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ORIGINAL PAPER

Serological marking of *Pnigalio agraules* (Hymenoptera: Eulophidae) for field dispersal studies

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Abstract The parasitoid wasp *Pnigalio agraules* (Wlk.) is a key natural enemy of the horsechestnut leafminer *Cameraria ohridella* Deschka and Dimić (Lepidoptera: Gracillariidae). As a basis for mark-release-recapture studies, aimed at investigating the dispersal of this parasitoid in the field, adults of *P. agraules* were marked using a vertebrate-specific immunoglobulin (IgG). The marker was later detected by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). The IgG was either applied externally by spraying or internally by feeding an IgG-enriched diet. Different concentrations of the marker were used and the influence of abiotic (climatic conditions, time elapsed between marking and marker examination) and biotic factors (sex and age of the parasitoids) on the detection of the immunomarker was tested. External marking by spraying led to more homogeneous labelling than feeding the marker. Parasitoids labelled with 0.25 mg rabbit IgG per ten individuals contained enough immunomarker to be easily distinguished from unmarked ones. Neither the climatic conditions nor the sex or age of the insects had an influence on the detection of the marker. The IgG remained well detectable during the entire lifespan of the parasitoids, which was not negatively affected by the marking procedure. Serological marking can be used to investigate the dispersal behaviour of beneficial insects within mark-release-recapture studies.

Keywords Insect marking · *Pnigalio agraules* · Chalcidoidea · Eulophidae · *Cameraria ohridella* · Gracillariidae · Mark-release-recapture · Biological control · ELISA

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Introduction

The chalcidoid *Pnigalio agraules* (Wlk.) is one of the most important natural enemies of the horsechestnut leafminer *Cameraria ohridella* Deschka and Dimić (Lepidoptera: Gracillariidae) (Grabenweger and Lethmayer 1999; Hellrigl and Ambrosi 2000; Grabenweger 2003; Stojanović and Marković 2004). *C. ohridella* is an invasive pest of unknown origin that was first observed in Macedonia in 1986 (Deschka and Dimić 1986). Since its first detection has spread rapidly over most of Europe (Heitland et al. 1999; Skuhravy 1999; Augustin et al. 2004; Lupi 2005) and become the most prominent pest of the white-flowering horsechestnut tree, *Aesculus hippocastanum* L. (Tomiczek and Krehan 1998; Skuhravy 1999; Lupi 2005). Outbreaks

of *C. ohridella* can cause extensive damage, resulting in early defoliation in summer and the total loss of foliage of heavily infested trees by midsummer, often followed by a second budding in late summer (Augustin et al. 2004).

More than 20 species of parasitic Hymenoptera native to Europe are known to be able to develop on the horsechestnut leafminer, mostly chalcidoids of the family Eulophidae (Grabenweger 2003; Stojanović and Marković 2004; Girardoz et al. 2006). However, parasitism of *C. ohridella* remains extremely low. In Central Europe percentage parasitism varies between 0 and 15% and normally does not exceed 5% (Heitland et al. 1999). In contrast, parasitism rates of more than 50% are commonly recorded from native European leafminers (amongst others Askew and Shaw 1979; Cornell and Hawkins 1993; Mey 1993).

The lack of phenological synchronisation between *C. ohridella* and its main parasitoids may explain the low level of parasitism (Grabenweger 2004). In spring, most of the parasitoids emerge from hibernation at least 4 weeks before the occurrence of the first suitable larvae of *C. ohridella* in the leaves of horsechestnut. Consequently, since no hosts are available when they emerge, the polyphagous parasitoids appear to leave the vicinity, focussing with their first generation on new hosts, e.g. *P. agraules* is considered highly polyphagous; there are 42 different host records so far (Noyes 2002). This affects negatively parasitism rate of the first generation of *C. ohridella*. However, there is no experimental proof for Grabenweger's theory yet.

In order to test this hypothesis, it is necessary to gain detailed knowledge of the biology of these parasitoids, especially their dispersal behaviour. Furthermore, knowledge of the mobility and dispersal of released parasitoids is an important factor in determining their efficiency as biological control agent. Generally, insect dispersal is determined by mark-release-recapture studies (Southwood 1978; Hagler 1997): the insects are initially labelled using a marker, then released into the field and finally collected at given time and distance intervals. There are various methods for marking insects. The most common to date are using paints or dyes, such as fluorescent dust, to mark insects externally, or feeding them with artificial diets containing trace elements, such as rubidium, to mark them internally. The method of choice for applying the marker depends on the specific insect, especially its size and life stage. The marker should not affect the normal behaviour, reproduction, growth or lifespan. Furthermore, the marker should be applied and clearly identifiable and detectable for a sufficient period of time (Hagler and Jackson 2001).

Neither paints, dyes nor trace elements could be used in this study with *P. agraules*: paints and dyes are not suitable for marking minute insects such as *P. agraules*, which reaches a maximum body length of about 2.5 mm. In addition, many are toxic and may inhibit normal dispersal behaviour (Hagler and Jackson 2001). Trace elements are useful for marking small insects but their retention in the marked insects may be very short-lived. In addition, the detection of the elements can be difficult, expensive and time consuming (Hagler and Jackson 2001).

Hagler et al. (1992) developed a marking technique that overcomes these drawbacks. They used vertebrate-specific immunoglobulines (IgG) to mark insects and then examined them for the presence of protein by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), using vertebrate-specific antibodies. To date, this method was used for marking plant bugs (*Lygus hesperus*; Hagler et al. 1992), ladybirds (*Hippodamia convergens*; Hagler 1997), parasitoid wasps (*Eretmocerus* sp.; Hagler et al. 2002) and pink bollworm (*Pectinophora gossypiella*; Hagler and Miller 2002).

Our study was carried out to examine the feasibility of using this technique to mark the chalcidoid *P. agraules*. Two methods to apply the marker were compared to identify the most suitable one, and the concentration of IgG needed for unambiguous results was determined. The influence of age and sex of the parasitoids, as well as climatic conditions and time on the retention of the marker, were tested.

Materials and methods

Test insects

Relatively little is known about the biology of *P. agraules*, most of the information has been generated by its association with its host *C. ohridella*. To obtain parasitoids dry horse chestnut leaves infested with *C. ohridella* were collected in the field in autumn and stored in darkness at 2°C. Previous experiments have shown that these leaves contain an adequate proportion of hibernating *P. agraules* (Moreth et al. 2001; Klug et al. 2008), which may be forced to cease diapause by warming the leaves at 25°C for about a week. The emerged parasitoids were kept in emty 1.2-l Tupperware® containers. Each container was fitted with a gauze-covered hole in the lid for provision of food and another one at the side to facilitate air exchange. *P. agraules* were fed on a solution of 20% honey in water that was replaced daily, nothing else was provided. All experiments were conducted with three replicates.

Marking procedure

Rabbit IgG [I5006, Sigma (Taufkirchen, Germany)] was applied once (a) externally by solely spraying inside the container's lids using an atomizer to avoid hitting the wasps directly, or (b) internally by feeding a honey solu-

tion containing the marker. Three different concentrations, 0.25, 1.0 and 5.0 mg IgG per ten individuals, were used. For external marking, the IgG was diluted in 3 ml dH_2O. This amount of dH_2O was found to coat the insides of the container without causing harm to the parasitoids. During container treatment wasps were in the cages but not on the treated lids. The containers with the control group were treated in the same way by spraying 3 ml dH_2O using an atomizer. Application of the IgG by feeding was carried out by daily offering 5 ml of honey solution containing 0.25, 1.0 and 5.0 mg IgG per ten individuals, respectively, whereas the control group was feed with unmarked honey solution.

Climatic conditions

The parasitoids were exposed to different climatic conditions in their containers, in order to check the effect of environmental conditions on the retention time of the immunomarker. One group ($N = 151$) was held for a maximum of 40 days in containers which were placed in an environmental chamber at 15°C, 75% RH and 16:8 h (L:D). Containers with another group were exposed to the natural climatic conditions for 24 days, with temperatures and relative humidity varying between +1 and +18°C and 35–100%, respectively. For a maximum of 40 days a third group of *P. agraules* was held at a constant temperature of 23°C during daytime (08:00–17:00 h) and was exposed to the natural climatic conditions during night to simulate night frost in spring/early summer, when temperatures and relative humidity varied between −3 and +12°C and 68 and 100%, respectively.

Detection of the marker

Individuals were tested for the presence of immunomarker by sandwich enzyme-linked immunosorbent assay. Each well of a 96-well ELISA microplate was coated with 100 µl of goat-anti-rabbit IgG (Sigma, R2004) diluted 1:1,000 in coating buffer and incubated for 3 h at 37°C. The IgG antibodies were discarded and 300 µl of 1% non-fat dry milk in phosphate buffered saline (PBS) was added to each well for 1 h at 37°C to block any remaining non-specific binding sites on the plate. The microplate was then washed three times, for 3 min with washing buffer. After individual insects were homogenised in 500 µl sample buffer, 100 µl of the homogenised samples were placed in the wells of the pre-treated assay plate and incubated overnight at 4µC. The samples were then discarded and the microplate was washed as described above. Anti-rabbit IgG (100 µl) conjugated to alkaline phosphatase (Sigma, A-3687) diluted 1:20,000 in conjugation buffer was added to each well for 3 h at 37°C. The plate was again washed as described above and 100 µl of substrate was added using 4p-nitrophenyl-phosphate. After a substrate incubation period of 1 h, the optical densities of the wells were measured with a microplate reader (Rainbow Thermo Spectra, Tecan) set at 405 nm. The samples are considered positive for the presence of the immunomarker if the adsorbance is twice that found with negative, unmarked healthy controls (Dijkstra and de Jager 1998).

Statistical analysis

All statistical analyses were performed using the SigmaPlot 11 program at the 1% probability level. Paired *t* tests were used to analyse differences between treatments and control. Mean ELISA absorbance values of *P. agraules* adults labelled by spraying different concentrations of rabbit IgG were submitted to a one-way analysis of variance (ANOVA) and mean comparisons between concentrations compared using Tukey's test ($P < 0.01$).

Results

Application method

Applying IgG by spraying inside the containers resulted in a marking that was more homogeneous than feeding the immunomarker (Fig. 1). All of the sprayed *P. agraules* adults scored positive (3.685 ± 0.197). The optical density values within this group exceeded those of the negative control by at least a factor of 20. Hence, marked parasitoids could be easily distinguished from unmarked ones. The optical density values for the insects that were fed on an IgG-enriched diet varied considerably (0.939 ± 1.986). Two-thirds of the fed *P. agraules* scored positive for the presence of the immunomarker, exceeding optical density values of the negative control at least by a factor of 4. However, 36% of the samples showed ELISA readings that were not significantly higher than that of the negative control. Hence, they remained undetectable.

IgG-concentration

All of the sprayed *P. agraules* adults scored positive for the presence of the immunomarker, regardless of the amount of protein applied (Fig. 2). Even at the lowest concentration of 0.25 mg rabbit IgG per ten individuals, the mean optical density values exceeded those of the negative control at least by a factor of 12. Although the intensity of the ELISA immunoreactivity increased when the marker was applied at a higher concentration, all of the marked individuals remained clearly detectable.

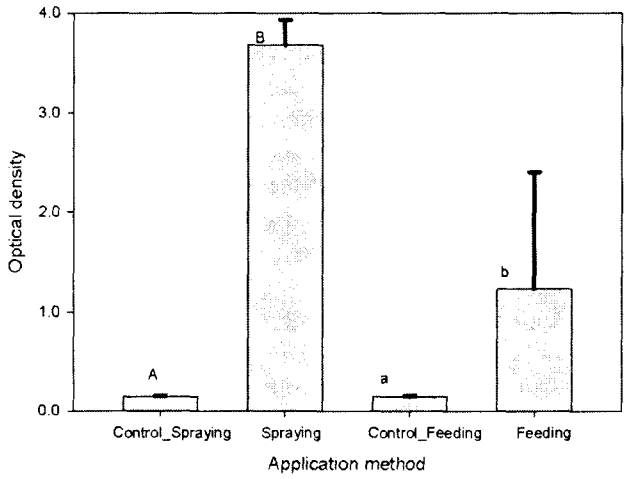

Fig. 1 Mean ELISA absorbance values of *Pnigalio agraules* adults labelled with rabbit IgG by either spraying the insides of their container boxes or by feeding an IgG-enriched diet, as well as their corresponding negative controls. The *vertical lines* indicate the standard deviation ($n = 56$; three replicates). Statistical differences are indicated with different *letters* within application methods between control and application

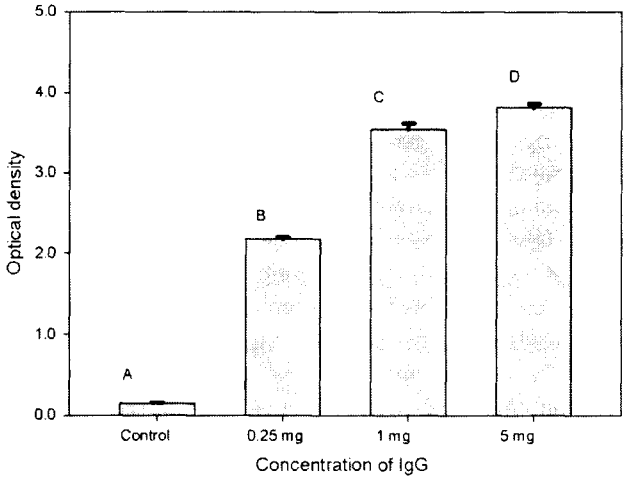

Fig. 2 Mean ELISA absorbance values of *Pnigalio agraules* adults labelled by spraying different concentrations of rabbit IgG. The *vertical lines* indicate standard deviation ($n = 151$, three replicates). Significant differences between concentrations are indicated with different *letters*

Sex and age of the marked parasitoids

Every sample of both sexes of marked *P. agraules* adults scored positive for the presence of immunomarker (Fig. 3). Even the lowest value measured exceeded the negative control values by a factor of 8 in males and a factor of 9 in females, respectively. Further, every sample scored positive for the presence of rabbit IgG, regardless of the age of the parasitoids at the time of marking (Fig. 4). The optical density values exceeded those of the negative control at least by a factor of 6.

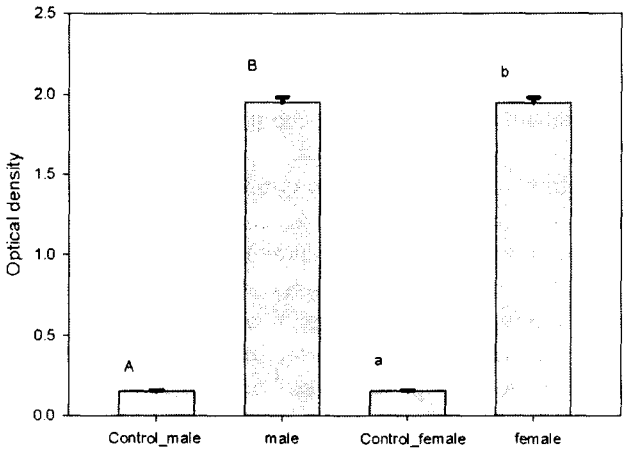

Fig. 3 Mean ELISA absorbance values of male and female *Pnigalio agraules* labelled by spraying with rabbit IgG, and their corresponding negative controls. The *vertical lines* indicate the standard deviation ($n = 76$, three replicates). Statistical differences are indicated with different *letters* within sex

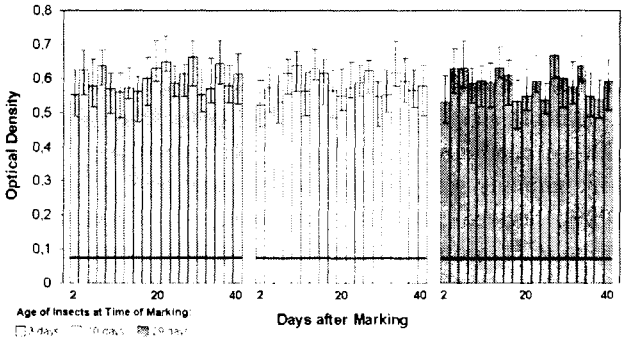

Fig. 4 Mean ELISA absorbance values of *Pnigalio agraules* adults that were labelled by spraying with rabbit IgG 3, 10 or 29 days after emergence. Samples were taken every second day over a period of 40 days. The *vertical lines* indicate the standard deviation. The *horizontal lines* indicate the mean ELISA readings of the corresponding unmarked negative control ($n = 57$, three replicates)

Retention period of the marker and climatic conditions

The level of the ELISA readings did not decrease with time (Fig. 4). Even 40 days after labelling, all samples retained as many quantities of rabbit IgG as on day 1 (0.589 ± 0.033). Therefore, the marker remained detectable over the whole lifespan of the parasitoids. The investigated chalcids lived up to 68 days, with a mean of 67 days (67 ± 0.002). No difference in lifespan was found between labelled and untreated individuals.

The climatic conditions to which the *P. agraules* were exposed, had no influence on the detection of the marker (constant: 0.570 ± 0.013; natural: 0.566 ± 0.022; constant/natural: 0.575 ± 0.016). Every sample of all three treatments scored positive for the presence of rabbit IgG, exceeding the negative control readings by 3.8 as a minimum (Fig. 5).

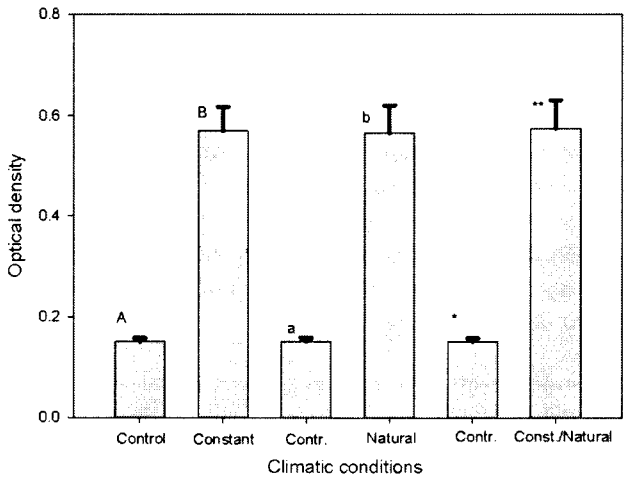

Fig. 5 Mean ELISA absorbance values of *Pnigalio agraules* adults labelled by spraying with rabbit IgG and exposed to different climatic conditions, as well as their corresponding negative controls. The *vertical lines* indicate the standard deviation (n = 151, three replicates). Statistical differences are indicated with different *letters/symbols* within a climatic condition and control. Constant: 15°C, 75% RH and 16:8 h (L:D) Natural: temperatures and RH varying between +1 and +18°C and 35 and 100%, respectively, constant/natural: constant temperature of 23°C during daytime (08:00–17:00 h) and nocturnal temperatures and relative humidity from −3 to +12°C and from 68 to 100%

Discussion

Marking insects with vertebrate-specific immunoglobulines and detecting the marker by DAS-ELISA, as developed by Hagler et al. (1992), proved to be applicable to native, chalcidoid wasps. This method led to unambiguous results in our model species, the eulophid *P. agraules*. All of the unmarked parasitoids examined, serving as negative controls, yielded low ELISA absorbance values similar to the PBS blanks. Consequently, none of the individuals was falsely deemed positive. Furthermore, the low ELISA values indicate that the anti-rabbit-IgG did not cross-react with any proteins of *P. agraules*. This proves the high degree specificity of the antibody and corroborates the results of Hagler (1997) and Hagler et al. (2002), who detected only a minimal cross-reactivity in *H. convergens* (Col., Coccinellidae) and no cross-reactivity in *Eretmocerus* sp. (Hym., Aphelinidae) when applying similar methods. Furthermore, the treatment with rabbit IgG seems to have no negative influence on the lifespan of the insects nor on the behaviour or flight physiology. The marked *P. agraules* lived up to 68 days, a time period which corresponds to the adult lifespan of unmarked *P. agraules* under laboratory conditions.

Applying the IgG by spraying resulted in a marking that was more homogeneous than the feeding of the immunomarker. One reason for the high variation in tests with the latter approach may be that *P. agraules* rapidly digests or excretes the protein. However, there is evidence that some parasitoid species are unable to digest the IgG (Hagler and Jackson 2001). It is therefore more likely that the test species took up different amounts of the honey solution, consequently leading to variation in IgG uptake. Besides the more reliable results, an application of the marker by spraying turned out to be easily practicable. The marking procedure requires only minimal personnel training and allows for marking of 100s of parasitoids simultaneously.

Generally, the intensity of the ELISA reaction increased when the protein was applied to the parasitoids at a higher concentration (Fig. 2). This confirms the results of Hagler et al. (2002) who achieved similar results with marking adult *Eretmocerus* sp. with different concentrations of rabbit IgG. However, even at the lowest concentration, the marker was unambiguously detected in our experiments. Consequently, a concentration of 0.25 mg rabbit IgG per ten individuals is sufficient for marking *P. agraules*.

It is remarkable that the IgG remained detectable on *P. agraules* during their entire lifespan at the same level. Even at 40 days after labelling, all of the individuals retained as many quantities of rabbit IgG as on day 1. In contrast, the retention of the immunomarker decreased rapidly in similarly labelled *H. convergens* (Hagler 1997) and *P. gossypiella* (Hagler and Miller 2002). Behavioural patterns of *P. agraules* may be one reason for the prolonged retention of the marker. Video-supported studies on the host-searching behaviour of *P. agraules* have shown that parasitoids spend a long time with cleaning their whole body with their tarsi. When foraging in the boxes which were treated with the marker, parasitoids took up the IgG with their tarsi and presumably dispersed the marker evenly over the whole body during cleaning. In addition, the marker may be worn-off more easily from smooth body surfaces, as in *H. convergens*, than from the roughly structured and hairy antennae, head, wings and mesosoma of *P. agraules*.

Neither the age of the insects at the time of marking nor their sex had an influence on the detection of the marker. The same was true for temperature and relative humidity. ELISA readings gave clear results for the discrimination of marked and unmarked parasitoids, regardless of the climatic conditions to which the parasitoids had been exposed. Recent field-release studies with marked *P. agraules* at temperatures between 4 and 30°C and relative humidities between 25% and almost 100% corroborated these results. In their experiments with *H. convergens*, Hagler (1997) found the IgG marker to be photo-stable, heat-tolerant and water-resistant, even under extreme field conditions.

The presented study indicates that the marking technique developed by Hagler et al. (1992) is an excellent method to study the field dispersal of eulophid parasitoids. However, the costs for the substances required and the time-consuming

detection of the marker limit the feasibility of this method. Approximately 20 h of incubation are needed to run the ELISA as described here. However, a reduction of the time needed for the ELISA procedure may be possible by lowering the incubation periods. Hagler et al. (2002) conducted a DAS-ELISA, using incubation intervals that ranged from 5 to 60 mins per incubation, for detecting *H. convergens* labelled with rabbit IgG. Although the ELISA reaction increased significantly with increasing incubation intervals, even the shortest incubation periods were sufficient for doubtlessly detecting the marker.

One way to improve the cost effectiveness of the system may be to further reduce the concentration of protein applied to the insects. Even the smallest amount of IgG used in this study led to optical density values that exceeded the negative control readings by a factor of 12 as a minimum, while optical density values twice as high as the control would be sufficient for the detection of the marker. The costs of marking could also be significantly reduced if the purified rabbit IgG marker could be substituted with a less-expensive protein. Rabbit IgG is a component of normal rabbit serum which has been isolated from the whole serum. Jones et al. (2006) applied chicken egg albumin, bolvine casein and soy protein to mark insect populations such as pear psylla (*Cacopsylla pyricola*) and codling moth (*Cydia pomonella*). These markers detectable by ELISA led to a decrease of costs of at least 2,000% compared with purified vertebrate IgG. In his experiments with *H. convergens*, Hagler and Naranjo (2004) achieved the highest optical density values with beetles that were marked with purified rabbit IgG. However, *H. convergens* labelled with some of the more concentrated rabbit serum dilutions also yielded significantly high ELISA absorbance values, providing a clear detection of the marker. It is likely that an acceleration of the ELISA procedure and the use of lower concentrations of IgG or normal rabbit serum are also possible for marking and detecting parasitic eulophids.

The most difficult part in such studies is probably the provision of a sufficient amount of test parasitoids for release. Hagler et al. (2002) released between 27,000 and 117,000 *Eretmocerus emiratus* in their field experiment and recaptured between 1.2 and 1.7% of them. Field experiments in these dimensions may be conducted with a very limited choice of commercially produced parasitoids only. However, a recent study with a few thousands of *P. agraules* released into a tree nursery resulted in a recapture rate of about 1.4%. This amount of test specimens was reared with self-made and low-cost equipment from fallen leaves, demonstrating the feasibility of such MRR-studies at reasonable cost and work load. The immunological insect marking technique described here will, therefore, substantially facilitate the research on the field biology of *P. agraules* and other important natural enemies of *C. ohridella*. Since *P. agraules* has been identified out of 16 parasitoid species known to attack *C. ohridella* in Berlin, Germany (Jäckel et al. 2006). This wasp will be further studied as a suitable candidate for biocontrol experiments. Spatially correlated environmental noises, dispersal and trophic interactions have been considered by ecologists as the causes of spatial synchrony (Gao et al. 2006). The described mark-release-recapture technique can help to identify parasitoids which are spatially synchron with *C. ohridella* in the future.

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