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Article

An immunomarking method to investigate the flight distance of the Japanese beetle

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With 2 figures and 4 tables

Abstract: *Popillia japonica* Newman (Coleoptera: Scarabaeidae), a devastating pest to many crops and other plants, has been detected in the European mainland for the first time in 2014 in Northern Italy. Official measures have been immediately implemented to contain the spread of this insect, and studies to evaluate its dispersion rate have been rapidly launched. The flight activity of beetles has been studied in 2017–19 using a protein immunomarking technique (PIT), by spraying either chicken egg white (marker: albumin) or cow milk (casein) on infested plants. Sampling of potentially marked beetles occurred up to a distance of 12 km with double lure baited traps, and markers were identified via two indirect ELISAs assays (anti-egg and anti-milk). Females were dissected to assess egg load and relate it to distance travelled. Data were handled with geostatistical methods (indicator kriging, IK). Collectively, about 1600 beetles were captured and analyzed. Rates of marked beetles ranged 7–15% for albumin and 2–22% for casein. Mean distances travelled ranged 1–7 km from sources; however, a few marked beetles have been found to disperse up to 12 km (maximum distance investigated). Geostatistical analyses showed that the spatial dependence of marked beetles occurred up to 2.3 km distance. Egg load in marked females was not related to distance travelled. The results of this research, examined together with field surveys carried out by the Plant Protection Service, could provide useful guidance in establishing an effective radius for the demarcated area.

Keywords: *Popillia japonica*, Scarabaeidae, quarantine pest, dispersal, mark-capture, ELISA, indicator kriging

1 Introduction

The Japanese beetle (JB), *Popillia japonica* Newman (Coleoptera: Scarabaeidae), introduced into the USA in 1916 (Fleming 1972), was reported for the first time in the European mainland in Northern Italy (Pavesi 2014). Until then, the only report in Europe concerned the Azores Islands (Potter & Held 2002). In Europe, the JB is confined to the Lombardy and Piedmont regions, on both sides of the Ticino river (Lessio et al. 2019), although a few specimens have also been found in Switzerland, where it is claimed to be close to eradication (EPPO 2019b). The European Plant Protection Service (EPPO) has placed it in the A2 List (a list of species recommended for regulation as quarantine pests) (EPPO 2019a), and the European Food Safety Authority (EFSA) gathered useful data about the impact of the Japanese beetle on European agriculture and landscape (Bragard et al. 2018).

Popillia japonica has a single generation per year (every two years in cold climates). Larvae (grubs) live in the soil, generally on meadows and lawns. They feed on roots, causing yellowing and stunting of turfs. Third instars overwinter, and pupae appear in May. Adult beetles emerge in early summer and are present until the middle of September, peaking at mid-July. They feed on more than 300 plant species (79 families), including corn, soybean, fruit trees, grapevine, forest trees (e.g. oak, linden, elm, and maple), roses, and many others, crowding on the leaves and causing intense defoliation (Fleming 1972, Potter & Held 2002). During its lifespan, each female lays up to 40–60 eggs in the soil, preferring sunny areas, dense and short (low) turfs, high relative humidity, and a sandy-loam soil texture (Fleming 1972; Régnière et al. 1979, 1981a, 1981b).

Flight activity is crucial for an insect pest to colonize new environments, and a correct knowledge of natural spreading capability is therefore a key point to put in place regulatory measures. To date, a buffer area having a 10 km extension from the border of the JB infested area has been adopted. However, flight capabilities of the JB are not yet well quanti-

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fied. Therefore, the application of more reliable methods to assess dispersal are needed.

Marking is often used to assess the flight capability of an insect, and may be performed using dusts, dyes, radioisotopes, paints, mutilation, and protein immunomarking technique (Hagler & Jackson 2001). The last technique has many advantages such as low impact on insect behavior, low costs, and high reliability, allowing the marking of large numbers of insects by spraying the markers directly on host plants (indirect marking, or mark-capture) (Hagler 2019). In the past, mark-release-recapture techniques have been applied to the JB using paints (Holmes & Barrett 1997), fluorescent dusts (Polivka, 1949), and lacquers (Polivka 1949), but the number of insects marked and released was always low (300–1000 specimens) compared to the population size, making it difficult to recapture a considerable proportion of the population. Moreover, both studies were conducted at a plot size scale, and did not discriminate between males and females. Long-range dispersal, up to 8 km or more, of JBs was reported by Fleming (1972), but without quantifying mean distance covered, or proving it with mark-capture experiments. The aim of the present research was to calculate the within-season flight distance of JB males and females. Moreover, we wanted to test if the egg load could affect to some extent the flight activity of females, in order to quantify the risk of spread of the JB over an unexploited area.

2 Materials and methods

2.1 Insect marking and capturing

The study was conducted from 2017 to 2019 in NW Italy. Two plots (henceforth referred to as Plot 1 and 2) subject to recent reforestation were chosen as sources: Plot 1 was in the district of Turbigo (MI) (45.545315°N, 8.742356°E) and consisted of a 2000 m^2 area planted with oak (*Quercus* sp.), and hawthorn (*Crataegus* sp.), located within a corn growing area. Trees were planted in stands having a 3 × 4 m spacing, resulting in a density of 8.3 plants per 100 m^2. Other plants growing were wild grapevine (*Vitis* sp.), *Parthenocyssus* spp., wild hop (*Humulus lupulus* L.), dog rose (*Rosa canina* L.), and poplar (*Populus* spp.). Plot 2, in the district of Vanzaghello (MI) (45.601135°N, 8.786983°E), was located within an industrial area close to a railway, and had an area of approximately 1500 m^2. Plants included hornbeam (*Carpinus betulus* L.), hazelnut (*Corylus avellana* L.), hawthorn, oak, elder (*Sambucus nigra* L.), and bramble (*Rubus fruticosus* L.), covering the whole plot. The distance between plots was approximately 7 km.

Woody plants growing within each plot (therefore covering the whole plot) were sprayed with a tap water solution of either 10% vol. chicken egg whites (Plot 1), or 20% whole fat cow milk (Plot 2) to provide indirect marking of JB adults, according to Lessio et. al (2014). Additionally, as a great number of beetles aggregated on trees and shrubs at the moment of spray, many of them may have been marked directly. The equipment used consisted of 1–2 Garden 12® hand-jet sprayers with a 12 L tank (manufacturer: Di Martino SpA, Casoni, VI, Italy). Within both areas, the plants were sprayed abundantly until runoff, targeting upper parts with heavy defoliation, as beetles tend to concentrate higher in the crowns (Fleming 1972, Potter & Held 2002). Spraying was carried out between the end of June and the end of July, with a 7–8 days difference between consecutive spraying events. We sprayed three times in 2017, and five times in 2018 and 2019. Spraying was carried out in the morning, usually between 10:00 am and 12:00 pm.

Potentially marked beetles were captured 24 h (over the three years), and seven days (in 2018 and 2019 only) after each spray. A pattern of 10 georeferenced sampling points, falling inside meadows, field margins etc., was arranged around each treated area (sources): five points fell within a 2.5 km radius, and the other five within a 5 km radius. Moreover, in 2018 and 2019 we added three points between the two sources. Therefore, on the whole, 20 points were sampled in 2017, and 23 in 2018 and 2019 (Fig. 1A). The maximum distance from a treated point and a sampling point was 12 km.

In order to avoid any cross-contamination of protein markers in captured beetles, we modified a standard Pherocon® JB trap (Trecé Inc., Adair, Oklahoma), by removing the body from the top funnel and replacing it with a 50 mL plastic Falcon vial (length 11 cm; diameter: 28 mm) fitted into the hole of the funnel itself. Bait consisted of standard JB dual lure (Trecé Inc., Adair, Oklahoma), to attract both males and females (Potter & Held 2002). Sampling was made by moving from point to point, placing the trap on an iron post (1 meter from ground level), and leaving it in place either for 10 minutes or until 10 beetles were attracted and captured. Each time a single beetle was captured, the vial was immediately replaced. Before moving to another point, the trap funnel was cleaned with alcohol to avoid contamination by potentially marked beetles landing on it. Usually, the whole sampling lasted one day from 10:00 am to 5:00 pm, during the daily window of beetle flight activity. Beetles were killed with a drop of ethyl acetate inside the vial, which was closed with two plugs (cotton inside, and plastic outside), labelled, and placed into a cool bag until transferred to our lab facility in Grugliasco (TO), Italy (45.066472°N, 7.588618°E), which is located about 150 km far away from the treated plots, in a completely JB-free zone. There, the vials were stored in a freezer (–20°C) until analysis.

2.2 Detection of markers

An indirect ELISA was performed to detect protein markers acquired by the beetles. Our equipment consisted of 96-well microplates (Nunc Polysorp, Nalge Nunc, Naperville, IL, USA), and an LT –3000 micro-plate washer and a LT-4000 micro-plate scanner (both from Labtech International Ltd, Uckfield, UK).

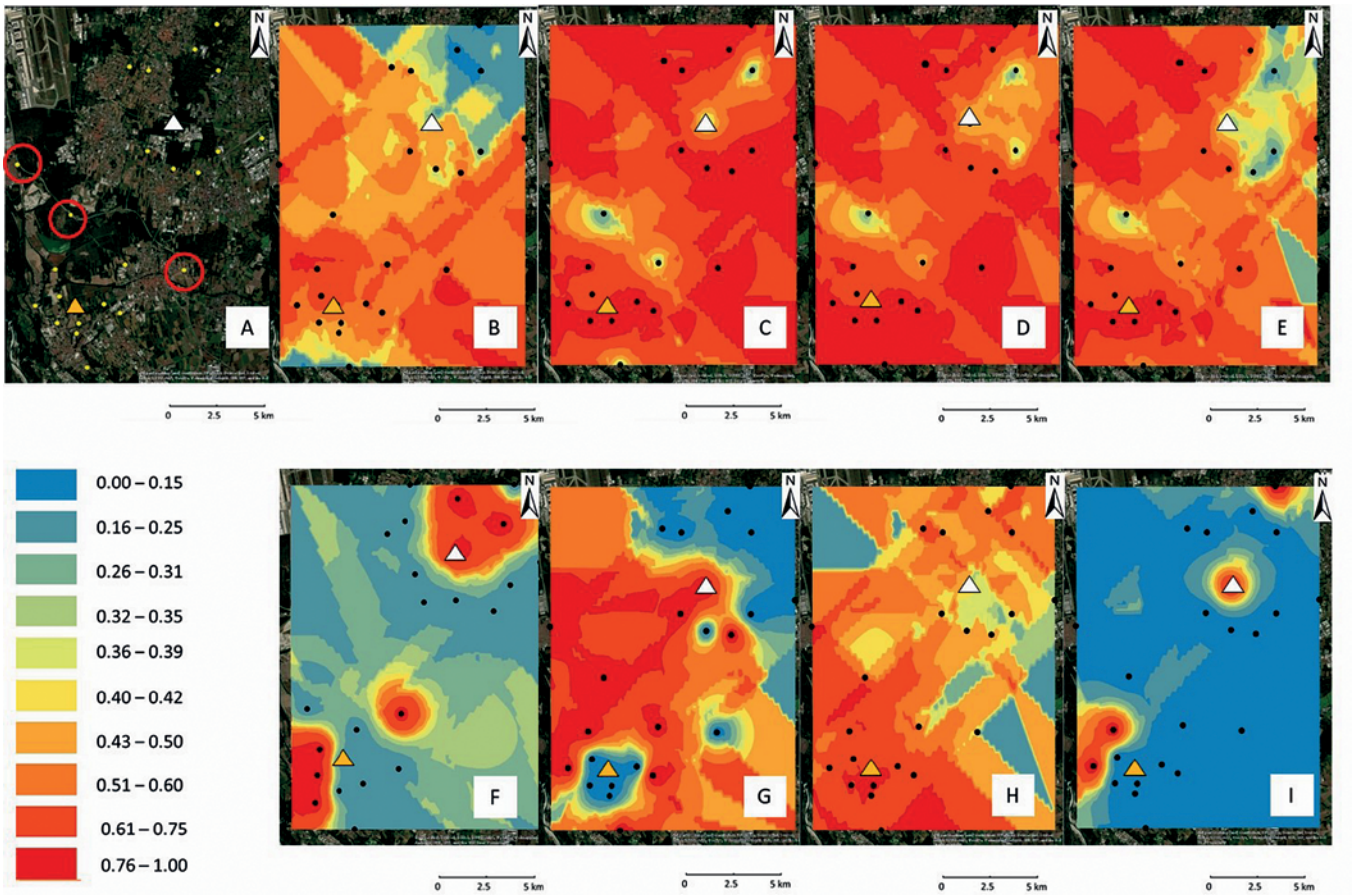

Fig. 1. Displacement of sampling points and treated areas, and results of spatial interpolation. **A:** map showing the displacement of treated plots (triangles: yellow for egg, white for milk), and sampling points (yellow dots; the three dots surrounded by red circles represent the sampling points added in 2018), over the study area. B–I: interpolation maps generated by indicator kriging, and showing the probability of exceeding a given cutoff value *c* of marked Japanese beetles; the areas in different colors (see legend) represent the probability that the rate of marked JBs exceed the given cutoff (*c*); sampling points are here given in black. **B:** year 2018, egg-marked, captures after 24 h, *c* = 0.05; **C:** year 2019, egg-marked, captures after 24 h, *c* = 0.05; **D:** year 2019, egg-marked, total captures (after 24 h + 7 days), *c* = 0.05; **E:** year 2019, egg-marked, total captures (after 24 h + 7 days), *c* = 0.10; **F:** year 2017, milk-marked, captures after 24 h, *c* = 0.20; **G:** year 2018, milk-marked, captures after 24 h, *c* = 0.05; **H:** year 2018, milk-marked, total captures (after 24 h + 7 days), *c* = 0.05; **I:** 2018, milk-marked, total captures (after 24 h + 7 days), *c* = 0.20.

Reagents included:

- TBS – EDTA: Tris Buffered Saline (pH 8.0) + 0.3 g/l sodium ethylenediamine tetra acetate (Sigma-Aldrich)
- PBS – BS: Phosphate Buffered Saline + 20% Bovine Serum (Sigma-Aldrich)
- PBSS – BS 20: Phosphate Buffered Saline + 20% Bovine Serum (PBS-BS) (Sigma-Aldrich) + 1300 ppm; Silweet L-77 (Chemtura Manufacturing, Manchester, UK)
- PBST: Phosphate Buffered Saline + 0.09% Triton X-100 (Sigma-Aldrich)
- PBS – SDS: Phosphate Buffered Saline + 2.3 g/l Sodium dodecyl sulfate
- sulfuric acid (H_2SO_4) 2 N;
- immuno – pure ultra 3,3',5,5' – tetramethylbenzidine (TMB) substrate (Pierce Biotechnology, Rockford, IL, USA).

Primary antibodies were: rabbit anti egg (C6534, Sigma-Aldrich, St. Louis, MO, USA) for the egg assay; and rabbit anti casein (GeneTex, Inc., Irvine, CA) for the milk assay. The secondary antibody was peroxidase conjugated donkey anti-rabbit IgG (H + L) (Bethyl laboratories, Inc., Montgomery, TX), for both egg and milk. All antibodies were diluted in Phosphate Buffered Saline + 20% Bovine Serum (PBSS – BS20) as follows: rabbit anti-egg: 1:4000 (2 µl in 8.0 ml); rabbit anti-casein: 1:500 (16 µl in 8.0 ml); donkey anti-rabbit: 1:6000 (1.4 µl in 8.4 ml).

The whole ELISA protocol assay was as follows: each insect was soaked into 1 ml Tris-Buffered Saline + sodium ethylenediamine tetra acetate (TBS-EDTA) inside a 1.5 ml Eppendorf tube for three minutes at room temperature (25°C); the tube was shaken in a vortex for 3 seconds; three aliquots of 80 µl each were taken from the tube, and poured

into three distinct microplate wells; the plate was incubated for 2 h at T = 37°C and then emptied; the plate was rinsed 5 times with 300 µl of Phosphate Buffered Saline + 0.09% Triton X-100 (PBST) each; 300 µl of PBSS-BS 20 (blocker solution) was added; the plate was incubated for 1 h at T = 37°C, then rinsed 2 times with 300 µl Phosphate Buffered Saline + 0.09% Triton X – 100 (PBST) each; the first antibody was added: 2 µl rabbit anti-egg diluted in 8 ml PBSS – BS 20 (1:4000), and 16 µl rabbit anti-casein diluted in 8 ml PBSS – BS 20 (1:500) for the egg and milk assay, respectively; the plate was incubated for 30 minutes at T = 37°C; the first antibody was discarded, and the plate was rinsed 5 times with 300 µl Phosphate Buffered Saline + 0.09% Triton X – 100 (PBST) each; the second antibody was added: 1.4 µl of donkey anti-rabbit in 8.4 ml PBSS – BS20 (1:6000) for both assays; the plate was incubated for 2 h at T = 37°C; the second antibody was discarded; the plate was rinsed three times with 300 µl PBS-SDS and three times with Phosphate Buffered Saline + 0.09% Triton X-100 (PBST); 80 µl of ultra tetramethylbenzidine (TMB) was added; the plate was incubated in the dark, rotating, at room temperature for 10 minutes; the reaction was stopped with 80 µl sulfuric acid (H_2SO_4), and the optical density was red.

Plate scanning occurred at wavelengths of λ = 450 and 492 nm (reference standard), according to Jones et al. (2006). The mean optical densities of each sample (ODS) taken at 450 and 492 nm were calculated from the readings of the three previously described aliquots, and the difference calculated: $ODS_{(450\text{-}492)} = ODS_{450} - ODS_{492}$; and the same equation was applied to the optical density of the negative control: $ODN_{(450\text{-}492)} = ODN_{(450)} - ODN_{492)}$; and the optical density of the blank control: $ODB_{(450\text{-}492)} = ODB_{450} - ODB_{492}$. Finally, the optical densities of corrected (blanked) sample (ODCS) and negative control (ODCN) were obtained as $ODCS = ODS_{(450\text{-}492)} - ODB_{(450\text{-}492)}$ and $ODCN = ODN_{(450\text{-}492)} - ODB_{(450\text{-}492)}$, respectively. A sample was considered marked when the ODCS was greater than the mean ODCN added plus 4 times its standard deviation (SD): ODCS > ODCN + 4SD, providing additional protection against false positives (Jones et al. 2006). The beetles which were marked with both egg and milk were excluded from statistical analyses, as we could not be sure if this was due to long-range movements or cross contamination.

Positive standards consisted of adults of the leafhopper *Euscelidius variegatus* (Kirschbaum) (Hemiptera: Cicadellidae) reared on oat (*Avena sativa* L.) under laboratory conditions in our facility in Grugliasco. Filter paper disks (diameter 15 cm) were sprinkled with the marker and placed inside a Petri dish. Leafhoppers were then put inside the dish and left in place for 30 minutes. Afterwards, they were retrieved, killed by freezing and preserved at –20°C before analyses. Some untreated leafhoppers were used as negative controls, and extraction buffer alone served as the blank control. We preferred using leafhoppers mainly for a practical reason, because it is strictly forbidden to carry viable JBs away from the infested zone: the capture and handling of great numbers of JBs for many times per year would have been too dangerous. In order to test if *E. variagatus* is an appropriate negative control, a preliminary ELISA test was conducted: we made six plates, testing for both egg and milk, on 45 leafhoppers and 45 JBs. Data were analyzed with a Student *t* test ($p < 0.05$), using the average optical density $ODS_{(450\text{-}492)}$ of each species within the same plate as a replication.

2.3 Egg load in females

After ELISA analysis, the beetles were removed from vials and sexed by observing under a stereomicroscope the shape of the tibia and the length of the first tarsomeres in the first leg pair (Fleming,1972). Afterwards, while males were discarded, females were dissected for egg load quantification: each specimen was placed into an excavated glass block (40 × 40 × 15 mm) into a phosphate buffered saline (PBS) solution for ovary dissection. Egg load was assessed by dissecting each female under a stereomicroscope at 15× magnification. The abdomen was separated from the rest of the body using a couple of needles. Ovaries were then removed from the abdomen and spread for egg load counts as described in Picciau et al. (2017, 2019). Mature eggs were defined by the presence of a complete yolk deposition, clear chorion definition, and no nurse cells. While sexing of beetles was made for all the three years of study, the dissection of females was conducted only in years 2018 and 2019: on the whole, 402 females were dissected.

2.4 Data analysis

To calculate the dispersal of marked beetles, sampling points were included into circular zones having the same distance from the treated point(s), with a tolerance of 500 m. Mean dispersal indices were then calculated as follows:

$$I = \frac{\sum_{i=1}^{n} D_i \cdot \frac{P_i}{N_i}}{\sum_{i=1}^{n} \frac{P_i}{N_i}}$$

Being *i = 1, 2,., n* the set of zones from source, *D* the distance (expressed in km) of each zone from source, *N* the number of sampling points within the same zone from source, and *P* the total number of positive beetles captured within the same distance from source. Moreover, the distances traveled corresponding to the 50th, 75th, and 90th percentiles of captured beetles were calculated.

Data of egg load in marked females were analyzed with a generalized linear model (GLM) with a Poisson distribution (counts) and a *Log* transfer function. Egg load levels were ranked as follows: R1: no eggs; R2: 1–5 eggs; R3: 6–10 eggs; R4: >10 eggs. The number of marked females was the dependent variable, whereas the number of dissected females was the weight variable. The following interactions between

predictors were tested: egg load × distance; egg load × plot; egg load × distance × plot, whereas the sampling year was not considered. Both analyses were run using SPSS 26.0® statistical package (IBM corp., Armonk, NY).

Data of captured JB (after 24 h, seven days, and both) were analyzed with ESRI ArcGIS 10.1® geographic information system (ESRI Italia, Roma, Italy), applying the indicator kriging (IK) geostatistical interpolation method (Liebhold et al., 1993, De Luigi et al. 2011). IK transforms count data into binary response values (that is, indicators), on the basis of a cut-off value (c):

$$x \geq c \Rightarrow I_c = 1$$
$$x < c \Rightarrow I_c = 0$$

In the present research, cutoff values were chosen equal to 5% (cutoff: c = 0.05), 10% (c = 0.10), and 20% (c = 0.20) of marked adults, after having excluded the double-marked specimens. Experimental semivariograms were calculated using a *lag* distance of d = 200 m, with a number of lags n = 25 (2 to 5 neighbors, 45° offset), with isotropy (no directionality). Spatial correlation was therefore investigated up to 5 km distance (200 × 25), which is about half of the sampling space considered according to Liebhold et al. (1993). For each of the *lag* values, at least n = 30 pairs were available. Data were fitted to an exponential theoretical model, and a cross-validation was performed to test the semivariogram goodness-of-fit. The final outputs were interpolation maps showing the cumulative distribution of marked JB over the study area.

3 Results

Overall, 1794 specimens of JB were collected over three years of study: 247, 780, and 761 in 2017, 2018, and 2019, respectively. The sex ratio (males vs females) was 2.38 : 1. Optical densities of marked and unmarked JBs, along with negative controls (*E. variegatus*), are shown in Table 1. The average blank-corrected optical densities of positive beetles were 0.07 for egg and 0.05 for milk, which is seven-fold and five-fold higher than the unmarked specimens. The average value of cutoff was 0.02 for both egg and milk.

The average unblanked optical densities of negative *E. variegatus* and JBs (mean ± SD) were: 0.005 ± 0.001 and 0.004 ± 0.002 for the egg assay (t = 7.82, p = 0.08); 0.006 ± 0.006 and 0.004 ± 0.003 for the milk assay (t = 0.89; p = 0.54). As no significant differences were detected, it may be concluded that the use of unmarked leafhoppers as a negative control has not invalidated the ELISA assays performed on field-collected JBs.

After having excluded 181 double-marked specimens, 1613 beetles were available for data analyses. The rates of marked beetles over the three years depending on marker, sex, and time elapsed are given in Table 2. The number of marked specimens clearly decreased along with an increasing distance from Plot 1 (marker: egg), whereas this trend was not evident in beetles coming from Plot 2 (marker: milk) (Fig. 2). The distances travelled from source (mean ± standard error, and 25th, 50th, 75th percentiles) calculated in different years are listed in supplementary file S1. Regardless to sex, the mean distances covered by egg-marked speci-

Table 1. Optical densities (ODs) of field-collected marked and unmarked Japanese beetles, and relative threshold values. ODS: optical density of unblanked sample; ODCS: optical density of corrected (blanked) sample; threshold value is equal to the sum of the mean plus 4-fold the standard deviation (SD) of either unblanked (ODN) or blank corrected (ODCN) negative control.

Marker	Category	Blank correction	mean	SD
Egg	Negative (N = 1418)	Unblanked (ODS)	0.010	0.010
		Blanked (ODCS)	0.001	0.016
	Positive (N = 376)	Unblanked (ODS)	0.077	0.204
		Blanked (ODCS)	0.069	0.205
	Threshold (N = 64)	Unblanked (mean + 4•SD ODN)	0.032	0.035
		Blanked (mean + 4•SD ODCN)	0.021	0.024
Milk	Negative (N = 1401)	Unblanked (ODS)	0.012	0.015
		Blanked (ODCS)	0.003	0.014
	Positive (N = 309)	Unblanked (ODS)	0.061	0.096
		Blanked (ODCS)	0.052	0.096
	Threshold (N = 61)	Unblanked (mean + 4•SD ODN)	0.032	0.028
		Blanked (mean + 4•SD ODCN)	0.021	0.024

Table 2. Rate of marked Japanese beetles depending on plot, sex, and time elapsed from spray to capture (double-marked specimens are excluded).

Plot (marker)	Year	Time elapsed	Sex	Captured	Marked	Rate
Plot 1 (egg)	2017	24 h	Females	71	5	7.0%
			Males	142	20	14.1%
		Total		213	25	11.7%
	2018	24 h	Females	73	11	15.1%
			Males	173	8	4.6%
		7 days	Females	129	3	2.3%
			Males	320	28	8.8%
		Total		695	50	7.2%
	2019	24 h	Females	141	21	14.9%
			Males	338	76	22.5%
		7 days	Females	59	2	3.4%
			Males	167	10	6.0%
		Total		705	109	15.4%
	Total			1613	184	11.4%
Plot 2 (milk)	2017	24 h	Females	71	15	21.1%
			Males	142	33	23.2%
		Total		213	48	22.5%
	2018	24 h	Females	73	7	9.6%
			Males	173	10	5.8%
		7 days	Females	129	16	12.4%
			Males	320	32	10.0%
		Total		695	65	9.3%
	2019	24 h	Females	130	7	5.4%
			Males	319	4	1.3%
		7 days	Females	56	1	1.8%
			Males	115	3	2.6%
		Total		620	15	2.4%
	Total			1528	128	8.4%

mens ranged 3.97–4.76 km after 24 h and 1.37–2.39 km after 7 days, respectively. Males and females flew at distances ranging from 3.24 km to 5.06 km and from 3.11 km to 3.77 km, respectively. Finally, the 75th and 90th percentiles of egg-marked JBs (without considering distinctions in sexes or time elapsed) were captured within distances ranging from 5 km to 9 km and from 6.5 km to 11 km, respectively. Mean dispersal of milk-marked specimens ranged 5.21–6.39 km, without considering sex and time elapsed. After 24 h, dispersal of both sexes ranged 4.63–6.39 km, whereas after 7 days it was 6.49–7.04 km. As for differences between sexes, males dispersed 5.77–6.57 km, and females 4.45–6.23 km. Overall, in milk-marked specimens the 75th and 90th percentiles ranged 6.25–9 km and 7.5–11 km, respectively. The maximum distance covered by single egg-marked specimens was up to 12 km for males, and 9 km for females. Milk-marked beetles flew up to 10 km (both males and females), and up to 8.5 km and 7.5 km after 24 hours and 7 days, respectively.

The results of geostatistical analyses on marked JBs are listed in Supplementary files S2 (egg) and S3 (milk), whereas some of the most interesting among the correspondent spatial interpolation maps are shown in Fig. 1B–I. As stated in the methods section, indicator kriging was used: therefore, each map shows the probability that, at a given position, the rate of marked JBs exceeds a given cutoff value (blue: lowest probability; red: highest probability). In other terms, an area in the same color measures the probability that a given rate (5%, 10%, or 20%, depending on cutoff value) of the JBs are marked, originating therefore from the treated point. While most of the variograms obtained out of egg-marked specimens showed a pure nugget effect (no sill) with a range of 5 km, in some cases a clear spatial correlation was observed:

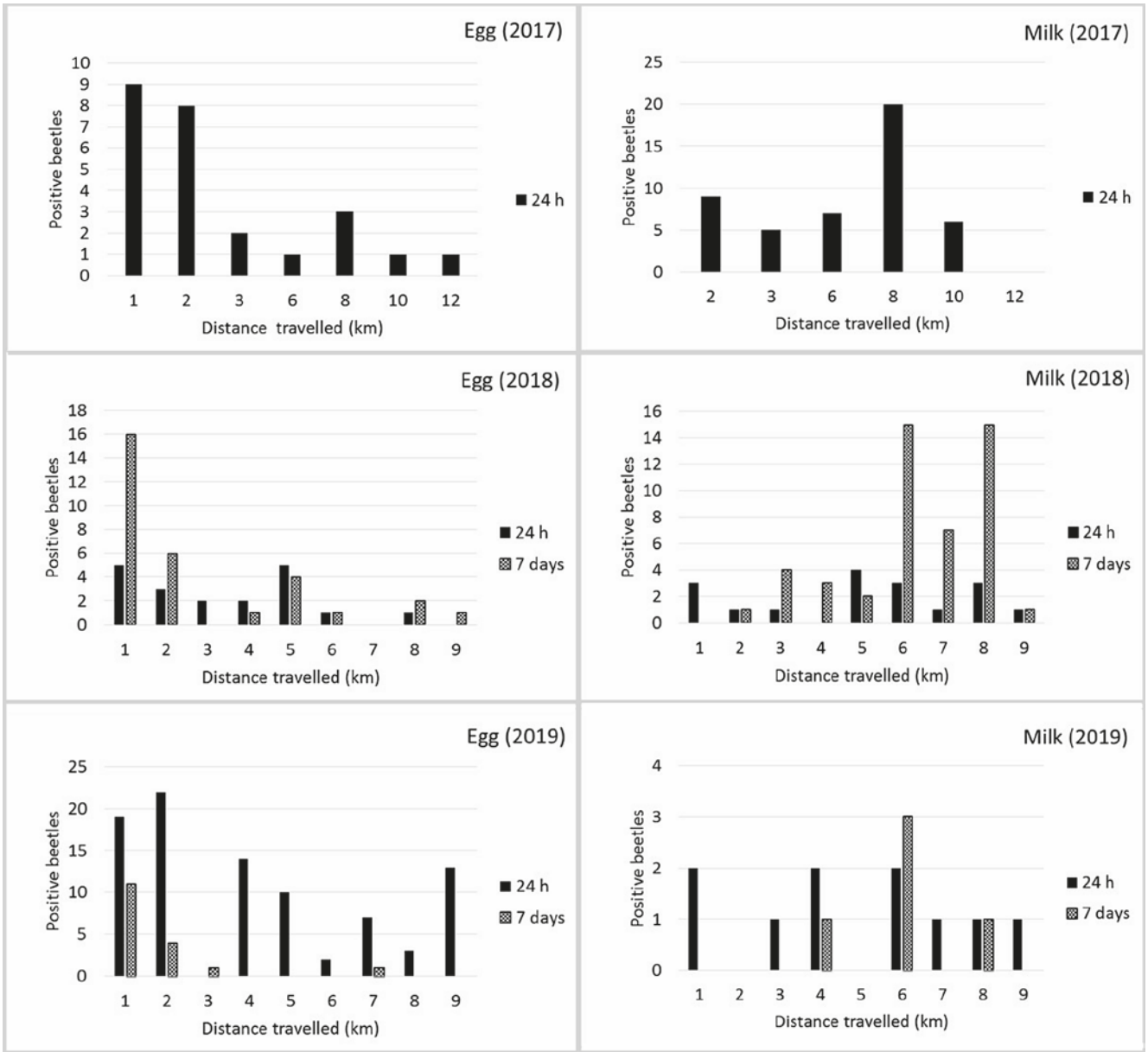

Fig. 2. Number of marked Japanese beetles captured at different distances from the treated plots.

in 2018 for cutoff $c = 0.05$ and captures after 24 h; in 2019 for $c = 0.05$ and $c = 0.10$ (captures after 24 h + 7 days), and for $c = 0.05$ and captures after 24 h (supplementary file S2). All of these interpolations showed a range of 2.1–2.3 km and a real sill, and absolute values of mean errors were between 0.01 and 0.06. In biological terms, this means that within this distance the aggregations of JBs are mutually related, and therefore the density of marked *P. japonica* is homogeneous (either low or high). In 2018 after 24 hours (Fig. 1B), the probability of exceeding the cutoff $c = 0.05$, indicating the probability that more than 5% of the beetles in this area are potentially marked, originating therefore from the treated point, ranged 40 –75% within almost all of the sampling area, having however a patchy pattern indicating a shattered dispersal. In 2019, considering the overall movement of JBs, the probabilities of exceeding cutoffs $c = 0.05$ (Fig. 1D) and $c = 0.10$ (Fig. 1E) were instead much higher, being often >75% with few "empty spots". Moreover, in 2019 the probability of exceeding cutoff $c = 0.05$ was almost the same for captures within 24 hours (Fig. 1C) and overall (Fig. 1D), indicating therefore a strong movement of beetles within a relatively small window of time. Compared to egg-marked ones, milk-marked beetles showed more often a clear spatial correlation with a proper sill, the range being between

1.9 km and 3.3 km (supplementary file S3). In 2017, after 24 hours three isolated hot spots were identified for cutoff c = 0.20, showing the shortest range of 1.9 km (Fig. 1F). In 2018, a very similar pattern was observed for the interpolation with cutoff c = 0.20 (Fig. 1I), whereas a wide area with a probability over 75% was observed for cutoff c = 0.05 after 24 hours (Fig. 1G), and overall (Fig. 1H). All of these interpolations showed a range of 2.1 km. In 2019, as very few specimens were marked with milk only, all interpolation maps showed only single hot spots.

Egg load was determined in 402 dissected females (2018: n = 202; 2019: n = 200). Mean egg count (± standard errors) in different ranks (R) were as follows: R2 = 2.37 ± 0.10; R3 = 7.69 ± 0.18; R4 = 16.93 ± 0.83 (maximum value: 38 eggs). Values of R1 are not given, as it refers to females with no eggs. Overall, 68 females were marked with either egg or milk. Distances travelled were higher in milk-marked females, in terms of both mean values and percentiles. The 75th percentiles were 5.5 km in egg-marked and 7.25–7.50 km in milk-marked females, whereas the 90th percentiles were 6–7.75 km in egg-marked and 8 in milk-marked females (Table 3). The maximum distances travelled were 10 km by milk-marked females (a single specimen with no eggs), and 9 km by egg-marked females (two females with no eggs, two with 1–5 eggs, and two with >10 eggs). However, no significant differences were detected with respect to interactions between factors (GLM, χ^2 = 37.63, df = 37, p = 0.44) (Table 4).

4 Discussion

Overall, the protein marking yielded a great number of marked specimens of *P. japonica*. This is also the first study which used protein marking to study the dispersal ability in a scarab beetle: generally, mark-capture and mark-release-recapture studies on coleopterans have been conducted using different kinds of markers. Studies involving longhorn beetles (Cerambycidae) involved non-toxic paints applied to single insects (Tikkamäki & Komonen, 2011; Rossi de Gasperis et al., 2016), whereas the dispersal of the spruce-bark beetle *Ips typographus* (L.) (family Scolytidae) has been studied by means of fluorescent powders (Dolezal et al., 2016). The results of all of these studies, which involve species belonging to families whose biology is quite different from scarabs and use different markers, are hardly comparable to our study on JB. Other protein markers, namely rabbit IgG and chicken IgY, were used instead to mark the gut content in the ground beetle *Harpalus pensylvanicus* de Geer (Carabidae) (Blubaugh et al., 2016). An immunomarking study conducted on the lady beetle *Hippodamia convergens* Guérin-Méneville using egg and milk showed a higher rate of marking for both markers (over 90%), but this was not a dispersal study, as the beetles were retained on the marked point and not allowed to move away (Hagler et al. 2014). Another immunomarking study concerns the emerald ash borer, *Agrilus planipennis* Fairmaire (Buprestidae), where chicken egg white at different concentrations (0, 10,

Table 3. Distances covered (km) by marked Japanese beetle females having a different egg load (years 2018 and 2019).

Marker	Egg rank	N	Mean	Standard error	Percentiles		
					50%	75%	90%
Egg	R1 (no eggs)	16	3.61	0.77	2.10	5.50	6.25
	R2 (1–5 eggs)	8	3.81	1.28	2.10	5.50	7.75
	R3 (6–10 eggs)	6	2.67	0.91	1.50	5.50	6.00
	R4 (> 10 eggs)	7	4.41	1.34	2.10	5.50	7.75
Milk	R1 (no eggs)	13	5.84	0.61	6.00	7.50	8.00
	R2 (1–5 eggs)	8	6.23	0.85	5.50	7.25	8.00
	R3 (6–10 eggs)	4	7.13	0.43	6.00	7.25	8.00
	R4 (> 10 eggs)	6	4.87	0.95	6.00	7.50	8.00

Table 4. Interactions between egg load and other factors in marked Japanese beetle females.

Source of variation	Wald χ2	Df	P
intercept	3.12	1	0.08
egg load × marker	1.76	3	0.62
distance × egg load	29.75	29	0.43
distance × egg load × marker	0.99	1	0.32

50, and 100% volume) was sprayed on infested logs placed into cardboard tubes with a plastic collecting cup, and adult borers self-marked while exiting the holes: at a 10% dilution, the marking rate was about 35% and the mean optical density about 0.18 (Gula et al., 2020). These data are slightly higher than ours, but differences in methodology should be taken into account: emerald ash borers emerging from sprayed logs inside sealed tubes had a greater chance to be marked than JBs flying up to a 12 km distance from the treated point. On the other hand, a mark-capture study involving exactly the same methodology and the same markers as the present one was conducted on the brown marmorated stink bug (BMSB), *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) (Bosco et al., 2020). *H. halys* is similar in size to the JB, and displays a crowding behavior as well, both during feeding and overwintering: therefore, although we are talking about species belonging to different orders, the results may be comparable to a certain extent. Rates of marked BMSB (about 600 insects analyzed) were 30% for albumin, 14% for casein, and 9% for both markers (Bosco et al., 2020): these values are similar to those obtained for *P. japonica* which confirms the reliability of the method used, given also the greater number of JBs tested (about 1600).

Many specimens were double-marked: this may be due either to medium-long range roaming beetles from one marked plot to another, or to cross-contamination due to a crowding behavior (Fleming 1972). Not being sure about it, we preferred to exclude these specimens from analyses. More beetles were marked with egg rather than with milk: this may be due to a higher marking efficiency of albumin compared to casein, in accordance with Jones et al. (2006). On the other hand, the time elapsed did not always affect the rate of marked specimens. It is known that the protein markers used can be retained up to three weeks (Jones et al. 2006). On the other hand, heavy rain occurred on some occasions and may have decreased the markers' persistence to some extent. Some differences in marking rates of males and females have been detected; however, they were not consistent over years: sometimes more females resulted marked, some other the marking rate was higher in males, and in other cases it was the same for males and females.

The mean flight range of beetles was between 1.37 km and 7.04 km, and 75% of the marked specimens were captured at distances between 5 km and 9 km, depending on plot source, sex, and time elapsed. Since these distance values have been calculated over a maximum time of seven days, and some specimens were found up to 12 km away after 24 hours only, these results are consistent with the spread rate of JB in North America, which occurred at rates of 8–11 km/year (Allsopp 1996). In this research the mean distance travelled by marked beetles was sometimes greater after 24 h than after 7 days. It is worth noting that, while flying, JBs tend to wander about rather than following a straight line, therefore the time spent flying may not be related to the distance covered. Previous mark-release-recapture experiments conducted on JB with powders and dyes showed a short-range dispersal up to 300–500 m (Polivka 1949). Other studies reported by Fleming (1972) stated that flight distances were recorded up to 1.6–4.4 km; however, these results seem to underestimate the spread capabilities of the JB as shown in the present research, where mean flight distances were between 1.5–6 km and single beetles flew up to 12 km in one day. This, also, is consistent with an anecdotal report of beetles driven by wind, flying over the sea and alighting on a boat about 8 km offshore (Fleming 1972).

The distribution pattern of marked specimens has been often quite patchy. It is worth noting that spatial correlation showed greater range values starting from the milk-marked spot, settled within an urbanized area. Probably, in this case the beetles had to move farther away in order to find suitable resources. In fact, land use is a driving factor for JB distribution within 500 m radius from sampling point (Hamilton et al. 2007). On the other hand, the egg-marked point was set inside a more rural area, which may have led to a much more crowded behavior of beetles. It must be noticed that spatial correlation of marked specimens occurred up to 2–3 km, whereas correspondent mean flight distances recorded were generally higher. This may be due to the restless flight activity of beetles returning often on their tracks and not following consistently a straight line. However, while the greatest part of the marked specimens remained within a 2–3 km range, some individuals flew up to 10–12 km away.

The spread capabilities of JB are much more important concerning females bearing eggs, which are responsible for spreading the infestation. However, in our research, the distance covered by flight had no relationship with egg load. A problem with the JB is the phenomenon of spillover, with beetles being attracted by lures but landing in proximity of the trap and not being captured (Switzer et al, 2009). The same research has demonstrated that females attracted to traps tend to have fewer eggs than not attracted ones (Switzer et al. 2009), therefore, our data may have been biased by this behavior. Besides, it would have been difficult to sample JB by sweep net or other means without having contamination issues. Another study on *H. convergens* showed that cross-contamination during sweep-net samplings on alfalfa occurred at a 0.7 and 0.8% rate for egg and milk, respectively (Hagler et al., 2015), however the crowding behavior in *P. japonica* is considerably greater.

In the aggregation dynamics of *P. japonica,* the first specimens reaching a new food source are mated females (pioneers), followed by males and unmated females (joiners) (Kowles & Switzer 2012). Sara et al. (2013) also found that females with heavier egg loads tend to move away from the edges (that is, away from aggregations). Therefore, mated females bearing eggs are the most likely to disperse from beetle bunches. However, female JBs tend to oviposit in places located close to their own food sources (e.g., in meadows and lawns close to broadleaf trees, or such); if none are present, then females increase their flight range to find

other ones (Potter & Held 2002). The absence of correlation between distance travelled and egg load may therefore be the result of these behaviors, along with a patchy environment that leads beetles to travel along wandering pathways rather than following straight lines. Long-range travelling by females with heavy egg load recorded in this research may be due to aggregation points far away from oviposition sites. The great spread capability of females independently from egg load is therefore a threat for beetle-free areas.

As a conclusion, this research conducted with protein immunomarking technique has confirmed that *P. japonica* is a species with a great spread potential: some specimens, albeit rarely, may fly up to 12 km away, rapidly expanding year-to-year the infested area. The results of this research, along with those of surveys carried out in fields by the Plant Protection Service, could provide useful guidance in establishing an effective radius for the demarcated area.

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Appendix

Table S1. Distances travelled (km) from source by Japanese beetles (mean, standard error, 50th, 75th, and 90th percentile).

Year	Marker	Group	N	Mean	Standard error	50%	75%	90%
2017	Egg	24 h	25	4.76	0.38	6.00	9.00	11.00
		males	20	5.06	0.57	6.00	9.00	11.00
		females	5	3.77	1.58	3.00	7.00	8.00
	Milk	24 h	48	6.39	0.32	6.00	8.00	9.00
		males	32	6.57	0.34	7.00	9.00	11.00
		females	15	5.88	1.49	6.00	8.00	9.00
2018	Egg	Total	50	3.20	0.06	2.60	6.00	8.00
		24 h	19	4.01	0.47	3.75	6.25	6.50
		7 days	31	2.39	0.14	2.20	6.00	8.00
		males	36	3.26	0.04	2.20	6.00	8.00
		females	14	3.11	0.12	2.20	6.00	6.50
	Milk	Total	65	5.42	0.41	4.25	6.75	7.75
		24 h	17	4.90	0.50	4.25	6.25	7.50
		7 days	48	6.49	0.64	5.00	7.00	7.75
		males	42	5.77	0.53	4.25	6.75	7.75
		females	23	6.23	0.56	4.00	6.75	7.75
2019	Egg	Total	174	3.33	0.06	4.25	6.25	7.75
		24 h	131	3.97	0.36	3.50	6.25	8.00
		7 days	40	1.37	0.14	2.20	5.00	7.50
		males	123	3.24	0.06	3.50	6.25	8.00
		females	48	3.60	0.06	3.50	6.25	7.50
	Milk	total	77	5.21	0.48	4.25	5.50	7.75
		24 h	51	4.63	0.47	4.25	6.25	7.75
		7 days	26	7.04	1.21	6.25	7.25	8.00
		males	44	5.84	0.63	4.00	6.25	7.75
		females	33	4.45	0.32	4.00	6.25	7.75

Table S2. Results of geostatistical analysis on egg-marked Japanese beetles.

Year	Time elapsed	Cutoff	Nugget	Sill	Range (km)	Mean error
2017	24 h	0.05	0.11	0.13	5.00	0.03
		0.10	0.24	0.00	5.00	0.02
		0.20	0.15	0.00	5.00	–0.03
2018	Total	0.05	0.26	0.00	5.00	0.00
		0.10	0.20	0.00	5.00	0.04
		0.20	0.09	0.00	5.00	0.04
	24 h	0.05	0.21	0.05	2.10	0.01
		0.10	0.22	0.00	5.00	0.02
		0.20	0.12	0.00	5.00	0.04
	7 days	0.05	0.27	0.00	5.00	0.01
		0.10	0.22	0.00	5.00	0.03
		0.20	0.04	0.00	5.00	0.03
2019	Total	0.05	0.11	0.12	2.31	–0.06
		0.10	0.13	0.14	2.31	0.01
		0.20	0.19	0.07	5.00	0.01
	24 h	0.05	0.08	0.16	2.31	–0.03
		0.10	0.27	0.00	5.00	–0.02
		0.20	0.26	0.00	5.00	0.01
	7 days	0.05	0.15	0.00	5.00	0.02
		0.10	0.14	0.00	5.00	0.04
		0.20	0.08	0.00	5.00	0.02

Table S3. Results of geostatistical analysis on milk-marked Japanese beetles.

Year	Time elapsed	Cutoff	Nugget	Sill	Range (km)	Mean error
2017	24 h	0.05	0.15	0.09	5.00	–0.02
		0.10	0.26	0.00	5.00	–0.02
		0.20	0.00	0.23	1.91	0.03
2018	Total	0.05	0.24	0.01	2.10	–0.02
		0.10	0.24	0.00	5.00	–0.02
		0.20	0.00	0.15	2.10	–0.08
	24 h	0.05	0.00	0.25	2.10	- 0.03
		0.10	0.23	0.00	5.00	–0.04
		0.20	0.00	0.15	2.10	–0.08
	7 days	0.05	0.26	0.00	5.00	- 0.05
		0.10	0.23	0.00	5.00	–0.04
		0.20	0.00	0.15	2.10	–0.08
2019	Total	0.05	0.00	0.15	3.28	0.03
		0.10	0.00	0.11	5.00	0.04
		0.20	0.00	0.11	5.00	0.04
	24 h	0.05	0.00	0.09	5.00	0.00
		0.10	0.00	0.06	5.00	0.04
		0.20	0.00	0.06	5.00	0.04
	7 days	0.05	0.02	0.06	2.31	–0.06