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Compatibility of the Invasive Alien *Lemna minuta* and Its Potential Biocontrol Agent *Cataclysta lemnata*

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Received: 21 August 2020; Accepted: 27 September 2020; Published: 29 September 2020

Abstract: The American duckweed *Lemna minuta* is invasive in freshwater habitats across much of Europe, often causing serious ecological impacts. To date, few studies have addressed how to halt its expansion. However, encouraging empirical evidence of *L. minuta* control by the aquatic herbivorous larvae of the insect *Cataclysta lemnata* is emerging. To better understand the biocontrol capacity of *C. lemnata*, information on overlap in the phenology and the growth conditions in nature of both species is fundamental. In this study, *L. minuta* and *C. lemnata* populations were analyzed in the field to define (i) their phenological features, (ii) the main environmental characteristics where the two species occur, and (iii) any overlap or difference in phenology and ecological requirements. The seasonal occurrence of the two species and environmental data were collected from 31 wetlands in central Italy. The two species showed a large phenological overlap and ecological similarities. Populations of *L. minuta* and *C. lemnata* were found all year long, although abundances were greater in spring and summer. Both species preferred waters that were shallow, circumneutral, with moderately high conductivity and trophic level and with low dissolved oxygen. The phenology and ecology of the two species were shown to be compatible, suggesting the insect could be released in natural sites invaded by the alien *L. minuta* where could act as potential biocontrol agent of it.

Keywords: aquatic plant invader; duckweed; aquatic herbivorous larvae; augmentative biocontrol; phenological and ecological suitability; wetland

1. Introduction

Globally, alien plant invasion is a growing environmental problem [1] and is considered one of the most serious threats to biodiversity conservation [2]. The occurrence of plants outside their normal ranges may be due to natural or anthropic events, such as global trade, transport, and tourism, which can overcome natural barriers to species dispersion [3]. When in a new area, the invader loses the inhibitory effects of competitors and natural enemies that normally restrict its population growth [4]. Therefore, the invader's growth can be unchecked, and this can seriously compromise the ecological functioning and the biodiversity of the invaded habitats [5–8].

Freshwater environments are particularly vulnerable to biological invasions that can often threaten their integrity [6,9] and because of this, active mitigation or amelioration strategies become extremely important in the management of these invaded habitats. The American duckweed *Lemna minuta* Kunth (Araceae: Lemnoidae) has proven to be one of the most invasive species of freshwaters across most of Europe, including Italy [10,11]. Since its arrival in Italy in the late 1980s [12], *L. minuta* has rapidly spread as shown by multiple records in recent decades e.g., [13–15].

With a high vegetative growth rate [16,17], *L. minuta* can quickly colonize the whole surface of waterbodies [18], forming thick free-floating mats. If left unchecked, the coverage of these mats can limit light penetration and reduce air-water gas exchange, causing severe negative impacts on aquatic animal and plant communities [19–22]. In addition, *L. minuta* has proven to be highly competitive with other aquatic plants, including the congeneric native *L. minor*, which it is partially replacing [23,24].

Controlling the growth of this aquatic alien species by chemical or physical methods has proved to be only partially effective [16,18] and/or an environmental health risk. Therefore, some form of biological control of this species could be a highly attractive and ecologically friendlier option. Some recent exploratory studies have revealed the possibility of using aquatic herbivorous larvae of the European insect *Cataclysta lemnata* Linnaeus 1758 (Lepidoptera: Crambidae, Acentropinae) for the biocontrol of the alien duckweed. Indeed, these larvae seem to be able to remove large quantities of *L. minuta* using the fronds of the plant as a trophic resource and a construction material for the protective cases for larvae and pupae [25,26]. However, the information on this interaction is based on studies carried out only under laboratory conditions and, therefore, suffer from certain limitations, such as small volumes and low environmental complexity, when compared to natural conditions [27,28].

Therefore, in order to fully evaluate the possibility of using the larvae of this insect to control *L. minuta*, it is fundamental to understand the degree of overlap of the respective phenologies and ecological requirements of both plant and insect. In fact, mismatches in phenology and ecology, i.e., no seasonal synchrony or spatial co-occurrence between the control agent and target plant in nature, would undermine any biological control effort. However, surprisingly, on this issue, there are no specific studies in the literature but only passing considerations that highlight the importance of this compatibility between the biocontrol agent and invasive plant [29–31]. Commonly, biocontrol methods include the introduction of a natural enemy of the invader from its native range (classical biological control), and therefore a biological control agent that has co-evolved together with the invader, having spatially and temporally coexisted in nature [32]. However, an alternative biocontrol method exists, which is based on new non-co-evolved associations between the alien pest and a native natural enemy [33]. This type of control (augmentative biological control) consists of rearing the native control agent, and then releasing it into the site invaded by the alien plant [32]. It is proposed that the containment of the alien *L. minuta* by adopting an augmentative biocontrol using *C. lemnata* could be considered as a viable biological method.

Thus, having ascertained under laboratory conditions that there is an actual trophic interaction between *C. lemnata* and *L. minuta* [34], it is now necessary to verify whether they share in nature the same temporal and spatial conditions in order to implement this type of biocontrol. To this end, the dynamics of *C. lemnata* and *L. minuta* populations were followed through field surveys to define (i) the phenological features of both species, (ii) the main environmental factors characterizing habitats where the two species naturally occur, (iii) any overlap or difference in their phenology, and ecological requirements.

2. Materials and Methods

2.1. Biological Sampling

Sampling of *L. minuta* and *C. lemnata* populations was carried out from May to October 2019, during the optimal period of growth for both the insect [35] and the duckweed [16]. Sampling was carried out in 31 aquatic sites ranging from permanent ponds to canals or tanks around 30–100 cm deep, all of which were located in Latium region (central Italy), with the exception of three locations in the Basilicata region. The sites were selected based on the occurrence of *L. minuta* and/or *C. lemnata* populations. The geographical coordinates (UTM coordinates) of the sampling sites are reported in Table S1.

At each site, the occurrence and abundance of *L. minuta* and *C. lemnata* were estimated within a standard area of 4 m^2, which was considered adequate for quantifying both species. The abundance

(% coverage) of *L. minuta* was made visually and the average thickness (mm) of its free-floating mats was determined by measuring the mat thickness at three randomly selected points using a precision digital caliper. The estimation of *C. lemnata* abundance was made by searching for larvae among free-floating aquatic plants; care was made to normalize this procedure by applying the same sampling effort at each site (2 operators, 40 min/4 m^2). The larvae, enclosed in their cases made by plant portions, are free-floating on the water surface and, according to the larval stage, have dimensions ranging from 2 to 3 mm (early stages) to 6 to 15 (mid to advanced stages) in length [34]. Larvae abundances were categorized based on the following classes: 1 = 1 individual; 2 = 2–10; 3 = 11–30; 4 > 30. In addition, in the sites with *C. lemnata* the occurrence and abundance of the dominant aquatic plants other than *L. minuta* were noted.

Specimens of *L. minuta* and *C. lemnata* were transported to the laboratory to be identified; for *L. minuta*, this activity is considered particularly important as it can be easily confused with the native congeneric *L. minor* [36]. Both *L. minuta* and *C. lemnata* specimens were identified at the stereo microscope (Leica MZ6, Leica Microsystems GmbH, Wetzlar, Germany), using Ceschin et al. [36] for the duckweed, and Vallenduuk & Cuppen [35] and Speidel [37] for the insect.

To study the phenology of both *L. minuta* and *C. lemnata*, a subset of eight sites (four sites for each species) were monitored once each season. These sites were selected based on them having a lower anthropic disturbance, allowing us to follow the phenological cycle of *L. minuta* and *C. lemnata* over time. In addition, these sites were easily accessible and therefore easier to monitor periodically.

2.2. Chemical and Physical Analyses

Water chemical and physical analyses were carried out for each sampling occasion. Temperature (T, °C), conductivity (C, µS/cm), pH (pH, pH unit), and dissolved oxygen (DO, mg/L) were measured in situ at about a 20-cm depth from the water surface, using the appropriate immersion probes (Hach-Lange HQ40d, Hach-Lange GmbH, Berlin, Germany). All plastic and glassware used for chemical analyses were pre-washed by soaking in phosphate-free detergent (ES 7X, MP biomedical, distributer DBA) and rinsed three times in double-distilled water. Water samples were collected at a depth of 15 cm in 50-mL tubes and stored in cool bags at around 6 °C to be transported to the laboratory. Analyses of nitrate (NO_3^-, mg/L) and phosphate (PO_4^{3-}, mg/L) concentrations were carried out colorimetrically (Hach Lange DR 2800, Hach-Lange GmbH, Berlin, Germany) using standard protocols (standard Hach Lange Method 8039 and 10209/10210, respectively).

In addition, the water depth (D, cm) of the waterbodies was measured by graduated shaft, while the shading level (S, %) on the water surface of each site due to emerging and bank plants was visually estimated by giving shading percentage values in the field.

2.3. Statistical Analyses

Statistical analyses were carried out on datasets of environmental variables and site-specific abundance of *L. minuta* and *C. lemnata*. Mann–Whitney U tests were carried out to determine any differences between the environmental variables of sites with *L. minuta* and those with *C. lemnata*. Principal component analysis (PCA) was used to identify environmental variance and to define the variables that may help explain the distribution of *L. minuta* and *C. lemnata* (ecological driving forces). Given the heterogeneity of the environmental variables, a transformation of data was performed (z-score transformation).

To evaluate differences between diverse site groups (*L. minuta*-only, *C. lemnata*-only, *L. minuta*-*C. lemnata* sites), analysis of similarities (ANOSIM) [38] was performed on an Euclidean distance matrix obtained from the principal component scores of PCA. ANOSIM provides a way to test statistically whether there is a significant difference between two or more groups of sampling units by an approach derived from the general Mantel test. The probability to reject the null hypothesis (no differences between groups) is calculated by a permutation method (permutation n: 9999). All these statistical analyses were conducted with Past software version 3.07 [39].

To verify the degree of ecological similarity between *L. minuta* and *C. lemnata*, ecological response curves were calculated for both species to the diverse environmental variables. Response curves were obtained applying the fuzzy set theory approach [40] to ecological data [41]. According to this method, the species are described as a fuzzy set, which models the response of the species along environmental gradients. The response function (expressed as belonging degree) ranges from 0 to 1 and helps define the *optimum* response of the two species along each ecological gradient [41]. The response curves with a threshold optimal value around 0.5 are considered as significant responses.

3. Results

3.1. Characterization of L. minuta and C. lemnata Populations in the Sampling Sites

Lemna minuta populations occurred in 20 out 31 sites (16 of which were *L. minuta*-only sites) with a coverage ranging from 60% to 100%. The mats were free-floating and multilayered, ranging in thickness from 5 to 21 mm (Table S1). Larvae of *C. lemnata* were found in 15 of the 31 sites (11 *C. lemnata*-only sites) where they had a strong variability of abundance, ranging from 1 to more than 30 individuals per site (Table S1). These populations were found co-occurring with various free-floating aquatic plants, such as the native *Lemna minor* L. and *Lemna gibba* L. and the alien *Azolla filiculoides* Lam. and *L. minuta*. In particular, *C. lemnata* larvae were found among monospecific mats of *L. minuta* (*L. minuta*-*C. lemnata* sites) at 4 sites out of 31; here, the largest abundances of the insect (mainly abundance class 4) and the lowest values of thickness of the *L. minuta* mat (on average, 6 mm vs. 11 mm in *L. minuta*-only sites) were found.

Based on the three-monthly monitoring of the populations of *L. minuta* and *C. lemnata* at the selected sites for phenological purposes, it emerged that both species were found in all seasons (Table 1). *L. minuta* had always formed multilayer populations whose thickness varied from a winter minimum of 8 mm (winter mean: 11.5 mm) to a spring maximum of 25 mm (spring mean: 17 mm). The percentage coverage of these populations was always high (> 80%), with an annual average of 95%. Throughout the year, active larvae of *C. lemnata* were always found, with the exception of one station in winter (WNU1_inv). High *C. lemnata* abundances occurred for the majority of the year (spring, summer, and autumn). The lowest abundances were in winter, recording only class 2 (i.e., 2–10 individuals per site).

Table 1. Phenological monitoring of populations of *L. minuta* and *C. lemnata* in a site-subset during different seasons. Site acronyms: FOG = Foglino wood; CAF 3, CAF 7 = Caffarella Valley; OST = Ostia Lido; MET 2 = Metaponto; PAQ 1 = Aqueduct Park; TMA 2 = Tor Marancia Park; WNU 1 = Water Nursery).

***Lemna minuta* Coverage (%), Thickness (mm)**				
Site	Winter	Spring	Summer	Autumn
FOG	80%, 10 mm	100%, 19 mm	100%, 15 mm	80%, 13 mm
CAF3	100%, 16 mm	100%, 25 mm	100%, 21 mm	100%, 18 mm
CAF7	100%, 12 mm	100%, 12 mm	100%, 14 mm	100%, 15 mm
OST	80%, 8 mm	100%, 12 mm	90%, 12 mm	85%, 10 mm
***Cataclysta lemnata* Abundance (Class)**				
Site	Winter	Spring	Summer	Autumn
MET2	2	2	3	2
PAQ1	2	2	2	1
TMA2	2	4	3	4
WNU1	0	2	2	2

3.2. Chemical and Physical Characterization of *L. minuta* Sites and *C. lemnata* Sites

A summary of the environmental variables at each of the sites where populations of *L. minuta* and *C. lemnata* were found is shown in Figure 1. In general, the environmental conditions of the sites of both species were on average similar ($p > 0.05$), yet variable; the only exceptions were temperature ($p < 0.05$) and dissolved oxygen ($p < 0.005$). Sites with *C. lemnata* were generally warmer and more oxygenated than sites with *L. minuta*.

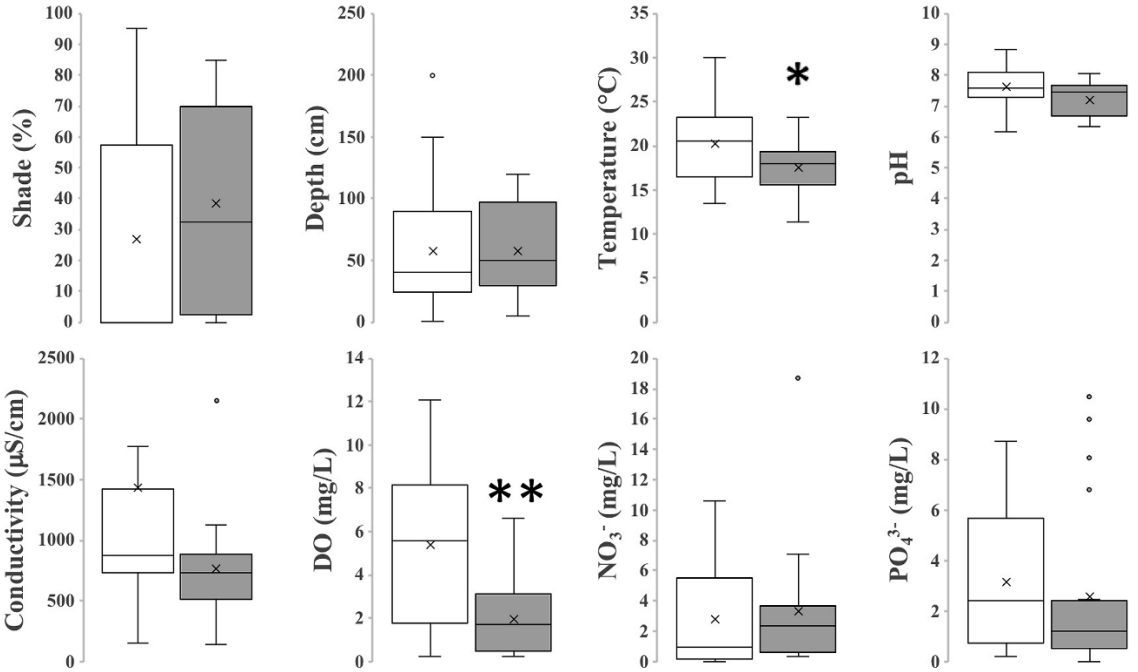

Figure 1. Ranges of the environmental variables at the sites with *C. lemnata* (white) and sites with *L. minuta* (grey). The plots show the median (line across the box), the mean (x), interquartile range (the upper and lower parts of the box), and variance beyond the quartile range (the whiskers) including the outliers (circles) (* and ** for the $p < 0.05$ and $p < 0.005$ significant differences).

3.3. Main Ecological Driving Forces Affecting *L. minuta* and *C. lemnata* Distribution

The cumulative percentage of variance for the first two components of PCA is about 50% (Figure 2). The first main component (pc-1) is interpreted as a combination of environmental factors mainly associated with conductivity and nutrients (NO_3^- and PO_4^{3-}). The variability associated with pc-2 is mainly explained by pH and DO. Environmental variables, such as shading, temperature, and depth, seem to explain less of the variance of the first two main components.

L. minuta-only and *C. lemnata*-only sites have a similar distribution across ordination space (Figure 2), but as the box plots showed, they seem to occupy a large space. Sites with either or both species are found to have negative and positive correlations with the environmental variables.

Based on the ANOSIM test, there are no significant differences between the different site groups (i.e., *L. minuta*-only sites vs. *C. lemnata*-only sites vs. *L. minuta*-*C. lemnata* sites) in the ecological space ($p > 0.05$) (Table 2).

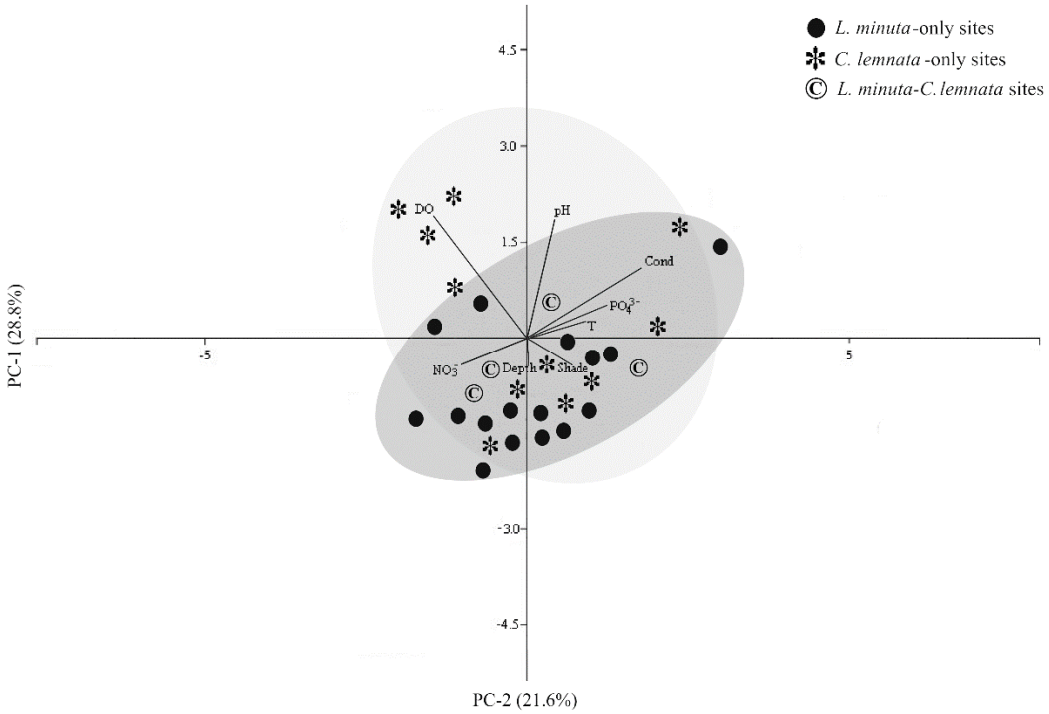

Figure 2. Ordination scattergram (PCA) based on environmental variables measured at sites with only *Lemna minuta* (*L. minuta*-only sites), with only *Cataclysta lemnata* (*C. lemnata*-only sites), and plant and insect co-occurring at the same time (*L. minuta*-*C. lemnata* sites).

Table 2. Pairwise comparisons between site groups (*L. minuta*-only sites, *C. lemnata*-only sites, *L. minuta*-*C. lemnata* sites) by the ANOSIM test. Probability values related to similarity between site groups are reported ($p < 0.05$; significantly dissimilar groups).

	***L. minuta*-Only Sites**	***C. lemnata*-Only Sites**	***L. minuta*-*C. lemnata***
L. minuta-only sites	1.000	0.325	0.820
C. lemnata-only sites	0.325	1.000	0.683
L. minuta-*C. lemnata*	0.820	0.683	1.000

3.4. Ecological Response Curves

The ecological responses curves of *L. minuta* and *C. lemnata* along the analyzed environmental variables showed the ecological preferences of the two species and helped to identify the degree of overlap or difference of their ecological requirements (Figure 3). Taking the value of 0.5 as a significant response, the main environmental factors that better explained the ecological requirements of both species were water depth, pH, conductivity, dissolved oxygen, nitrates, and phosphates (Figure 3). Regarding depth, both species showed a preference for shallow waters (around 40 cm). The two species had the same *optima* for pH, conductivity, and nutrients, with pH values around 7.5, conductivity over 700 µS/cm, and nitrates and phosphates of 3 mg/L and 1.8 mg/L, respectively. As also shown in the ordination scattergram (Figure 2), *L. minuta* was more common to sites with very low dissolved oxygen concentrations (< 0.5 mg/L), while *C. lemnata* appeared to prefer slightly more oxygenated waters (around 3 mg/L).

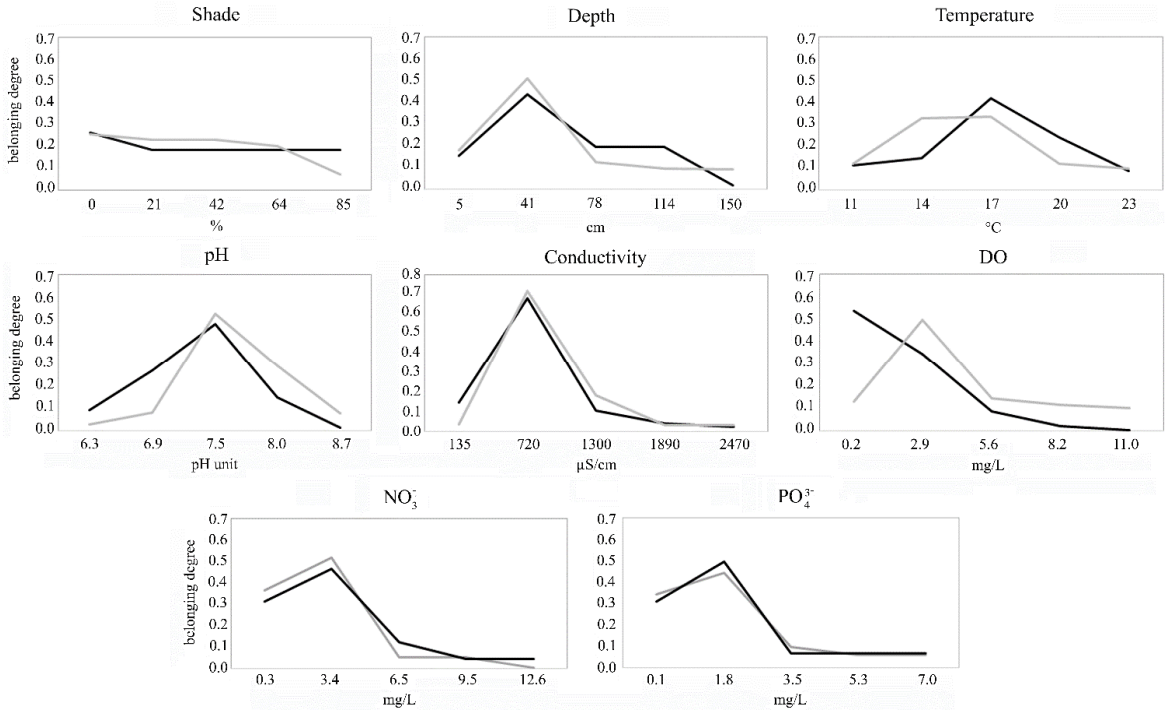

Figure 3. Ecological response curves of *L. minuta* (dark grey line) and *C. lemnata* (light grey line) populations to each environmental variable.

4. Discussion

The alien duckweed *L. minuta* was widespread and abundant in the study sites, where it formed thick free-floating mats on the water surface, that proved vital throughout the year. In fact, water surfaces usually had high coverages of *L. minuta* mats (> 80%), which were multilayered (around 15 mm). The findings here confirm the invasive character of this species out of its native range [11,13,42,43].

Current rates of the *L. minuta* spread indicate that its growth is continuing unchecked, and at present, there seems to be no effect of control strategies in place to manage this invader [22]. The use of herbicides is advised against in order to safeguard the health of aquatic ecosystems from any widespread and persistent contamination [44,45]. Physical control has been considered, but mechanical removal of the phytomass has proved ineffective and short-lived [18]. Indeed, the total removal of *L. minuta* populations is very difficult due to the smallness of the fronds (individuals of 1–2 mm) and the complexity and the discontinuities of the banks where the fronds can easily remain hidden. The rapid re-assumption of an invasion, even from a very few fronds, has already been demonstrated in the field [18].

In light of these restrictions to stymie the spread of *L. minuta*, the possibility of a biological control strategy is an attractive option. That *C. lemnata* larvae are able to use and consume fronds of the invasive species, both as food and as a material for the construction of protective larval cases [34], shows promise in using this insect in the containment of *L. minuta*. It should be noted that in the sites where the co-occurrence of insect and alien duckweed was recorded, the thickness of the plant mats was less than in the sites where the insect was absent (see Table S1). However, to counteract the rapid vegetative growth of the invasive duckweed, it would be necessary to increase the number of larvae in the field by adopting an augmentative biological control, which involves releasing large quantities of natural enemies at the invaded site [32]. This type of control could be restricted by the capacity to breed large quantities of the natural enemy in the laboratory; fortunately, the rates that *C. lemnata* can be reared have shown some promise [26]. The use of this insect for such purposes will require indoor and in situ studies to optimize the required number of *C. lemnata*, not only for *L. minuta* mat unit area but also for mat thickness.

As an effective control agent, larvae would need to have phenological characteristics and ecological requirements that match to some extent those of *L. minuta* and it has been shown here that a significant overlap in these requirements exists with *C. lemnata*. Both species are perennially occurring and can inhabit a variety of environmental conditions, even if they do seem to prefer waters that are shallow, with circumneutral pH, high conductivity and nutrient, and low dissolved oxygen levels. Thanks to this temporal and environmental compatibility, it is therefore possible to assume that *C. lemnata* larvae released in the site invaded by *L. minuta* could form stable populations that will self-perpetuate over time, and providing continuous control of the target plant.

However, as for water oxygenation, *C. lemnata* seems to colonize sites with waters that have slightly higher oxygen levels than the severely anoxic to which *L. minuta* is associated. However, it should be underlined that the oxygen concentrations recorded at *L. minuta*-only sites are severely affected by *L. minuta* free-floating mats themselves; the extensive coverage and thickness of these mats act as highly effective physical barriers that compromise gas exchange in the air–water interface [14,21,22,24,46,47]; it is highly likely that the *L. minuta* preference for very low oxygen levels may just be an artefact of this phenomenon.

It is also important to note that the release of a large population of *C. lemnata* larvae in the field would reverse the physical effects of *L. minuta* on water quality by the removal of phytomass to allow air and water gaseous exchange. However, if the *L. minuta* mat is too thick and dense, it may actually reduce the survival of the insect. Indeed, respiratory activity of *C. lemnata* could be limited under potential hypoxic conditions within the mats and the water column below them, a phenomenon already demonstrated empirically for other aquatic animals [21]. Thick *L. minuta* mats may also physically impede the insect's life cycle, which includes a larval phase that only occurs among the fronds free-floating on the water surface, and the subsequent emergence phase of winged adults [26].

These combined observations raise other questions that need to be resolved before the invasiveness of *L. minuta* can be controlled using biological methods. There is a need to understand the effective *ratio* of *L. minuta* and *C. lemnata* populations that is required to suppress the former. To do this, it is necessary to quantify in laboratory the *L. minuta* biomass consumption rate by the *C. lemnata* larvae both to optimize the larvae number to be released at an invaded site, and to determine the insect and invader growth rates for defining any follow-up procedures that may be needed. What is sure, is that before releasing any larvae into the invaded waterbody, it would be necessary to mechanically remove a large part of the duckweed biomass to allow optimal starting environmental conditions for the success of the released insect individuals.

5. Conclusions

After understanding that in the laboratory *C. lemnata* is able to consume *L. minuta* [32], it was necessary to determine if these two species actually co-exist at the same time and in the same space in order to investigate the possibility of adopting a biological control strategy for this invasive plant. The results emerging from this study showed that the herbivorous insect and the invasive alien duckweed are able to co-occur temporally and spatially in the field. This phenological and environmental overlap is a fundamental requirement for the success of an augmentative biological control strategy [31], since this type of control involves the encounter in nature between a biocontrol agent and an invasive target plant that are not evolutionarily associated. Based on this evidence, it is therefore possible to propose *C. lemnata* as a potential biocontrol agent of *L. minuta* to contain the spread of this alien invasive plant.

Supplementary Materials: The following is available online at http://www.mdpi.com/2073-4441/12/10/2719/s1, Table S1: Location of the sampling site.

Author Contributions: Conceptualization, F.M. and S.C.; Data curation, F.M. and S.C.; Formal analysis, N.T.W.E. and V.Z.; Investigation, F.M. and S.C.; Methodology, F.M. and S.C.; Project administration, S.C.; Resources, F.M. and S.C.; Software, V.Z.; Supervision, S.C.; Validation, F.M. and S.C.; Visualization, F.M. and S.C.; Writing—original

draft, F.M. and S.C.; Writing—review and editing, N.T.W.E. and S.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Grant to Department of Science, University of Roma Tre (MIUR-Italy, Departments of Excellence, ARTICLE 1, COMMA 314–337 LAW 232/2016).

Acknowledgments: The authors are grateful to Fabrizio Piccari of the Appia Antica Park for their help in sampling activities.

Conflicts of Interest: The authors declare no conflict of interest.

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