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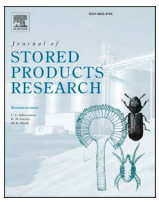

Pheromone-mediated mating disruption of the European grain moth *Nemapogon granellus* in ham factories

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ABSTRACT

Nemapogon granellus is a lepidopteran species that can cause significant damage to stored animal products such as meats, sausages and cheeses. In the warehouses where such products are stored, pheromone-based control approaches can avoid or reduce insecticide chemical treatments and be more effective than traditional control methods. This study aimed to evaluate the effectiveness of mating disruption (MD) techniques to control *N. granellus* populations in ham factories. Trials were conducted in two factories located in Northern Italy. In both locations two warehouses were selected: a warehouse test where dispensers, loaded with 10 mg of *N. granellus* pheromone, were deployed at a density ranging from 1 unit/22.5 m^3 (factory A) to 1 unit/25 m^3 (factory B), and a control warehouse left untreated. To assess the mating disruption efficacy, the reduction of the number of mated females in water traps, placed in control and treated warehouses, was used as main parameter. The results indicated a substantial reduction in mated females in the treated warehouses in comparison with control warehouses in both the sites of experiments. In detail, the total number of mated females sampled in water traps was above 90% in control warehouses, in warehouses treated with MD technique this percentage was below 50%. In addition, a "trap shutdown" effect was recorded in MD treated warehouses of both factories. These findings suggest that mating disruption is a promising technique that can be positively applied in the integrated pest management of *N. granellus* in ham factories.

1. Introduction

Nemapogon granellus (L.), also known as European grain moth, is a lepidopteran species belonging to the fungus moth family (Tineidae: Nemapogoninae), nearly cosmopolitan in temperate regions of the world (Trematerra and Lucchi 2014). This species can infest several vegetal stored foodstuffs as grains, garlic bulbs or dried mushrooms and also stored animal products (Blaeser et al., 2006; Anaclerio et al., 2013; Hrudová and Šafránková 2017). *Nemapogon granellus* is a species with moderate cold tolerance and can thrive at a temperature as low as 6.4 °C (Trematerra and Lucchi 2014), an aspect that determines its adaptability also to refrigerate conditions. In fact, European grain moth has also been reported as a pest in wine cellars, where larvae feeding activity causes damages to the corks of wine bottles with consequent aesthetic damage and alteration in the organoleptic properties of the products (Trematerra and Lucchi 2014). In northern Italy, *N. granellus* infestations are particularly dangerous on stored seasoning animal products (meats, sausages and cheeses) where, even modest attacks, are causing considerable economic damage (Anaclerio et al., 2013). During such infestations, larvae determine direct damage on the product with their trophic activity, by burrowing the surface and penetrating in the meat (or the cheese) to a depth of a few centimeters, causing severe loss to the products (Anaclerio et al., 2013).

In consideration of the dangerousness of this pest in such environments, an integrated pest management (IPM) should rely on prevention, monitoring and the use sustainable control tools. In this context the pheromone-based methods can give an opportunity to achieve proper early detection methods and provide alternative control tools. Pheromone-based control approaches are species-specific techniques that can reduce or avoid insecticide treatment and can be found preferable and possibly more effective than traditional chemical control methods (Campos and Phillips 2014). In the case of *N. granellus*, the sex

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pheromone emitted by females to attract males was identified and characterized by Ohshima et al. (1993) as (3*Z*, 13*Z*)-3, 13-octadecadienyl acetate. In recent years, the use of traps baited with this pheromone demonstrated to be a valid method for monitoring this species and, consequently, is crucial to include it among strategies for integrated production in food industries (Anaclerio et al., 2013).

Despite its effectiveness in detecting and monitoring pest populations, at the moment *N. granellus* pheromone has no application as an alternative method to chemical control, which nowadays is mainly carried out by fumigation with pyrethrum or synthetic insecticides (Servadei et al., 1972; Trematerra and Lucchi 2014). However, the use of any insecticide is not allowed in warehouses where hams and cheeses are cured, therefore, alternative methods to chemical treatments deserve to be evaluated, such as, for example the mating disruption (MD) technique. In food industries and warehouses, other stored products moths have been successfully managed using MD technique, by releasing high levels of synthetic pheromone so that a substantial reduction of the mating occurs (Savoldelli and Trematerra 2011). This technique is based on the disruption of male moth ability to find the female, either from masking of the female pheromone (odor) plume or from saturation of males' central nervous system (Cardé and Minks 1995; Campos and Phillips 2014). Mating disruption is especially applicable in enclosed ecosystems, such as stored product environments, where the technique can be effectively utilized due to the reduced impact of limiting factors that often hinder its effectiveness in agriculture pest management (Trematerra 2012). In fact, MD technique has been used successfully in both North America and Europe to reduce population density of storage moth in short term and long-term studies against *Plodia interpunctella* (Hübner), *Cadra cautella* (Walker) and *Sitrotoga cerealella* (Olivier) (Vick et al., 1978; Ryne et al. 2001, 2006, 2007; Shani and Clearwater 2001; Fadamiro and Baker 2002; Sieminska et al., 2009; Trematerra et al., 2011).

Among the different possible application methods of MD, the false-trail following, also known as disorientation or sexual distraction, represents a valid tool that can be applied in biological control programs and integrated pest management protocols (Rama et al., 2002). This method consists in the setting up of several prevailing synthetic pheromone trails, released by an appropriate number of pheromone dispensers, able to compete with those emitted by the females and thus to distract males in their search for partners (Bartell 1982; Miller and Gut, 2015).

A critical issue in MD is how to determine the success rate (Ryne et al., 2006). The effectiveness of such technique on population levels has been successfully measured for some lepidopteran pests, such as *P. interpuncella* or *C. cautella,* by the presence of spermatophores in the females' *bursa copulatrix*: this is a good indicator of mating activity and is a straighter method than reducing catches in pheromone traps or oviposition in diet cups (Fadamiro and Baker 2002; Savoldelli and Trematerra 2011; Trematerra and Savoldelli 2013).

Aim of this work is then to evaluate the possibility to use the MD technique to control *N. granellus* populations through bioassays carried out in two ham factories located in Northern Italy. Specific objectives were: 1) to establish the *N. granellus* population level in ham maturing warehouses by a pheromone based monitoring system; 2) to assess the MD efficacy by the reduction of number of mated females in the treated areas; 3) to understand the MD mechanism of activity, evaluating if males would spend time and energy following the pheromones false sources rather than the females present in the treated warehouses; 4) to estimate the pheromone emission rate from dispenser during the period of the trial.

2. Materials and methods

2.1. Study warehouses

Tests were performed in two production ham factories (factory A and factory B) located in the North of Italy. Factory A was about 6500 m^2 with a production of 130,000 hams/year, factory B was about 4500 m^2 with a production of 75,000 hams/year. In each factory, two well delimited zones of ham maturing were selected: in one the MD technique (test) was applied while the other was left untreated (control). Factory A is organized on four floors: ham maturing warehouses are at ground floor and third floor; offices and shipping area on the ground floor; the refrigerators are on the first floor; the fresh produce processing area on the second floor. Factory B is organized in five floors: ham maturing warehouses are at basement, second, third and fourth floors; offices and shipping area at ground floor; on the first floor there are the fresh produce processing area and refrigerators. In the factory A, the test warehouse was 900 m^3 (15 m × 15 m x 4 m) at third floor and the control warehouse of 1520 m^3 (20 m × 19 m x 4 m) at ground floor; in the factory B the test warehouse was 378 m^3 (10 m × 10.5 m x 3.6 m) at third floor and the control warehouse of 1008 m^3 (18 m × 14 m x 4 m) at basement. Selected warehouses in both factories were compartmentalized and separated from the other plant departments, and the access doors have always been kept closed, except to allow the passage of personnel.

2.2. Monitoring of male flights

To monitor *N. granellus* populations in ham maturing warehouses, a pheromone trap system was established in both ham factories. Twenty-four pheromone traps were employed in factory A and twenty traps in factory B. Cardboard delta traps [154 mm × 100 mm x 160 (H) mm] with "dust-proof" glue were used (Easy trap, Gea SrL, Settimo Milanese, Milan, Italy). Pheromone traps were distributed in both factories, excluding the MD test warehouses. The pheromone traps were baited with a cylindric silicon dispenser loaded with 1 mg of (3*Z*, 13*Z*)-3, 13-octadecadienyl acetate. Traps were suspended at a height of 1.8–2 m and changed every 30 days. Monitoring activity was carried out from February to December 2021.

2.3. Evaluation of mating disruption

A total of 40 and 15 cylindric silicon dispensers (Gea SrL, Settimo Milanese, Milan, Italy) loaded with 10 mg of *N. granellus* pheromone, were applied in the test warehouses of factory A and B to achieve the density of a dispenser per 22.5 and 25 m^3, respectively. Pheromone dispensers (changed every 90 days) were placed at a height of approx. 2 m on ham hangers, spaced to uniformly cover the test warehouse. Trials were conducted from January to December 2021 in factory A and from January to July 2021 in factory B. Environmental conditions varied between 15 and 20 °C and 61–70% RH in factory A and 16–21 °C and 63–72% RH in factory B.

In each factory, two water traps made from a plastic box (50 cm × 40 cm × 15 cm), filled with about 8 cm of water (Trematerra and Savoldelli 2013) were placed on the floor at a distance of 10 m apart to each other in both the test and control warehouse. Trapped insects were collected monthly using a colander (∅ = 15 cm) and transferred with an entomological tweezers into centrifuge tubes of 50 mL filled with ethyl alcohol 70%. In laboratory, insects were sexed under stereomicroscope, and female moths were then dissected to determine their mating status by observing the presence or absence of one or more spermatophores in the *bursa copulatrix* (Ryne et al., 2001) (SM1). Finally, females were also examined to verify the presence or absence of eggs inside their ovaries.

In addition, to evaluate the reduction in trap catches determined by MD treatment, a comparison among the mean number of captured males per trap in control and tested areas was carried out in both factories. To achieve this, in factory A, three pheromone traps (the same type described above) were placed in MD test warehouse and nine traps in the control warehouse; in factory B, three pheromone traps in MD test warehouse and seven traps in the control warehouse. Male captures were scored monthly from March to December in factory A and from

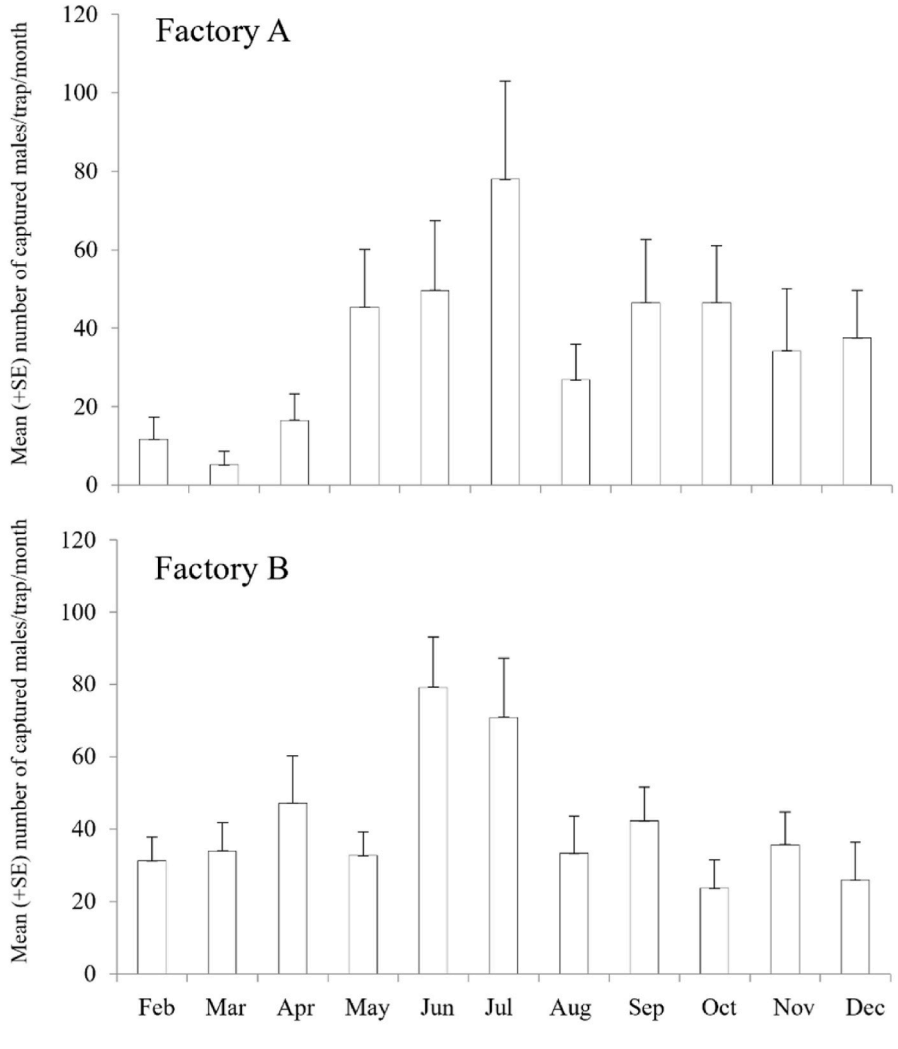

Fig. 1. Mean (+SE) number of captured males of *Nemapogon granellus* in monitoring traps placed in the factories A (n = 24) and B (n = 20), excluding the MD test warehouses (February–December 2021).

March to July in factory B.

2.4. Evaluation of MD activity mechanism

To the best of our knowledge, is not known if *N. granellus* MD acts by masking the female's pheromone plume by a false trail activity, or by saturation of males' central nervous system. In case of false trail activity, the males should be able to locate and being distracted by the MD pheromone dispensers, differently an inability to locate the dispenser would be meaning a saturation of males' sensilla. To establish this, an experiment testing traps loaded with 1 mg pheromone dispenser, commonly used for *N. granellus* monitoring, vs traps loaded with 10 mg pheromone dispenser, the same amount used in the test to evaluate MD technique, was carried out in both the test warehouses of factory A and B. Three traps loaded with 10 mg pheromone dispenser and three traps loaded with 1 mg pheromone dispenser were placed in test warehouse of factory A and in test warehouse of factory B. Traps were inspected monthly from March to December in factory A and from March to July in factory B, and the number of males was counted. At each inspection traps were changed, while the pheromone dispensers substituted every two months.

2.5. Pheromone emission estimation

The pheromone emission rate from the dispenser was analyzed by the headspace method using solid phase micro-extraction (SPME) in static air using a methodology similar to Lo Verde et al. (2020). The stationary phase used as the coating was carbowax-divinyl benzene (CW-DVB, 65 μm) obtained from Supelco, Bellefonte, PA, USA. A manual SPME holder from the same manufacturer was used for injections. Fibres were conditioned in a gas chromatograph injector port as recommended by the manufacturer. The dispensers, loaded with 10 mg of (3*Z*, 13*Z*)-3, 13-octadecadienyl acetate, were opened and kept in climatic chamber at (18 ± 1 °C and 40 ± 5% RH), resembling the factory conditions. SPME extractions were performed in the same room. The release rate of the dispensers was measured during weeks 1, 2, 3, 4, 6, 8, 10 and 12 after their openings. Measurements were carried out during the 3rd and 4th day of each week, using three dispensers sampled by three SPME fibres for each day. Experiments were replicated six times for each week of sampling. For pheromone collection, the dispensers were singly placed into 22 mL vials, which were sealed with a poly (tetrafluoroethylene) silicon septum-lined cap (Supelco, Bellefonte, PA, USA). An SPME needle was then inserted through the septum and headspace volatiles were absorbed on the exposed fibre for 24h. To estimate pheromone emission a known amount of an internal standard (IS)

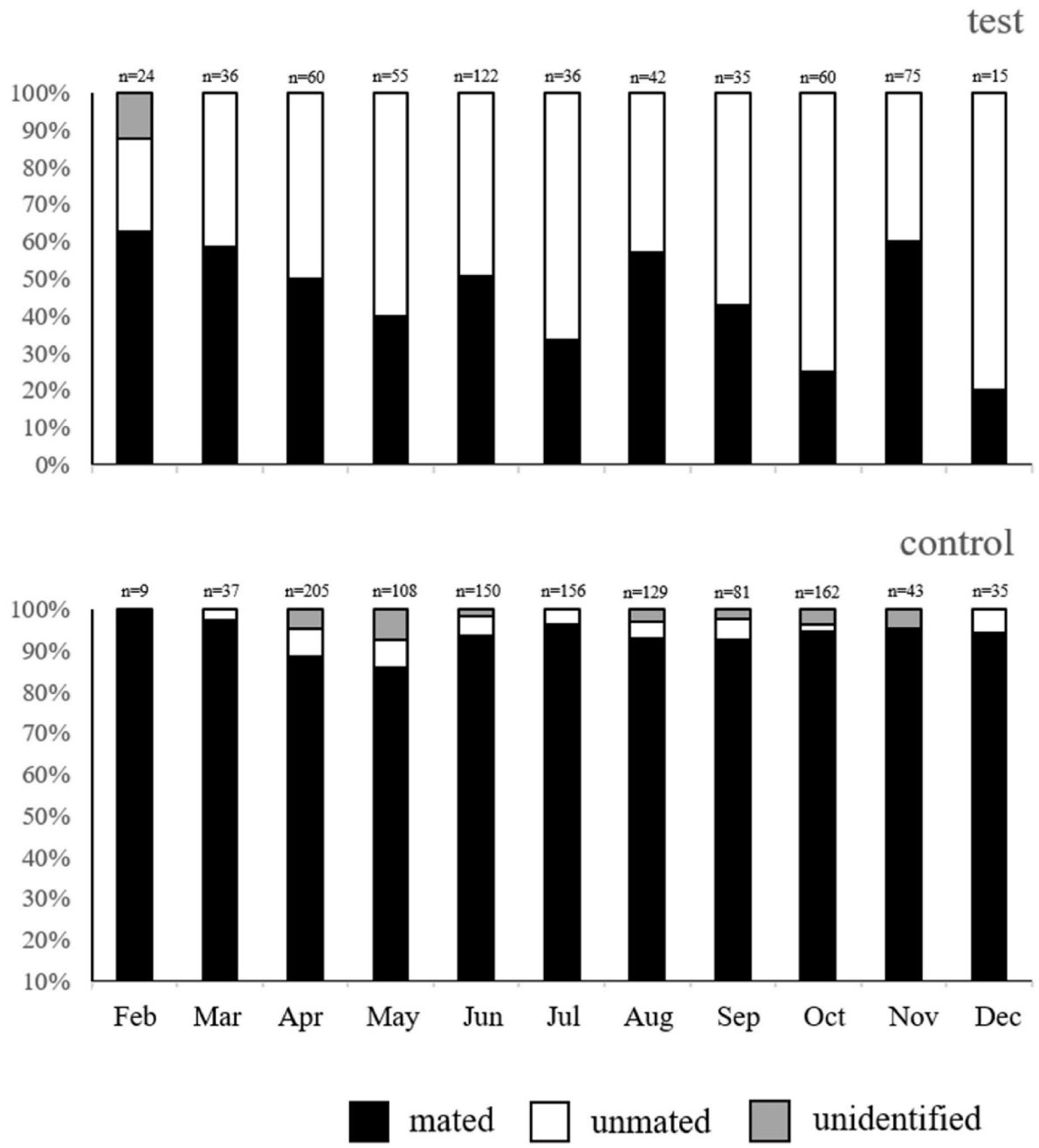

Fig. 2. Factory A: percentage of mated and unmated females captured in water traps of the warehouses treated with mating disruption (test) and untreated (control) (n = number of females). Overall test (observed) versus control (expected); Chi-Square = 2853.60; df = 1; $p < 0.001$.

was added before starting the collection. In detail, 20 ng of hexadecane (purity 99%, Sigma-Aldrich, Milan, Italy) diluted in 2 μL of hexane were pipetted inside the vial. The estimation of the pheromone amount emitted was calculated by comparing the pheromone peak integration with the one of the IS. Immediately after the end of the sampling time, the loaded fibre was desorbed for 1 min in the inlet port of an Agilent 6890 GC system interfaced with an MS5973 quadruple mass spectrometer. Injector and detector temperatures were 260 °C and 280 °C, respectively. The GC oven temperature was set at 40 °C and then increased by 10 °C/min to 250 °C, with initial and final hold times of 5 and 20 min, respectively. A DB5-MS column was used in splitless mode with helium as carrier gas at 1 mL min^{-1}. Electron impact ionisation spectra were obtained at 70 eV, recording mass spectra from 40 to 550 amu. After each sampling the dispensers were removed from the vials and placed again in climatic chamber.

2.6. Statistical analysis

The total number of mated females captured in water traps, recorded during all experimental period, in test and control warehouses of each ham factory was compared by using contingency table and Chi-square analysis. The mean number of captured males per trap per month in pheromone baited traps observed in test and control warehouses of factory A and B was compared by using a one-way ANOVA. The mean number of males captured per trap per month in standard and overloaded pheromone traps was root square transformed and evaluated using one-way ANOVA. The mean of the estimated amounts (ng) of pheromone obtained from headspace SPME pheromone collection of dispensers with different age was compared by using one-way ANOVA, followed by Fisher LSD test ($p < 0.05$).

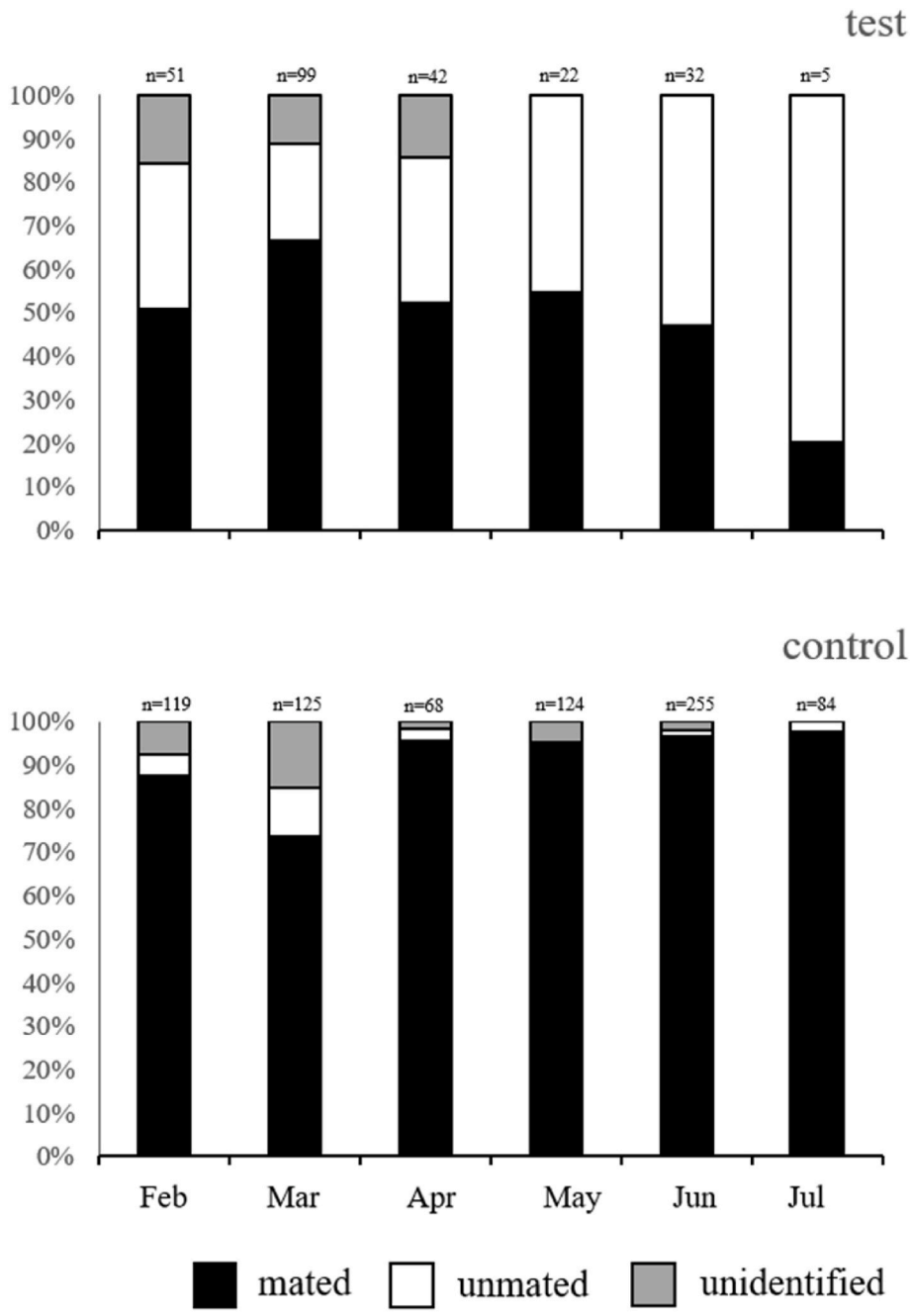

Fig. 3. Factory B: percentage of mated and unmated females captured in water traps of the warehouses treated with mating disruption (test) and untreated (control) (n = number of females). Overall test (observed) versus control (expected); Chi-Square = 688.35; df = 1; $p < 0.001$.

3. Results

3.1. Monitoring of male flights

The number of males captured during the moths of the experiments in factory A and factory B is reported in Fig. 1. *Nemapogon granellus* was present in both ham factories during the entire monitoring period. In factory A, the mean number of captured males per trap per month was minimum in March, 5.15 ± 3.54, and maximum in July, 78.00 ± 24.96 individuals. In factory B, the mean number of captured males per trap per month was minimum in October, 23.70 ± 7.82, and maximum in June, 79.08 ± 13.95 individuals.

3.2. Evaluation of mating disruption

During the trials a total number of 1675 and 1026 females were captured in water traps respectively in the factory A and B, while males captured were correspondingly 544 and 227.

The females' mating status was assessed for 1644 and 961 individuals in factory A and B respectively, while for the remaining ones the mating status remained unidentified as individuals were damaged. Among mated females the number of the ones presenting mature eggs was above 90% in both the factories.

Overall, the frequency of mated females captured was significantly reduced in test warehouses in comparison with control warehouses in both factories A (Chi-Square = 2853.60; df = 1; $p < 0.001$) and B (Chi-Square = 688.35; df = 1; $p < 0.001$). More in depth, the percentage of

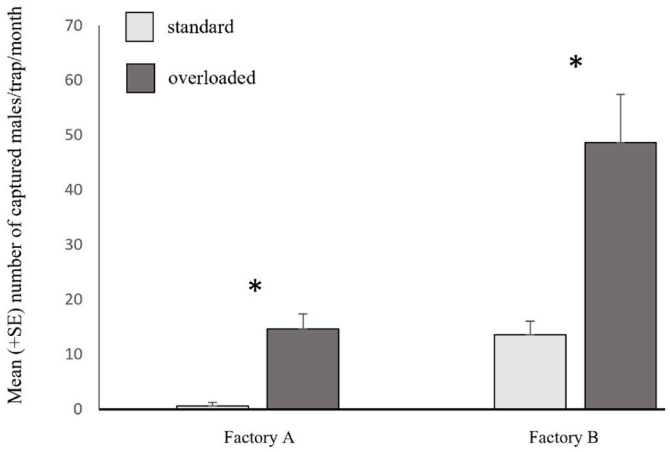

Fig. 4. Mean (+SE) captured males of *Nemapogon granellus* per trap per month in traps loaded with standard (1 mg) and overloaded (10 mg) of pheromone in factory A and B. Asterisks indicate difference among treatments for $p < 0.001$ (ANOVA).

mated females was 95.30% in control warehouse of factory A and 96.19% in control warehouse of factory B, while these percentages were respectively 47% in factory A and 69% in factory B. The frequencies of the presence of mated and unmated females in test and control warehouses of factory A and B during the months of trials are reported in Figs. 2 and 3.

The comparison of the captured male means in test and control warehouses exhibited differences with a higher number of catches in traps of the control warehouses in both the factory A ($F_{1,118} = 32.87$; $p < 0.001$) and B ($F_{1,48} = 6.02$; $p < 0.05$). In detail in factory A, the mean number of captured males per trap per month was 0.6 ± 0.2 in test warehouse and 84.10 ± 8.38 in control warehouse. In factory B, the mean number of captured males per trap per month was 13.60 ± 1.90 in test warehouse while 50.51 ± 9.75 in control warehouse.

3.3. Evaluation of MD activity mechanism

The mean number of *N. granellus* males captured in traps loaded with 10 mg and 1 mg pheromone dispenser is reported in Fig. 4. Regardless of the pheromone dispenser used, the number of captured males exhibited marked differences among the two sites of experiment with a greater number of captures observed in factory B rather than in factory A.

In factory A, the mean (±SE) number of captured males per trap lured with 10 mg dispenser was 14.66 (±2.77), significantly higher than in 1 mg dispenser whose captured 0.60 (±0.21) males ($F_{1,58} = 78.33$; $p < 0.0001$; ANOVA) (Fig. 5). Similarly, in factory B, the mean (±SE) number of captured males per trap lured with 10 mg dispenser was 48.70 (±8.73), significantly higher than in 1 mg dispenser whose captured 13.60 (±2.51) males ($F_{1,18} = 22.40$; $p < 0.001$; ANOVA) (Fig. 5).

3.4. Pheromone emission estimation

The mean release rates of pheromone from dispensers, measured by SPME in static air, are shown in Fig. 5. The estimated pheromone emission ranged from 5.36 (±1.98) ng after one week from the dispenser opening to 2.11 (±0.76) ng at the dispenser end of use (12 weeks). The amount of pheromone emitted during the different times did not differ statistically ($F_{7,37} = 0.74$; $p = 0.65$; ANOVA). However, difference in the pheromone amount emitted between the 1st and the 12th (and last) week of the dispenser exposure was marginally not statistically significant ($p = 0.06$; ANOVA followed by LDS test).

4. Discussion

The results of the monitoring activity for *N. granellus* showed the presence of the pest throughout the entire monitoring period in both factories. This study's findings, for the first time, demonstrate that the use of MD techniques can significantly reduce the number of mating in *N. granellus* and can be considered a tool positively exploitable for the management of this pest. This reduction of mated females observed in our experiments was recorded in both the sites of experiments. More in depth, while the total number of mated females sampled in water traps was above 90% in control warehouses, in warehouses treated with mating disruption this percentage was below 50%. The values obtained in our study are comparable with the ones obtained from Trematerra and Savoldelli (2013); the latter study evaluated MD technique in a confectionary factory infested by *C. cautella* and observed a reduction in the number of mated females recovered in water traps ranging from

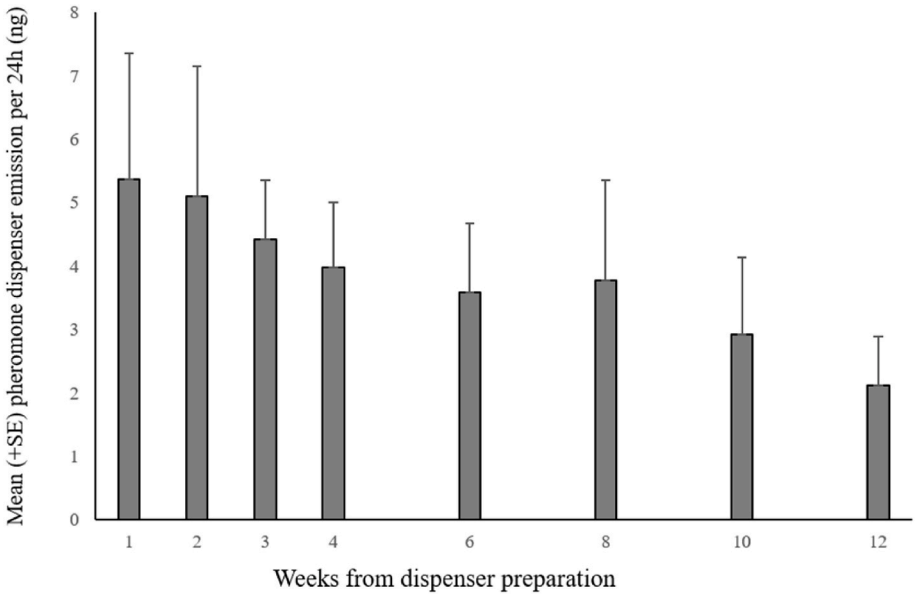

Fig. 5. Mean (+SE) release rates of *Nemapogon granellus* pheromone from dispensers in relation to the internal standard (20 ng of hexadecane), measured by collecting the volatiles by using a SPME head-space method at 18 ± 1 °C.

27.77% to 73.52%.

The data obtained in our study confirmed the suitability of the water traps for the estimation of mating decreases in MD treated warehouses. Furthermore, in consideration of the consistent number of individuals captured, with a large majority of mated females, we can suggest that these traps, cheap and easy to apply, can find application for monitoring and mass trapping *N. granellus*. In detail, the percentage of females captured was above 75%, a percentage higher than the one observed in other studies using water traps to monitor *C. cautella* (Ryne et al., 2006; Trematerra and Savoldelli 2013). The higher number of females rather than males captured in water traps, could be linked with the fact that, similarly to other lepidopteran pests of stored products, water is biologically more important for females than for males (Ryne et al., 2004).

In consideration that in the present study was not measured the reduction of the damages on the products in MD test warehouses, we cannot conclude that these percentages of mating reduction observed can have produced an effective damage reduction on the stored commodities. However, it is reasonable that the decrease in mating observed can trigger a long-term reduction of the damage determined by *N. granellus* in ham factories and that such tools can be positively implemented in a structured strategy of integrated pest management, including also other control methods (Trematerra et al., 2017).

The results of the experiments that compared the number of males caught in pheromone traps in test and control warehouses showed an evident "trap shutdown" effect determined by the MD treatment in both the factories. The reduction of male catches in pheromone traps of treated areas is another important index of the effectiveness of MD treatment as observed in other studies (Ryne et al., 2006; Trematerra et al., 2011; Athanassiou et al., 2016; Sharon et al., 2016).

The measurement of pheromone emission by SPME evidenced that dispensers released the *N. granellus* pheromone along the experimental period. Although SPME method cannot give a precise measure of real quantity of pheromone emitted, we evidenced that the amount estimated, in comparison with internal standard, was relatively constant during all the period of observations. Our estimation indicated lower amounts of pheromone emitted than the one observed in other studies testing MD dispenser for other lepidopteran stored product pest (Sieminska et al., 2009; Trematerra and Savoldelli 2013). This can be determined by several factors as the relatively low temperature of headspace SPME collection used in our study and the higher molecular weight of the *N. granellus* pheromone, (3*Z*, 13*Z*)-3, 13-octadecadienyl acetate, in comparison with *P. interpunctella* or *C. cautella* sex pheromones. This result can in perspective bring to consider also different types of dispensers that might augment the pheromone emission rate as observed in other studies (Guarino et al., 2020, 2022). Such dispenser improvement might result in an increase in dispensers' field longevity while reducing the amount of expensive pheromone active ingredient that is used per dispenser as suggested by Baker et al. (2016).

The relatively low emission rate from the dispensers observed in our study, can contribute to explain the higher number of males captures in traps loaded with overloaded pheromone dispenser in mating disruption treated warehouses in comparison with standard ones. The latter data suggest that, in the tested condition, males spend time and energy following such false sources rather than the females. Barclay and Judd (1995) defined the attraction of males away from virgin females as "false trail following". This behavior is also known as competitive mating disruption (Miller et al., 2006a). A literature review carried out by Miller et al. (2006b) examined different experiments on mating disruption across a range of moth species and evidenced that competitive attraction is the mechanism occurring in the majority of cases. In such cases, once males can respond to females and pheromone traps, the ratio of dispensers to females and traps is extremely consequential and makes the control pest-density-dependent (Miller and Gut, 2015). In consideration of the data obtained in our study, it seems the mating disruption effectiveness toward *N. granellus* is strictly related to the number of false traces used, so the application of a larger number of pheromone dispensers should be considered in future studies for the optimization of this technique to the management of this pest (Stelinski et al., 2005).

To conclude, mating disruption appears to be a promising alternative to chemical control methods for managing *N. granellus* population in ham factories. This is particularly noteworthy in consideration that chemical treatments such as methyl bromide or other fumigants, have been phased out or encounter increasing restriction for their use in the majority of the countries worldwide (Savoldelli et al., 2020; Hasan et al., 2021).

5. Conclusions

The data presented in this work indicate that mating disruption is a promising technique that can be effectively included in the integrated pest management of *N. granellus* in ham factories. Future research will focus on optimizing the dose and type of pheromone dispenser used, as well as the dispenser density, to increase the amount of pheromone in the environment and improve the technique's effectiveness. Additionally, further studies will aim to estimate the reduction of damage to products in warehouses treated with mating disruption, in order to assess the cost-benefit for producers.

Author contribution

SS, SG conceived the idea and set up protocols and methodology; SS, DL, CJ, SM, EP and SG collected the data; SS and SG analyzed the data and wrote the manuscript; all authors contributed critically to the draft and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jspr.2023.102117.

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