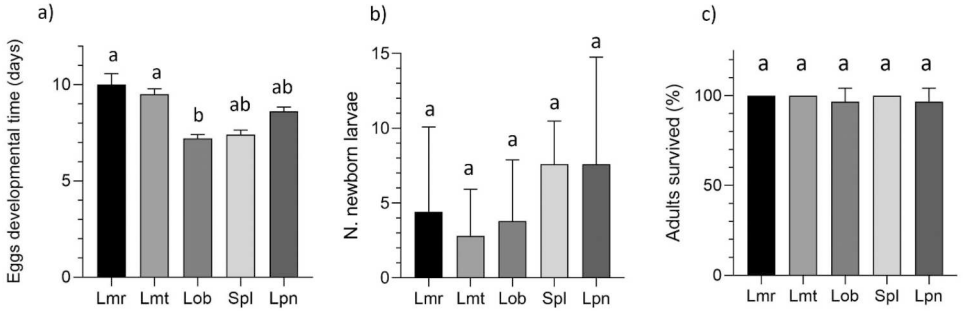

Figure 2. (a) Eggs developmental times on five different duckweed species (b). Number of neonate larvae emerged from the oviposition of *Tanysphyrus lemnae* on five different duckweed species. (c) Number of adult weevils that survived after exposure to five different duckweed species for 96 h. Within each graph, different letters indicate significant differences ($p < 0.05$). The x-axis displays the duckweed species utilised as test plants, denoted by the following abbreviations: Lmr = *Lemna minor*; Lmt = *Lemna minuta*; Lob = *Lemna obscura*; Spl = *Spirodela polyrhiza*; Lpn = *Landoltia punctata*.

There were no significant differences in the number of neonate larvae ($F = 10.10$; d$f = 4$, 20; $p = 0.4067$; Figure 2(b)) or adult survivorship ($\chi^2 = 5.79$, d$f = 4$, $p = 0.2156$; Figure 2(c)) among host plants. Literature provided an early indication that *T. lemnae* may be a generalist among the duckweeds, with reported host plants in the genera *Lemna* and *Spirodela* (Cummins & Merritt, 1996; Center et al., 2002) but no information existed previously on which duckweed species were suitable hosts. Our laboratory experiment confirmed field observations indicating that *L. minuta* is part of the host-range of *T. lemnae*. Moreover, feeding and oviposition were found to occur on all the species tested, including *Landoltia punctata*, thus expanding the known host-range of this weevil to a new genus. Based on our results, *T. lemnae* is not sufficiently host specific to be considered for biological control of *L. minuta* in Europe.

3.4. *Life history of* Lemnaphila scotlandae

Laboratory trials confirmed field observations, that *L. minuta* is readily attacked by *L. scotlandae* in the native range. However, when comparing several life history variables, *L. scotlandae* exhibited different performance among duckweed genera. Specifically, the number of eggs laid on *L. minor* and *L. minuta* was higher than on *Spirodela* (Table 2) ($\chi^2 = 15.36$; d$f = 4$; $p = 0.0040$) (Figure 3(a)). Eggs laid on *Spirodela* and *Landoltia* did not hatch (Table 2), and the adults exposed to these genera did not survive (Figure 3(b)).

Although a similar number of eggs were laid on all *Lemna* species, the percentage of eggs that developed to the adult stage was higher on *L. minuta* and *L. minor* ($\chi^2 = 21.34$; d$f = 4$; $p = 0.0003$), with about 50% of the eggs developing to the adult stage, compared to 9% on *L. obscura* and none on *L. punctata* and *S. polyrhiza* (Table 2). The observed preference for *L. minuta* and *L. minor* does not seem to be influenced by plant size, as the number of eggs laid on different species showed no consistent correlation with plant size. While our data support ovipositional discrimination among hosts, they challenge the hypothesis suggesting insects prefer larger host plants with more vigorous growth (Price, 1991; Cornelissen et al., 2008).

Differences in ovipositional preference likely stem from the diverse physical and chemical cues of plants (Nottingham, 1988; Hilker & Meiners, 2011). Limited egg laying and adult surviving were observed on *Landoltia* and *Spirodela* (Figure 3), probably due to the failure to feed on these plants, as there were no leaf gouges. In a study of Smolders et al. (2000) investigating secondary metabolites in macrophyte species, *S. polyrhiza* exhibited more than four times the phenolic content of *L. minor*. *Landoltia punctata* also has a high content of apigenin and luteolin derivatives (Pagliuso et al., 2020), potent antiherbivore compounds (Cipollini et al., 2008). The elevated content of phenolic compounds likely rendered these two species unpalatable to *L. scotlandae*, shedding light on the observed differences in ovipositional behaviour and mortality. Our field observations indicated that the alien *L. punctata* is widespread in Louisiana, occurring in 50% of the sites surveyed. The invasion success of *L. punctata* may be facilitated in part by physical and chemical defenses that limit the use by native insect herbivores, which might otherwise provide greater biotic resistance in these aquatic systems.

Table 2. Biological data and developmental time of *L. scotlandae* on different duckweed species (Lmt = *L. minuta*; Lmr = *L. minor*; Lob = *L. obscura*; Spl = *S. polyrhiza*; Lpn = *L. punctata*).

Test plant	Eggs number			Eggs develop. time (days)			Larvae develop. time (days)			Pupae develop. time (days)			Adults emerged number			Range of emergence (days)			Eggs developed to adults	
Lmt	107	±24.3	**a**	6.0	±0.0	**a**	7.0	±0.6	**a**	7.2	±0.5	**a**	46.4	±5.2	**a**	10.8	±0.6	**a**	52%	**a**
Lmr	108.8	±22.6	**a**	9.4	±0.9	**a**	10.0	±1.5	**a**	7.2	±0.7	**a**	48.2	±6.9	**a**	8.2	±0.6	**a**	51%	**a**
Lob	77.2	±30.9	**ab**	7.0	±0.4	**a**	9.0	±0.8	**a**	6.3	±0.6	**a**	8.5	±1.9	**b**	6.8	±0.9	**a**	9%	**ab**
Spl	3.2	±0.9	**b**	–	–		–	–		–	–		0		**c**	–	–		0%	**b**
Lpn	23.2	±5.2	**ab**	7.5	±0.5	**a**	–	–		–	–		0		**c**	–	–		0%	**b**

Number of eggs laid, duration of each life cycle stage, number of adults that emerged from the eggs and percentage of eggs that completed development to the adult stage are reported for each duckweed species. Mean values ± 1 SE followed by distinct letters (a, b) within each column indicate statistically significant differences between test plants, as determined by multiple comparison tests (Kruskal-Wallis and Dunn's test), with rows sharing the same letter indicating no significant difference ($p > 0.05$).

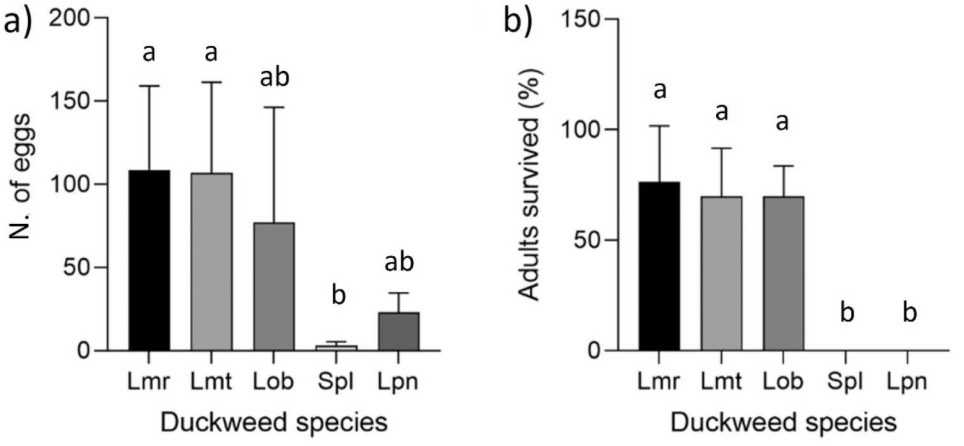

Figure 3. (a) Number of eggs laid by females of *L. scotlandae* on five different duckweed species. (b) Number of adult flies that survived after exposure to five different duckweed species for 96 h. Within each graph, different letters indicate significant differences ($p < 0.05$). The x-axis displays the duckweed species utilised as test plants. For the explanation of duckweed species abbreviations see caption of Figure 2.

3.5. Feeding damage of L. scotlandae

In larval no-choice tests, no significant differences were observed in the number of damaged *L. minor* versus *L. minuta* plants (*L. minor*: 41.6% ± 3.2 (percentage of damaged plants ± SE); *L. minuta*: 42.8% ± 9.4) ($\chi^2 = 2.93$, d$f = 1$, $p = 0.0870$) (Figure 4 (a)). There was no significant difference in relative growth rate between the two species when exposed to larval feeding (*L. minor*: 0.015 ± 0.016; *L. minuta*: 0.015 ± 0.006) or compared to the control group where larvae were not added (*L. minor* control: 0.009 ± 0.005; *L. minuta* control: 0.017 ± 0.007) ($F = 1.39$; d$f = 3, 12$; $p = 0.2930$).

These results suggest that, within the analyzed time frame, herbivory by the larvae did not sufficiently influence plant growth. No difference was also found between the two species in the number of individuals that completed the larval stage and pupated within the plants (*L. minor* 80% ± 8.0; *L. minuta*: 88% ± 7.1; $\chi^2 = 2.39$, d$f = 1$, $p = 0.1221$) (Figure 5). Similarly, even under choice conditions, no significant difference was found in the number of damaged plants of the two species ($t = 0.4282$; d$f = 8$; $p = 0.6798$) (Figure 4(b)). These data indicate that the plant species does not play a decisive role in larval migration. Once they have finished consuming a plant, the larvae migrate to the nearest adjacent one (Figure 1(b-3)), regardless of the duckweed species. Conversely, the host plant species appeared to influence the choice of pupation site for the larvae, displaying a preference for pupating inside *L. minor* plants compared to *L. minuta* plants (3.78 ± 0.3 in *L. minor*; 0.33 ± 0.2 in *L. minuta*; $t = 7.75$, d$f = 8$; $p < .0001$) (Figure 5(b)). This preference might be attributed to the larger size of *L. minor*, which likely offers the puparium comprehensive protection within the plant mesophyll.

With regard to adult feeding, under no-choice conditions there was no difference in the consumption of the two species for any of the factors analyzed: number of damaged plants (*L. minor*: 89.4% ± 1.0; *L. minuta*: 85% ± 1.87; $\chi^2 = 4.36$, d$f = 1$, $p = 0.0639$)

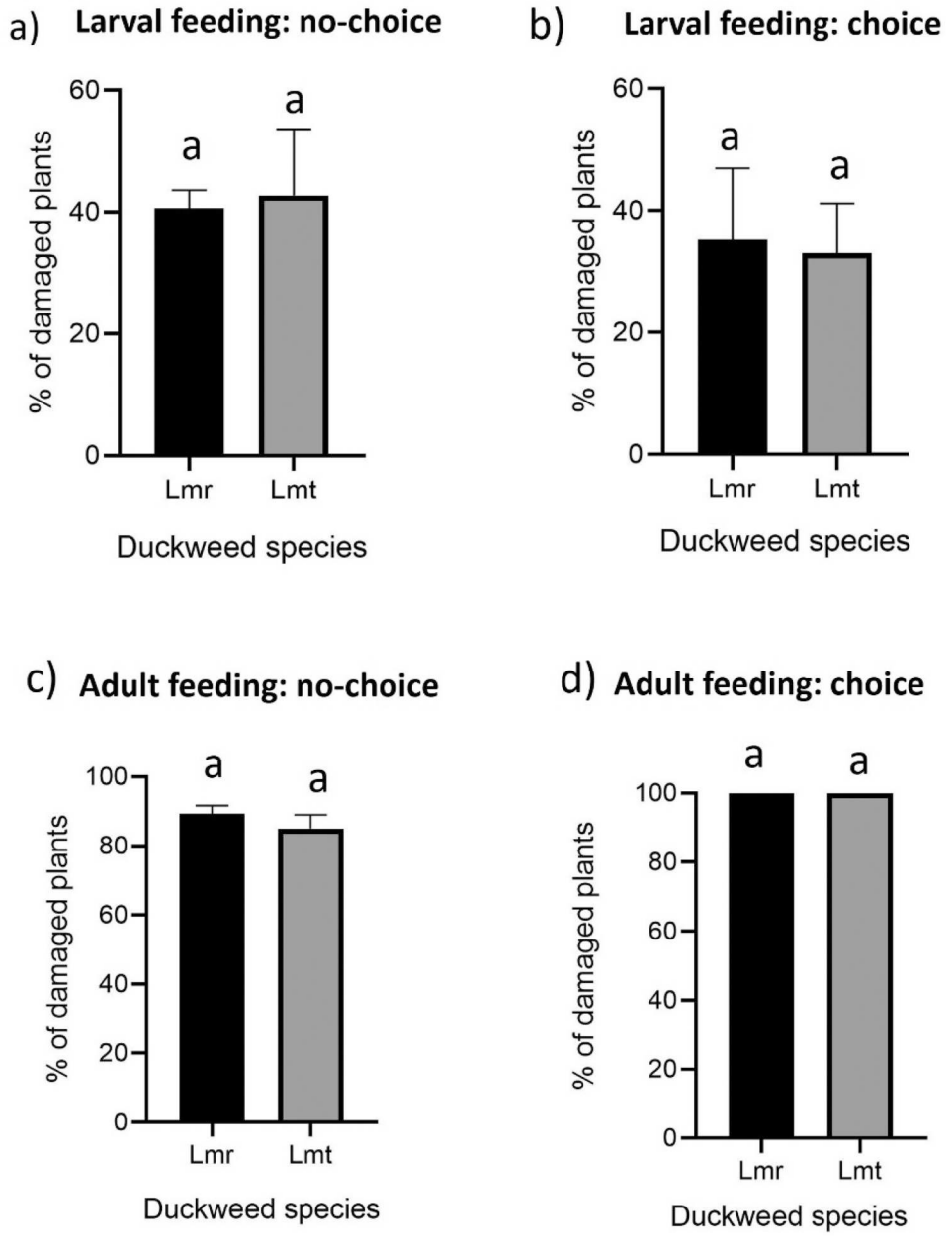

Figure 4. Feeding damage by larvae of *L. scotlandae* when exposed to two species of *Lemna* under both no-choice (a) and choice conditions (b), and by adult flies under no-choice (c) and choice conditions (d). Different letters within each graph indicate significant differences ($p < 0.05$). Duckweed species abbreviations: Lmr = *Lemna minor*; Lmt = *Lemna minuta*.

(Figure 4(c)) and relative growth rate of the species exposed to adult feeding (*L. minor*: 0.057 ± 0.003; *L. minuta*: 0.053 ± 0.008; $t = -0.1349$, $df = 8$, $p = 0.8960$). In the choice test, all plants had the gouges indicative of feeding (*L. minor*: 100% ± 0; *L. minuta*: 100% ± 0) (Figure 4(d)).

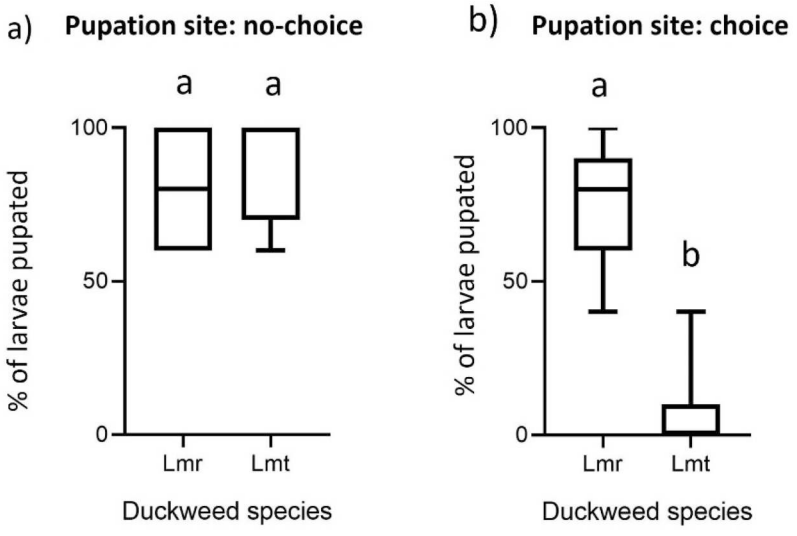

Figure 5. Percentage of larvae that pupated inside *L. minor* and *L. minuta* plants under no-choice (a) and choice conditions (b). The box plots show the median (line across the box), the upper and lower quartiles (the upper and lower parts of the box), values outside the quartiles (the whiskers). Different letters within each graph indicate significant differences ($p < 0.05$). Duckweed species abbreviations: Lmr = *Lemna minor*; Lmt = *Lemna minuta*.

Results from both no-choice and choice conditions demonstrated that larvae and adults of *L. scotlandae* can feed on *L. minor* and *L. minuta*. Similar results were found in previous studies by Mariani et al. (2020a), where aquatic lepidopteran larvae of *C. lemnata* were found to consume and use as host plants both *L. minor* and *L. minuta* without distinction, despite *L. minuta* being an alien, 'novel' species for the European lepidopteran. In this case, both species of duckweeds and the dipteran originate from the United States. As a result, the insect finds both equally palatable, likely due to a shared history of co-evolution. Moreover, both *L. minor* and *L. minuta* have a nutrient-rich parenchyma and high nitrate and phosphate content (Ceschin et al., 2020b; Nesan et al., 2020), potentially rendering them highly palatable to insects (Loader & Damman, 1991; Tabashnik, 1982).

4. Conclusions

In native range surveys, several herbivorous insects were found attacking *L. minuta*. However, the weevil *T. lemnae* was deemed unsuitable for biological control due to its overly broad host-range, which includes various *Lemna* species, as well as other genera like *Landoltia* and *Spirodela*. On the other hand, the dipteran *L. scotlandae* displayed some selectivity at the genus level for *Lemna* species but fell short of meeting criteria for further consideration as a biological control agent for *L. minuta*. While it doesn't attack the European native *S. polyrhiza*, it shows a preference for *L. minor* over the target duckweed *L. minuta*. This poses a risk as *L. minor* and *L. minuta* often co-occur in European water bodies. Overall, *L. scotlandae*'s host-range, including *L. minor*, is too broad to be a suitable biological control agent for *L. minuta* for Europe.

Despite not identifying a suitable biocontrol agent for invasive *L. minuta* in Europe, our findings confirmed that the dipteran *L. scotlandae* could potentially be considered suitable for biological control efforts in other parts of the world where alien *Lemna* spp. pose a threat to freshwater ecosystems, and where there are possibly no native species within the same genus to preserve. Additional native range surveys and associated studies are necessary to identify candidate biological control agents that could effectively limit the growth of this noxious duckweed in Europe.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Appendix

Figure A1. *Tanysphyrus lemnae*on mixed populations of *L. minor* (distinguishable by larger plant size) and *L. minuta* (smaller plant size) in Louisiana. Round holes indicative of adult weevil feeding damage are visible on both *Lemna* species.

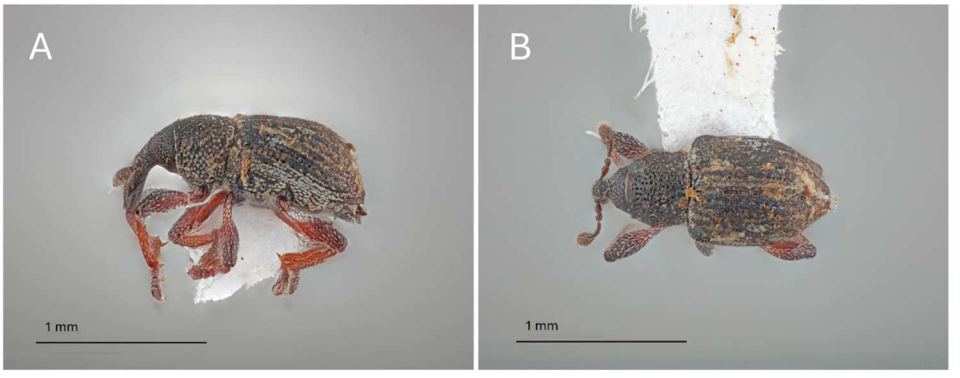

Figure A2. *Tanysphyrus lemnae*: lateral habitus (a) and dorsal habitus (b).

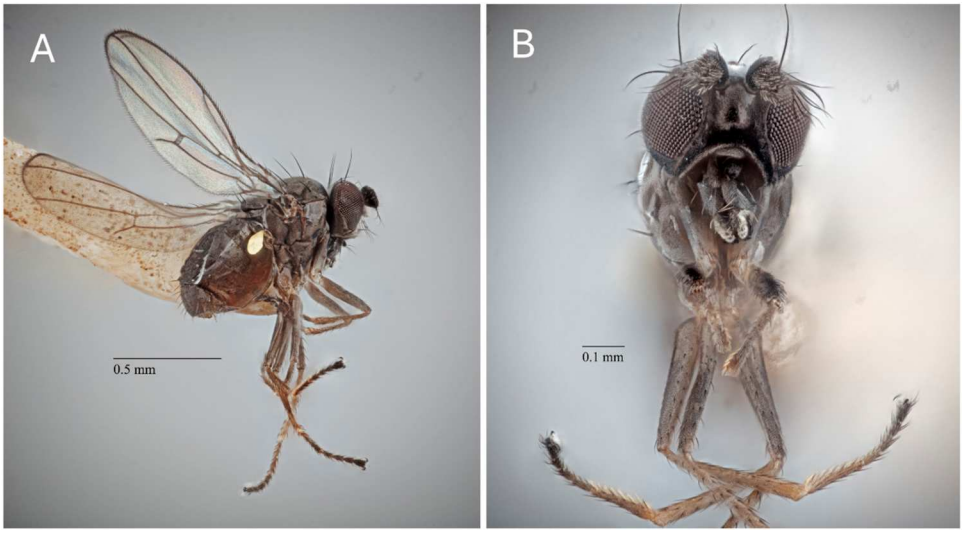

Figure A3. *Lemnaphila scotlandae*: lateral habitus (a) and anterior head (b)